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Differentiating effects of somatomedin-C/insulin-like growth factor I and insulin on Leydig and Sertoli cell functions

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Summary. Using an *in vitro* system of pig Leydig cells (LC) and Sertoli cells (SC) we have demonstrated that: 1) LC contained specific receptors for both somatomedin -C/insulin-like growth factor I (Sm-C/IGF-I) and insulin, whereas SC contained only Sm-C/IGF-I receptors ; 2) pretreatment of LC with insulin or Sm-C/IGF-I increased hCG receptor number and the cAMP and testosterone responses to this hormone. The enhanced steroidogenic capacity was related to an increased activity of several enzymes of the steroidogenic pathway. At physiological concentrations Sm-C/IGF-I was more potent than insulin, but the effects of the latter peptide at micromolar concentrations were similar to those produced by nanomolar concentrations of Sm-C/IGF-I. However, at maximal concentrations of both peptides, there was no additive effect ; 3) the specificity of the effect of Sm-C/IGF-I was proven by the fact that all the effects induced by this peptide, but not by insulin, were blunted by an anti-Sm-C/IGF-I antibody ; 4) pretreatment of SC with Sm-C/IGF-I at nM concentrations or with insulin (but only at µM concentrations) enhanced the stimulatory effect of FSH on cAMP production and the secretion of plasminogen activator ; 5) in both LH and SC, Sm-C/IGF-I had small mitogenic effects but potentiated the mitogenic action of fibroblast growth factor (FGF). The effect of insulin was observed only at µM concentrations ; 6) SC secreted a factor which had physico-chemical and biological properties similar to that of Sm-C/IGF-I. The secretion of this factor was stimulated by FGF and EGF.

Introduction.

It has been traditional to consider that the two principal testicular functions, androgen secretion and spermatogenesis, are mainly under the control of pituitary hormones. While it is clear that gonadotropins are absolutely required for the initiation and maintenance of testicular functions, recent data indicate that in addition to this endocrine regulation, other hormones and factors, coming from the blood or secreted locally, might also participate in the regulation of testicular function (reviews : Sharpe, 1984, 1986 ; Saez and Benahmed, 1985 ; Saez *et al.*, 1987).

The role of two hormones, insulin and growth hormone (GH), on testicular functions has long been suspected. Clinically, diabetes mellitus often results in disturbances in male reproductive function (Rodriguez-Rigau, 1980 ; Cameron *et*

al., 1985) and experimentally-induced diabetes in male rats produces a marked decrease in testicular luteotropin (LH) receptors and in Leydig cell steroidogenesis, alterations corrected by insulin treatment (Charreau *et al.*, 1978 ; Paz and Homonnai, 1979 ; Murray *et al.*, 1981). However, given the concomitant hypogonadotropism of diabetic rats (Adashi *et al.*, 1981 ; Murray *et al.*, 1981) and that insulin is an absolute requirement for the maintenance of normal plasma levels of somatomedin-C/Insulin-like growth factor I (Sm-C/IGF-I) (Maes *et al.*, 1986), it is not clear whether the observed changes in testicular functions in diabetic animals are a direct consequence of insulin deficiency at the testicular level or whether they are secondary to the low plasma gonadotropin or Sm-C/IGF-I levels or to a combination of the three.

Similarly, the role of GH on testicular functions has also been suggested by both experimental and clinical data. Administration of GH to hypophysectomized rats partially prevents the loss of testicular LH receptors and maintains steroidogenic responsiveness to acute stimulation by this hormone (Odell and Swerdloff, 1976 ; Zipf *et al.*, 1978). In humans, isolated growth hormone deficiency is very often associated with micropenis (Laron and Sarel, 1970), delayed puberty (Tanner and Whitehouse, 1979) and poor response to exogenous hCG (Kulin *et al.*, 1981), but treatment with GH very often improves both pubertal maturation and testosterone response to hCG administration (Tanner and Whitehouse, 1975 ; Kulin *et al.*, 1981). These data suggest therefore that GH is involved either directly or indirectly in the development and maintenance of gonadotropin responsiveness of Leydig cells. However, since most of the actions of GH *in vivo* are thought to be mediated by Sm-C/IGF-I (Froesch *et al.*, 1985 ; Underwood *et al.*, 1986), and since no study has reported a direct effect of growth hormone on cultured Leydig cells (despite the fact that Leydig cells from several species contain specific lactogenic receptors : Hughes *et al.*, 1985), it is likely that the action of GH on Leydig cell function may be mediated by Sm-C/IGF-I.

The present study concentrates mainly on recent work by our group on the characterization of somatomedin-C/insulin-like growth factor type I (Sm-C/IGF-I) and insulin receptors in somatic testicular cells and on the biological role of both peptides in Leydig and Sertoli cell functions using an *in vitro* system. In addition, the characterization and regulation of a Sertoli cell-secreted Sm-C-like peptide is presented.

Materials and methods.

Transferrin, bovine insulin, soybean trypsin inhibitor, vitamin E, fibrinogen, plasminogen, and 4-(2-hydroxyethyl)-1-piperazinethanesulfonate (Hepes) were purchased from Sigma Chemicals (St Louis, Mo), collagenase from Boehringer (Mannheim, Germany). Ham's F-12 medium and Dulbecco's modified Eagle medium in powder form, and trypsin-ethylenediaminetetraacetic acid (EDTA) were obtained from Grand Island Biological Co (Grand Island, NY). Porcine follicle-stimulating hormone (NIH-FSH-P_2) and human follicle-stimulating hormone (hFSH-2) were provided by NIADDK, National Pituitary Agency,

Na-^{125}I (carrier free) from Amersham. Epidermal growth factor (EGF) from mouse submaxillar gland was prepared as described (Savage and Cohen, 1972). Bovine brain basic fibroblast growth factor (FGF) was purified as described by Gospodarowicz *et al.* (1984) using heparin-Sepharose affinity chromatography. Somatomedin-C/insulin-like growth factor I (Sm-C/IGF-I) was purified from acromegalic plasma by a modification of the method described by Chernausek *et al.* (1985). The purified material migrates as a single narrow band on polyacrylamide gel in the same position as pure recombinant DNA Sm-C (Am-Gen Biologicals, Thousand Oaks, Ca) and has the same potency as pure plasma Sm-C in the specific Sm-C radioimmunoassay. Monocomponent porcine insulin was obtained from Novo Research Institute (Copenhagen, Denmark).

Preparation and culture of pig Leydig and Sertoli cells. — Leydig and Sertoli cells were prepared from piglet testes by the methods previously described (Bernier *et al.*, 1986b ; Perrard-Sapori *et al.*, 1987a ; Saez and Jaillard, 1986). The cells were suspended in Ham's F-12 medium/Dulbecco's modified Eagle medium (1 : 1) supplemented with 15 mM $NaHCO_3$, 20 mM Hepes pH 7.4, 150 U/ml penicillin, 75 µg/ml streptomycin, 5 µg/ml transferrin, 200 ng/ml vitamin E. These cells were plated either in plastic multiwell dishes or in dishes coated with extracellular matrices prepared from bovine endothelial cells (Gospodarowicz, 1984) ; they were then cultured at 33 °C in a controlled humidified atmosphere at 5 % CO_2 for 24 h ; at the end of that time, the medium was replaced by fresh medium.

All experiments with Leydig cells as well as those with Sertoli cells in which the secretion of Sm-C/IGF-I was studied, were performed using the primary culture. On the other hand, in experiments performed to study the mitogenic and trophic effects of several factors on Sertoli cells, second passage cells were used.

Treatment with the appropriate hormones and/or growth factors was initiated 36 h after the beginning of the primary culture for Leydig cells or 36 to 48 h after the beginning of the subculture for Sertoli cells. The growth factors were added every other day without changing the media and the incubation continued for 3 to 4 days for Leydig cells and 6 to 7 days for Sertoli cells. At the end of the experimental period the cells were washed twice with ice-cold saline. In addition, to determine the receptor number for a hormone or growth factor used during pretreatment, the cells were also washed with low pH glycine buffer to remove the membrane-bound hormone (Segaloff and Ascoli, 1981).

Binding studies. — Insulin, hCG and hFSH were radioiodinated and purified following previous procedures (Bernier *et al.*, 1986b ; Perrard-Sapori *et al.*, 1987a, b ; Saez and Jaillard, 1986). The specific activities were 160 to 200 µCi/µg, 80 to 100 µCi/µg and 60 to 80 µCi/µg respectively. ^{125}I-Sm-C (SA 200 to 300 µCi/µg) was a generous gift from Drs. Van Wyk and Underwood (University of North Carolina, Chapell Hill). Purified Sm-C/IGF-I was obtained from human acromegalic plasma as described (Chernausek *et al.*, 1985).

Binding of ^{125}I-hCG and ^{125}I-hFSH to the cells was used to assess the number of LH and FSH receptors, respectively, as described elsewhere (Bernier *et al.*, 1986b ; Saez and Jaillard, 1986). To assess the number of insulin or the

Sm-C/IGF-I receptors, cells were incubated in F-12/DME medium containing 0.5 % BSA, 0.1 % bacitracin and ^{125}I-Sm-C or ^{125}I-insulin ($\simeq$ 10-15 $\times$ 10^4 cpm/ml) and increasing concentrations of the corresponding unlabelled peptide at 20 °C for 4 h. At the end of the incubation, the cells were washed, solubilized in 0.4 % deoxycholate, 0.5 N NaOH and counted.

Affinity cross-liking was performed by a modification of the method described by Pilch and Czech (1980). Cells were incubated with ^{125}I-insulin or ^{125}I-Sm-C ($\simeq$ 1.5 $\times$ 10^6 cpm/ml) for 4 h at 20 °C. The medium was removed, the cells washed and incubated in fresh medium containing 1 mM disuccinimidyl suberate for 15 min at 4 °C. After addition of 0.1 ml of (100 mM) Tris-HCl, 10 mM EDTA to stop the reaction, the medium was removed, the cells washed and solubilized in 0.2 ml (0.1 M) Tris-HCl pH 7, 2 % sodium dodecyl sulfate buffer, 0.001 % bromophenol blue, 100 mM dithiothreitol and run on 0.1 % sodium dodecyl sulfate/7.5 % polyacrylamide gels.

Purification of Sm-C/IGF-I-like material from Sertoli cell-conditioned medium. — After 2 days in culture, the medium was removed, the cells washed three times with saline and cultured in fresh medium containing FGF (50 ng/ml) and ^{3}H-leucine (20 µCi/ml). After two days the medium was removed, acidified with acetic acid to a final concentration of 1 M and filtered (2 ml/min) through a Sep-Pak cartridge (Waters). Elution was performed with 2 ml of pure methanol and the eluate was lyophilized. The recovery of this step, monitored with ^{125}I-Sm-C tracer, was higher than 80 %. The dry residue was suspended in 2 ml of 50 mM phosphate buffer, pH 7.4, and loaded on a 3-ml affinity chromatography column of mouse monoclonal anti-Sm-C/IGF-I and eluted as previously described (Chernausek *et al.*, 1985). The fractions containing the peak of Sm-C/IGF-I-like material were pooled and dialyzed against 100 volumes of 0.01 %, trifluoro-acetic acid (TFA) for 6 h and then lyophilized. The dried sample was redissolved in 500 µl of 0.1 % TFA and injected into an HPLC µ-Bondapak C18 column (Waters) equilibrated with 20 % acetonitrile in 0.1 % TFA. Elution was performed with a 20 % to 60 % acetonitrile isocratic gradient in 0.1 % TFA, in 40 min.

A sample of the pooled fractions (30 to 32) of HPLC elution containing immunoreactive ^{3}H-leucine-Sm-C/IGF-I-like material was lyophilized and diluted in PBS, 10 % SDS, 10 % glycerol, 3 % 2-mercaptoethanol and treated at 100 °C for 3 min before running on 0.1 % SDS, 6 M urea, 18 % polyacrylamide gel. The gels were stained with silver nitrate, dried and exposed to Kodak films.

Sm-C/IGF-I content of fresh conditioned medium and of the different fractions during the purification procedure was determined by radioimmunoassay by the method previously reported (Chatelain *et al.*, 1983). Before the assay the fresh conditioned medium was acidified with ice-cold acetic acid to a final concentration of 1 M, lyophilized, and stored at − 20 °C until assay. The dried residue was suspended in assay buffer on the day of assay.

[^{3}H]-thymidine incorporation into DNA. — About 8 h after addition of growth factors, [^{3}H]-thymidine (1 µCi/ml) was added and the incubation continued for 18-20 h. At the end of this period, the medium was removed, the cells washed

three times with 5 % trichloroacetic acid, and solubilised in 0.5 N NaOH, 0.4 % deoxycholate. Counting was carried out for 10 min in a liquid scintillation spectrometer with a yield of 45 %.

Other methods. — cAMP, testosterone and pregnenolone contents of the media were determined by specific radioimmunoassay (Bernier *et al.,* 1986b). When pregnenolone was measured in the medium, cells were incubated in the presence of 0.06 mM WIN-24540 and 0.01 mM spironolactone to inhibit the metabolism of pregnenolone. In another series of experiments the conversion of pregnenolone to testosterone was measured by incubating Leydig cells with 0.02 mM pregnenolone for 4 h. Plasminogen activator activity was measured by determining plasminogen-dependent degradation of ^{125}I-fibrin (Lacroix *et al.,* 1977).

Statistics. — Statistical analyses were performed with Student's t-test to compare two groups or the analysis of variance with Dunnett's test was used to compare data from multiple groups. Differences were considered significant when p was < 0.05.

Results.

Binding of labelled Sm-C/IGF-I and insulin to Leydig and Sertoli cells.

Pig Leydig cells bound specifically both ^{125}I-Sm-C/IGF-I and ^{125}I-insulin (fig. 1). The concentration of Sm-C/IGF-I required to produce half-maximal displacement of bound ^{125}I-Sm-C/IGF-I was about 7 ng. The Kd of the binding reaction was $1.8 \pm 0.2 \times 10^{-9}$ M and the number of binding sites was $12\,200 \pm 3\,200$ sites/cell. Insulin was also able to inhibit the binding of ^{125}I-Sm-C/IGF-I but with a potency 500 to 600 times less than that of Sm-C/IGF-I. The binding of ^{125}I-insulin was inhibited by insulin, with half-maximal displacement at about 3 ng/ml. Scatchard analysis of the competitive binding (data not shown) demonstrated a curvilinear plot. For this reason the Kd and the number of binding sites could not be exactly calculated. The binding of ^{125}I-insulin was not inhibited by Sm-C/IGF-I at the maximal concentration used (200 ng/ml). These results indicate that the pig Leydig cells contained specific receptors for both peptides. Further characterization of both receptors was achieved by affinity cross-linking experiments (fig. 2). Under reducing conditions, ^{125}I-Sm-C/IGF-I and ^{125}I-insulin were cross-linked predominantly to a receptor complex with an apparent Mr $\simeq$ 125,000 similar to the α-subunit of Sm-C/IGF-I and insulin receptor observed in other studies (Rechler and Nissley, 1985 ; Czech, 1985). Unlabeled Sm-C/IGF-I (200 ng/ml) and high concentrations of insulin (30 µg/ml) (data not shown) inhibited the affinity labeling of Sm-C/IGF-I receptor, whereas insulin at low concentrations (200 ng/ml) had no effect. Similarly, the affinity labeling of insulin receptors was inhibited by unlabeled insulin, but not by Sm-C/IGF-I at identical concentrations.

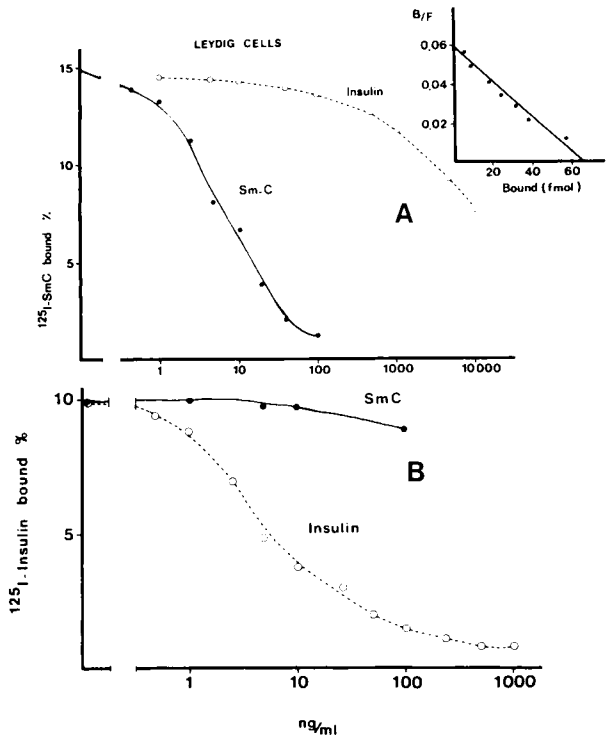

FIG. 1. — *Inhibition of binding of [^{125}I]-Sm-C/IGF-I* (A) *and [^{125}I]-insulin* (B) *to pig Leydig cells by increasing concentrations of Sm-C/IGF-I* (●) *or insulin* (○). Inset : Scatchard analysis of Sm-C binding.

Specific receptors for Sm-C/IGF-I were also demonstrated in pig Sertoli cells by binding (fig. 3) and cross-linking experiments (Perrard-Sapori *et al.*, 1987a). However, using these two approaches we were unable to demonstrate specific insulin receptors in pig Sertoli cells.

Biological effects of Sm-C/IGF-I and insulin on Leydig cell specific functions.

Previous work from this laboratory has shown that pig Leydig cells cultured in a serum-free medium containing micromolar concentrations of insulin maintain both the number of hCG receptors and responsiveness to this hormone at a fairly constant level for several days. In contrast, when Leydig cells are cultured in the absence of insulin, both the number of hCG receptors and the response (cAMP and testosterone) to this hormone declines dramatically within 3 days (Bernier *et al.*, 1986a, b). Treatment of insulin-deprived cells with insulin (5 µg/ml) and/or Sm-C/IGF-I (50 ng/ml) restored both hCG receptor number and responsiveness to this hormone within 3 days (table 1). At the concentration used, it can be seen that the effects of insulin and Sm-C/IGF-I were not additive.

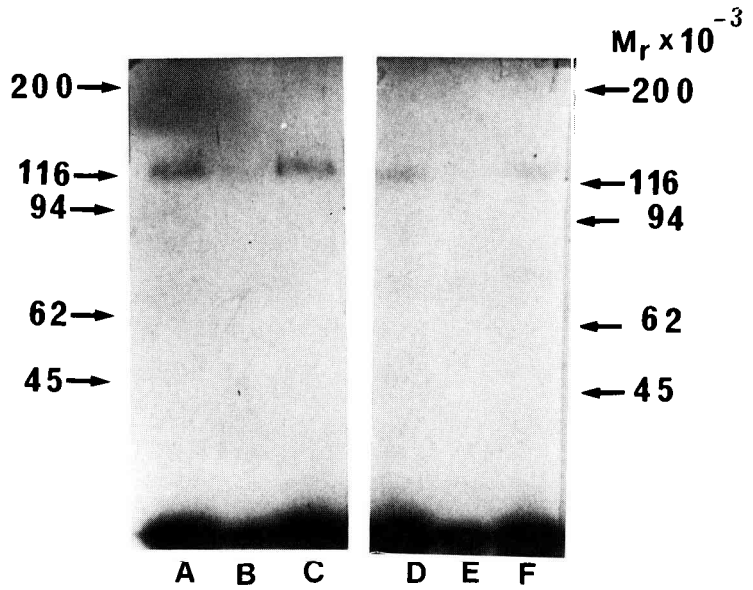

FIG. 2. — *Cross-linking affinity of [^{125}I]-Sm-C/IGF-I* (A-C) *and [^{125}I]-insulin* (D-F) *to Leydig cells examined under reduction conditions, in the absence* (A, D) *or in the presence of 200 ng/ml of Sm-C/IGF-I* (B, F) *or of 200 ng/ml of insulin* (C, E).

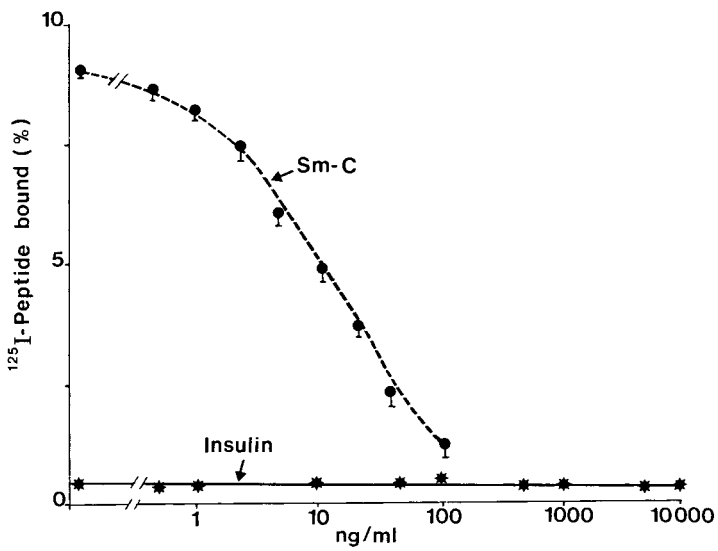

FIG. 3. — *Inhibition of binding of [^{125}I]-Sm-C/IGF-I* (●) *[^{125}I]-insulin* (*) *to pig Sertoli cells by increasing concentrations of unlabelled Sm-C/IGF-I and insulin, respectively.*

TABLE 1

Effects of insulin and Sm-C/IGF-I on Leydig cells.

Medium	[^{125}I]-hCG binding (cpm × 10^3)/10^6 cells	cAMP pmoles/30 min/10^6 cells	Testosterone ng/4 h/10^6 cells
Control	18 ± 2	7.1 ± 0.4	11 ± 2
Control + Insulin (5 µg/ml)	112 ± 8[a]	49 ± 4[a]	98 ± 6[a]
Control + Sm-C/IGF-I (50 ng/ml)	132 ± 4[a]	46 ± 3[a]	108 ± 7[a]
Control + Insulin + Sm -C/IGF-I	134 ± 8[a]	52 ± 5[a]	112 ± 8[a]
Insulin continuously	129 ± 7	50 ± 4[a]	109 ± 7[a]

Isolated Leydig cells were cultured for two days in F12/DME medium without insulin. The medium was removed and replaced by fresh medium without (control) or with the indicated concentration of insulin and/or Sm-C/IGF-I. The peptides were added daily and the incubation continued for 3 additional days. Insulin continuously refers to cells cultured with insulin (5 µg/ml) all the time. At the end of the experiment, the binding of [^{125}I]hCG and the cAMP and the testosterone responses to hCG (10^{-9} M) were measured. The results are the mean ± SD of four cultures.
a : $p < 0.01$ compared to control cells.

The effects of both peptides on Leydig cells were time- and dose-dependent (Bernier, 1986b). Treatment of insulin-deprived cells with increasing concentrations of Sm-C/IGF-I produced two opposite dose-dependent effects : down-regulation of Sm-C/IGF-I receptors and an increase in [^{125}I]-hCG binding (fig. 4). It is interesting to note that pretreatment of Leydig cells with hCG also produced two opposite dose-dependent effects : down-regulation of hCG receptors and enhancement of Sm-C/IGF-I receptors (Perrard-Sapori *et al.*, 1987b). All the above variations were due mainly to a change in the number of receptors without modification of the binding affinity.

Pretreatment of insulin-deprived Leydig cells with Sm-C/IGF-I produced enhanced cAMP and testosterone response to hCG, that was dose-dependent, without modification of the ED_{50} for hCG (data not shown). Interestingly, the cAMP response to forskolin was not enhanced in Sm-C/IGF-I or insulin-treated cells (fig. 5) but the potentiating effects of forskolin on hCG-induced cAMP production was increased (Bernier *et al.*, 1986b). These results suggest that both peptides not only increased hCG receptors but probably improved their coupling to adenylate cyclase.

The enhanced steroidogenic response of insuline or Sm-C/IGF-I pretreated Leydig cells to hCG was not only due to an improvement of the receptor-adenylate cyclase system but also to some modification of several steps of the steroidogenic pathway. This conclusion is inferred by the following results : a) testosterone response, but not cAMP response, to forskolin was higher in Sm-C/IGF-I- or insulin-treated cells than in control cells (fig. 5) ; b) pregnenolone production, under conditions where the metabolism of this steroid was blocked, was higher in Sm-C/IGF-I- or insulin-pretreated cells than in control cells (fig. 6) ; c) the conversion of exogenous pregnenolone to testosterone was also

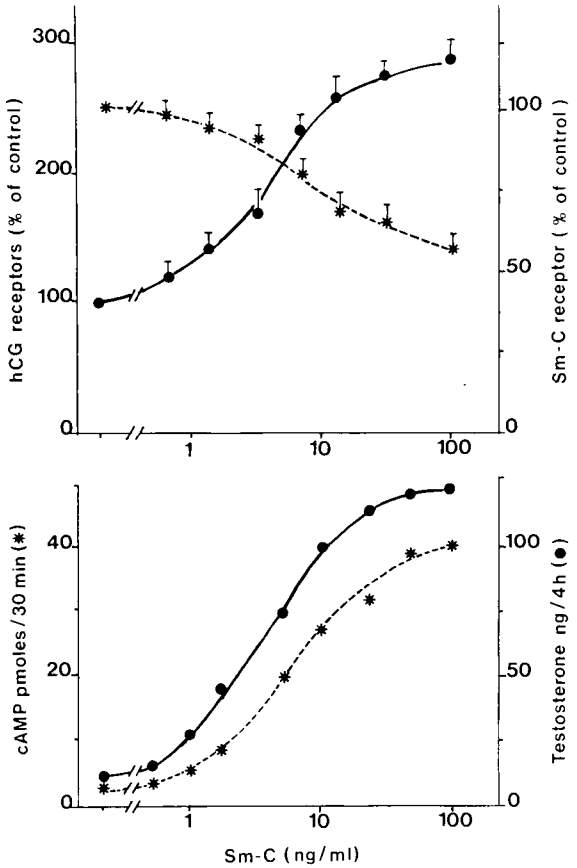

FIG. 4. — *Effects of pretreatment of Leydig cells with increasing concentrations of Sm-C/IGF-I for 2 days on hCG* (●) *and Sm-C/IGF type I* (*) *receptors* (top), *and on the cAMP* (●) *and testosterone* (*) *acute hCG (10^{-9} M) stimulation* (bottom).

significantly higher in peptide-pretreated cells than in control cells (fig. 6). These results suggest that both peptides enhanced the availability of cholesterol for steroidogenesis and/or the activity of the cholesterol side-chain cleavage and also the activity of some enzyme between pregnenolone and testosterone.

The specificity of the potentiating effects of Sm-C/IGF-I on Leydig cell function was proven by the fact that the effects were blunted when Leydig cells were treated simultaneously with Sm-C/IGF-I and an anti-Sm-C-antibody at a concentration which bound at least 95 % of the Sm-C/IGF-I (fig. 5 and 6). On the contrary, the antibodies had no effect on the stimulatory effects induced by insulin.

Biological effects of insulin and Sm-C/IGF-I on Sertoli cells.

Previous work from this laboratory has shown that when pig Sertoli cells are cultured in a chemically defined medium containing transferrin and insulin at

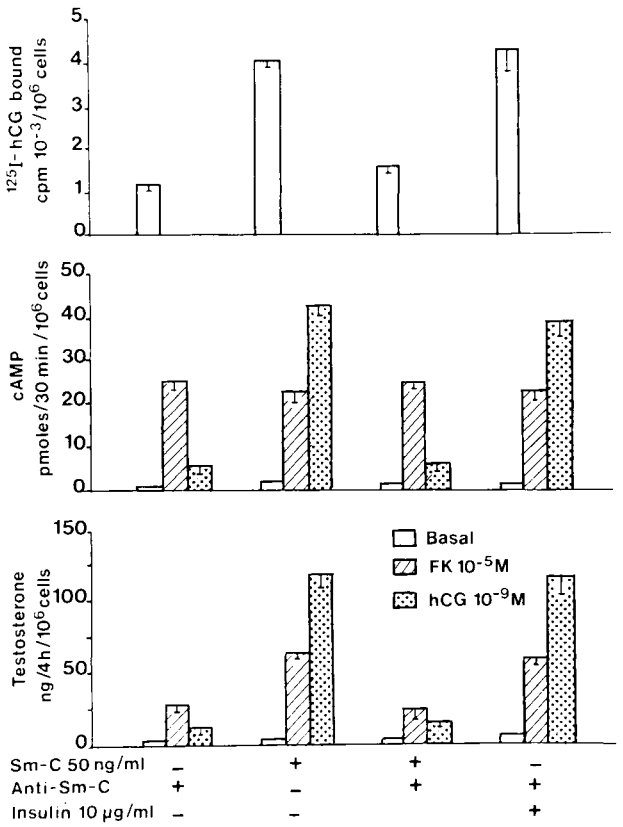

FIG. 5. — *Inhibition of the effects of Sm-C/IGF-I on Leydig cells by anti-(Sm-C) polyclonal antibodies.* Cells were cultured for 3 days without or with Sm-C (50 ng/ml) or insulin (10 µg/ml) in the absence or presence of anti-(Sm-C) polyclonal antibodies at a 1 : 1 000 final dilution. The binding of [^{125}I]-hCG and the cAMP and testosterone responses to forskolin (FK) and hCG were determined as described in Material and Methods.

micromolar concentrations, they maintain FSH binding sites and cAMP and plasminogen activator responses to FSH at a fairly constant level for at least two weeks (Saez and Jaillard, 1986). Table 2 summarizes the effect of several factors, alone or in combination, on pig Sertoli cell FSH receptors and responsiveness. FGF was the only factor able to increase the binding of [^{125}I]-FSH. Neither insulin nor Sm-C/IGF-I alone or together with FGF had a significant effect on this parameter. FGF was also the only factor which enhanced cAMP response to pFSH. However, pretreatment with FGF plus Sm-C/IGF-I (50 ng/ml) or insulin at 5 µg/ml, but not at 50 ng/ml, further enhanced cAMP response to pFSH. Under basal conditions, plasminogen activator activity was very low but pretreatment with FGF, Sm-C/IGF-I (50 ng/ml) or insulin (at 5 µg/ml but not at 50 ng/ml) produced a small but significant increase ($p < 0.05$) (data not shown).

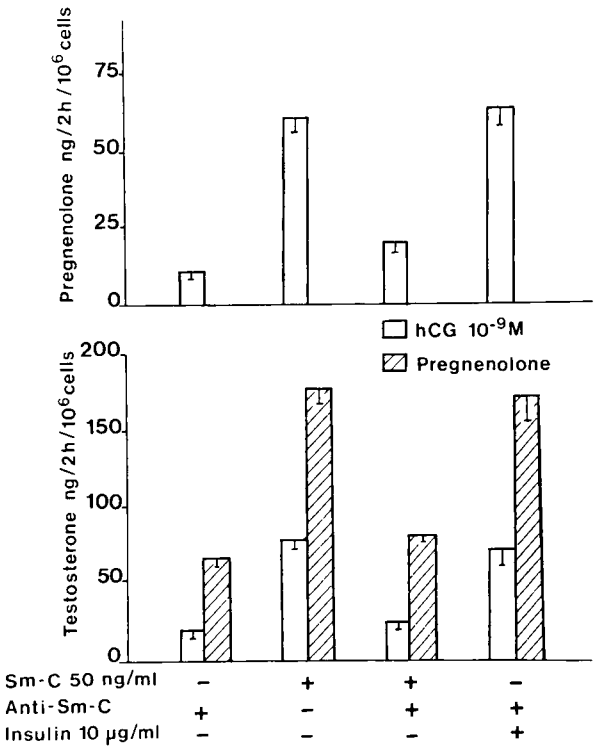

FIG. 6. — *Inhibition of the effects of Sm-C/IGF-I on Leydig cell steroidogenesis by anti-(Sm-C) polyclonal antibodies.* Same protocol as in figure 5. At the end of the culture, some dishes were incubated in fresh medium containing hCG (10^{-9} M), WIN-24540 (5×10^{-6} M) and spironolactone (10^{-5} M) for 2 h and the pregnenolone in the medium was measured (top). Other dishes were incubated with hCG (10^{-9} M) or pregnenolone (2.10^{-5} M) for 2 h and the testosterone in the medium was measured. The results are the mean ± SEM of triplicate determinations of three experiments.

TABLE 2

Effects of insulin, Sm-C/IGF-I and FGF on Sertoli cell function.

Culture media	FSH binding % of control		cAMP production % of control		Plasminogen activator % of maximum	
	− FGF	+ FGF	− FGF	+ FGF	− FGF	+ FGF
Control	100 ± 6	172 ± 8[a]	100 ± 4	149 ± 8[a]	2 ± 0.5	26 ± 4[a]
Insulin 50 ng/ml	102 ± 7	169 ± 6[a]	98 ± 7	151 ± 8[a]	3 ± 0.7	27 ± 5[a]
Insulin 5 µg/ml	107 ± 8	172 ± 8[a]	105 ± 6	187 ± 6[a,b]	7 ± 1.2[a]	65 ± 7[a,b]
Sm-C/IGF-I 50 ng/ml	108 ± 9	174 ± 9[a]	108 ± 6	198 ± 10[a,b]	8 ± 1[a]	78 ± 9[a,b]

a : $p < 0.05$ compared to control ; b : $p < 0.01$ compared to FGF alone.

Second passage Sertoli cells were cultured for 6 days in F12/DME medium without (control) or with the indicated concentrations of insulin or Sm-C/IGF-I in the absence (− FGF) or presence (+ FGF) of 50 ng/ml of fibroblast growth factor. At the end of the experiment, the binding of [^{125}I]-hFSH and the cAMP and the plasminogen activator activity responses to maximal concentrations of pFSH (1 µg/ml) were measured. The results, expressed as per cent of control cells, are the mean ± SEM of quadruplicate determinations of 3 to 8 different experiments.

The response to pFSH was enhanced by previous treatment with Sm-C/IGF-I, insulin (5 µg/ml) and FGF, but again FGF was several times more potent than the other two factors. Both Sm-C/IGF-I (50 ng/ml) and insulin at 5 µg/ml, but not at 50 ng/ml, potentiated the effect of FGF.

Mitogenic effect of Sm-C/IGF-I, insulin and FGF on Leydig and Sertoli cells.

The effects of these factors on Leydig and Sertoli cell DNA synthesis are shown in table 3. For both cell types, FGF was the most potent of the mitogenic factors studied, but its stimulatory effects on Sertoli cells were greater than on Leydig cells. Both Sm-C/IGF-I (50 ng/ml) and insulin (5 µg/ml) also stimulated DNA synthesis but, again, their action on Sertoli cells was higher than on Leydig cells. Moreover, Sm-C/IGF-I (50 ng/ml) and insulin (5 µg/ml) potentiated the stimulatory effect of FGF on both cell types. On the other hand, insulin at low physiological concentrations (50 ng/ml), either alone or with FGF, had no mitogenic action, suggesting that its mitogenic effects observed at micromolar concentrations on both cell types were mediated through Sm-C/IGF-I type I receptors.

TABLE 3

Effects of insulin, Sm-C/IGF-I and FGF on Leydig and Sertoli cell DNA synthesis.

Culture media	[^{3}H]-thymidine incorporation (% of control)			
	Leydig cells		Sertoli cells	
	− FGF	+ FGF	− FGF	+ FGF
Control	100 ± 5	220 ± 10[a]	100 ± 6	350 ± 15[a]
Insulin 50 ng/ml	108 ± 6	224 ± 12[a]	104 ± 7	358 ± 12[a]
Insulin 5 µg/ml	160 ± 12[a]	350 ± 12[a,b]	270 ± 10[a]	556 ± 25[a,b]
Sm-C/IGF-I 50 ng/ml	180 ± 15[a]	392 ± 10[a,b]	272 ± 12[a]	610 ± 20[a,b]

a : $p < 0.01$ compared to control ; b : $p < 0.01$ compared to FGF alone.

Isolated Leydig cells were cultured in dishes coated with extracellular matrices whereas second passage Sertoli cells were cultured in plastic dishes. After 3 days in culture in F12/DME medium containing only transferrin, the medium was removed and replaced by fresh medium without (control) or with the indicated concentrations of insulin or Sm-C/IGF-I in the absence or presence of FGF (50 ng/ml). About 8 hours later [^{3}H]-thymidine was added and the incubation continued for 18 to 20 hours. The incorporation of [^{3}H]-thymidine into DNA was measured as described in Methods. The results, expressed as % of control cells, are the mean ± SEM of quadruplicate determinations of 3 to 6 different experiments.

Secretion of Sm-C/IGF-I-like material by Sertoli cells.

Conditioned medium from Sertoli cells incubated with [^{3}H]-leucine contained a peptide which, through different steps of the purification procedure (see

Methods), behaved like [^{125}I]-Sm-C/IGF-I. Further characterization of the material in the pooled HPLC fractions 30-33 (fig. 7) was achieved by showing that it was able to specifically displace [^{125}I]-Sm-C/IGF-I in a parallel manner and with an apparent potency similar to that of pure plasma Sm-C/IGF-I in both Sm-C/IGF-I radioimmuno- and radioreceptor-assays (Chatelain *et al.*, 1987). Furthermore, it migrated in polyacrylamide gel electrophoresis to the same position as both purified human Sm-C/IGF-I and recombinant DNA Sm-C/IGF-I (Chatelain *et al.*, 1987).

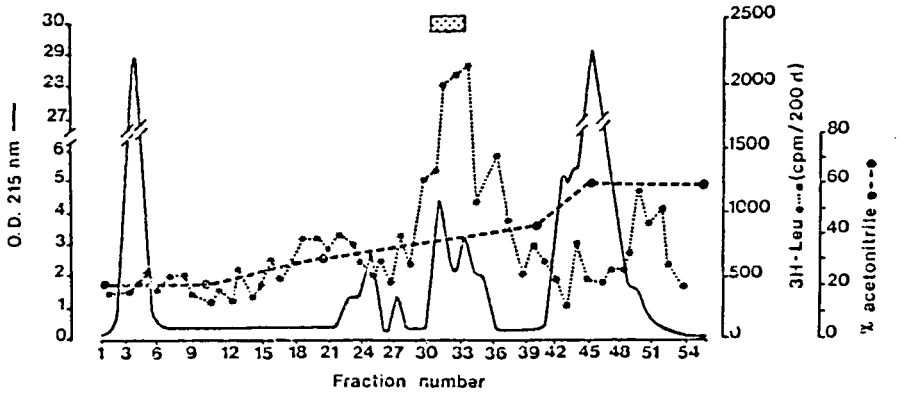

FIG. 7. — *Reverse-phase HPLC elution profile or ^{3}H-leucine Sm-C/IGF-I material secreted by cultured Sertoli cells.* The eluted fractions from the affinity chromatography column of monoclonal anti-Sm-C/IGF-I antibodies were pooled, dialysed and lyophilized. The dry residue was dissolved in 500 µl of 0.1 % TFA and injected in HPLC µ-Bondapak C18 column : (———) Adsorption at 215 nm ; (●---●) % acetonitrile ; (*---*) radioactivity in 100 µl ; ▨ immunoreactive Sm-C/IGF-I.

The secretion of Sm-C/IGF-I-like material by Sertoli cells was studied under several culture conditions. Sertoli cells cultured in plastic dishes secreted Sm-C/IGF-I, but this secretion fell steadily after the first two days of culture. Under our experimental conditions the only factor able to stimulate its secretion was FGF (Chatelain *et al.*, 1987). When the cells were cultured in extracellular matrices, they became more sensitive to the stimulatory effects of FGF or EGF, but neither hGH nor FSH was able to stimulate significantly Sm-C/IGF-I secretion (fig. 8). However, cells pretreated with FGF or EGF for 24 h became sensitive to the stimulatory effects of FSH.

Discussion.

The present results provide evidence that both Leydig and Sertoli cells from immature pigs contain specific IGF type I receptors which have properties similar

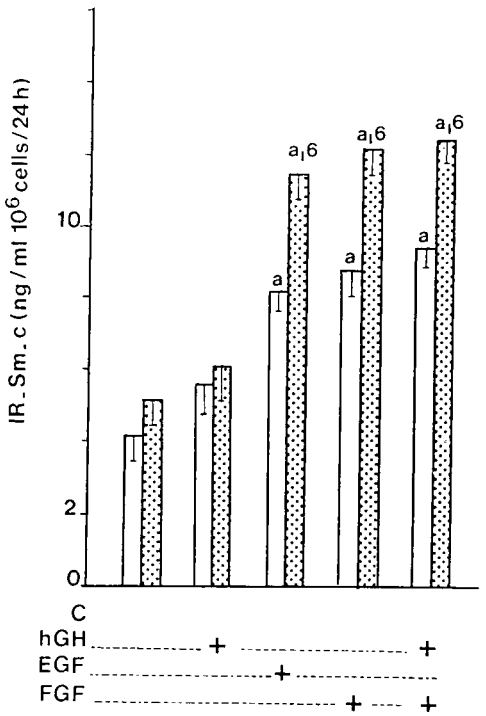

FIG. 8. — Secretion of Sm-C/IGF-I by Sertoli cells under several conditions. Cells were cultured in dishes coated with extracellular matrices for 2 days. Then the medium was removed and replaced by fresh medium alone (C) or with GH (2 µg/ml), EGF (50 ng/ml) or FGF (50 ng/ml). After 24 h the medium was removed and saved (open bars). Fresh medium containing the same factors plus pFSH (0.5 µg/ml) were added. After 24 h the medium was removed (hatched bars). The Sm-C/IGF-I content was measured by RIA. The results are the mean ± SD of triplicate determinations of four dishes. a : $p < 0.05$ compared to control values ; b : $p < 0.05$ compared to the first day under the same conditions.

to those described in most tissues (Rechler and Nissley, 1985). On the other hand, insulin receptors were demonstrated only in Leydig cells. Recent studies have also demonstrated the presence of specific IGF type I receptors (Handelsman *et al.*, 1985 ; Lin *et al.*, 1986a, b ; Kasson and Hsueh, 1987) and insulin (Lin *et al.*, 1986a, b) in rat Leydig cells. The presence of Sm-C/IGF-I receptors in immature rat Sertoli cells has also been reported (Borland *et al.*, 1984 ; Oonk and Grootegoed, 1987a,b), but contradictory results were obtained concerning insulin receptors. Borland *et al.* (1984) could not demonstrate such receptors in immature cultured rat Sertoli cells and in that model insulin at nanomolar concentrations had no biological effect. In contrast, Oonk *et al.* (1985, 1987a, 1987b) reported the presence of insulin receptors of high affinity in immature rat Sertoli cells ; these receptors had very low capacity characterized by binding and cross-linking experiments. They also showed that insulin at nanomolar concentrations was able to stimulate glucose metabolism.

In the present study both Sm-C/IGF-I (at nanomolar concentrations) and insulin (at micromolar concentrations, but not at nanomolar concentrations), had a small but significant mitogenic action on both Leydig and Sertoli cells. These results suggest that the growth stimulatory effect of insulin on testicular cells, as in other cell models (Froesch *et al.*, 1985), was mediated through Sm-C/IGF type I receptors. Our results also suggest that all the metabolic effects of insulin on pig Sertoli cells are exerted through Sm-C/IGF type I receptors.

The most interesting findings from the present study are the effects of Sm-C/IGF-I, alone or associated with other factors, on the specific functions of both Leydig and Sertoli cells, and the fact that pig Sertoli cells are able to secrete this peptide ; this suggests that Sm-C/IGF-I might play a paracrine and autocrine role in testicular growth and differentiation. However, an endocrine action of circulating Sm-C/IGF-I cannot be ruled out.

The present results and those previously reported (Bernier *et al.*, 1986a, b ; Perrard-Sapori *et al.*, 1987a, b) clearly show that the trophic differentiating effects of Sm-C/IGF-I on pig Leydig cells are exerted by several mechanisms : in membranes by increasing the number of hCG receptors and probably their coupling to the adenylate cyclase, and inside the cell by enhancing the activity of several enzymes of the steroidogenic pathway. Although these effects are also produced by insulin at physiological concentrations (through its own receptors), these effects of insulin are less pronounced. It should be pointed out that neither Sm-C/IGF-I nor insulin alone has a significant steroidogenic action, but they both enhance Leydig cell responsiveness to physiological (hCG) or pharmacological (forskolin, cholera toxin) steroidogenic effectors. Similar results have been reported recently concerning the effects of Sm-C/IGF-I and insulin on cultured rat Leydig cells (Lin *et al.*, 1986a, b ; Kasson and Hsueh, 1987).

The trophic differentiating effects of Sm-C/IGF-I have also been observed with other steroidogenic cells : granulosa cells and adrenal cells. Granulosa cells from both swine and rats contain specific IGF type I receptors (Veldhuis and Furlanetto, 1985 ; Adashi *et al.*, 1986) and this peptide at nanomolar concentrations exerts potent and specific differentiating effects on swine granulosa cell steroidogenesis (Veldhuis and Furlanetto, 1985 ; Veldhuis and Rodgers, 1987) and synergizes with FSH in the induction of rat granulosa LH receptors, progestin and estrogen synthesis (Adashi *et al.*, 1985). Similarly, bovine adrenal cells contain specific Sm-C/IGF-I receptors, and this peptide at nanomolar concentration increases the number of angiotensin-II receptors and the steroidogenic response to both angiotensin-II and ACTH (Penhoat *et al.*, 1988). Moreover, in both cell types, insulin at higher concentrations produces similar effects.

In both rat (Borland *et al.*, 1984 ; Mita *et al.*, 1985 ; Oonk *et al.*, 1987a, b) and pig (Jaillard *et al.*, 1987, present studies) Sertoli cells, Sm-C/IGF-I has minor mitogenic and major trophic differentiating effects. More important is the synergistic effect of Sm-C/IGF-I with FGF on both DNA synthesis and Sertoli cell specific functions. The interest of the effects of FGF on Sertoli cells is strengthened by recent data (Ueno *et al.*, 1987) showing that basic FGF is present in bovine testis and that cultured pig Sertoli cells are able to secrete a basic FGF-like material (O. Avallet and J. M. Saez, unpublished data).

The great interest of the effects of Sm-C/IGF-I, on both the male and female gonadic function described above, is strengthened by recent findings showing that swine (Hammond *et al.*, 1985 ; Hsu and Hammond, 1987) and rat (Davoren and Hsueh, 1986) granulosa cells and their purtative male homolog, Sertoli cells from the same species (Ritzen, 1983 ; Tres *et al.*, 1986 ; Smith *et al.*, 1987 ; Chatelain *et al.*, 1987), secrete Sm-C/IGF-I immunoreactivity in the medium. The Sm-C/IGF-I-like peptide purified from rat (Smith *et al.*, 1987) and pig (Chatelain *et al.*, 1987) Sertoli cell-conditioned medium has physicochemical and biological properties similar to those of pure human Sm-C/IGF-I and recombinant DNA Sm-C/IGF-I. The secretion of Sm-C/IGF-I-like peptide by swine granulosa cells was stimulated by gonadotropins and estradiol (Hsu and Hammond, 1987), the principal hormones involved in trophic regulation of the ovary, whereas the most potent stimulators in pig Sertoli cells were FGF and EGF. Surprisingly, neither GH nor FSH had a stimulatory effect when the cells were cultured on plastic dishes. However, their responsiveness to FSH appeared when the cells were cultured on extracellular matrices and pretreated with FGF or EGF. Tres *et al.* (1986) have also reported that the stimulatory effect of FSH and GH on Sm-C/IGF-I secretion by rat Sertoli cells was very little. These results suggest that the regulation of Sm-C/IGF-I gene expression in Sertoli cells, as in most non-hepatic tissues including testes (Clemmons *et al.*, 1981 ; D'Ercole *et al.*, 1984 ; Mathews *et al.*, 1986 ; Fagin and Melmed, 1987 ; Murphy *et al.*, 1987), is partially or not GH-dependent.

In vivo, the potential role of insulin on testicular function is suggested by the fact that human diabetes mellitus (Rodriguez-Rigau, 1980 ; Cameron *et al.*, 1985), as experimentally induced diabetes (Charreau *et al.*, 1978), results in disturbances of testicular function and that in experimental animals the alterations are corrected by insulin treatment. However, insulin is required for normal gonadotropin secretion (Adashi *et al.*, 1981 ; Murray *et al.*, 1981) and for maintenance of normal plasma levels of Sm-C/IGF-I (Maes *et al.*, 1986). Therefore, *in vivo,* the role of insulin on Leydig cells might be either direct or indirect. In addition, since insulin does not seem to be secreted by testicular cells (unpublished results) the paracrine/autocrine role of insulin can be excluded.

The physiological *in vivo* significance of the endocrine/paracrine/autocrine role of Sm-C/IGF-I on testicular growth and functions is suggested by the fact that in both humans (Tanner *et al.*, 1975 ; Kulin *et al.*, 1981 ; Laron, 1984) and experimental animals (Hochereau-de Reviers *et al.*, 1987) growth hormone deficiency is associated with delayed puberty. However, in both Laron dwarfism (probably due to a deficit of GH receptors : Geffner *et al.*, 1987) and in mouse Snell dwarfism (due to a low GH secretion : Hochereau-de Reviers *et al.*, 1987), testicular growth and function develop without any treatment but are delayed. This is consistent with the hypothesis that Sm-C/IGF-I production by the testis is not or only partially GH-dependent (D'Ercole *et al.*, 1984 ; Mathews *et al.*, 1986). Moreover, the potential role of Sm-C/IGF-I on human testicular function is also suggested by the recent findings of Vannelli *et al.* (1987). These authors, by an immunostaining technique using monoclonal antibodies against Sm-C/IGF-I and its receptor, have shown that Sm-C/IGF-I is preferentially localised in Sertoli

cells, while its receptors are located in both Leydig and Sertoli cells and in secondary spermatocytes and early spermatides.

In *conclusion,* our results and those of the literature indicate that Sm-C/IGF-I produced locally and/or coming from the blood stream might play an important role in the maturation and the maintenance of the differentiated function of both male and female gonads.

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Résumé. *Effets de la somatomédine-C/Insulin-like growth factor I et de l'insuline sur les fonctions différenciées des cellules de Leydig et de Sertoli.*

The rôle potentiel de la Somatomédine-C/Insulin-like growth factor I (Sm-C/IGF-I) et de l'insuline sur les fonctions des cellules somatiques testiculaires a été étudié en utilisant des cellules de Leydig (LC) et des cellules de Sertoli (SC) de porcelet maintenues en culture primaire dans un milieu chimiquement défini. Nous avons montré que : 1) Les LC contiennent des récepteurs spécifiques pour la Sm-C/IGF-I et pour l'insuline, tandis que les SC contiennent seulement des récepteurs pour la Sm-C/IGF-I. 2) La Sm-C/IGF-I et l'insuline augmentent le nombre de récepteurs à l'hCG et la réponse (AMPc et testostérone) à cette hormone des LC. A concentrations physiologiques (nM) la Sm-C/IGF-I est plus efficace que l'insuline, mais les effets de l'insuline à concentrations micromolaires sont similaires à ceux induits par la Sm-C/IGF-I à des concentrations nanomolaires. Par ailleurs, à des concentrations maximales, les effets des deux peptides ne sont pas additifs. 3) Les effets de la Sm-C/IGF-I, mais pas ceux de l'insuline, sur les LC sont bloqués par un anticorps anti-Sm-C/IGF-I. 4) Le traitement des SC avec la Sm-C/IGF-I (nM) ou avec l'insuline (µM) augmente l'action stimulante de la FSH sur la production d'AMPc et sur la sécrétion de l'activateur du plasminogène. 5) La Sm-C/IGF-I a une faible action mitogène sur les deux types de cellules, mais le peptide potentialise l'action mitogène du facteur de croissance fibroblastique (FGF). 6) Les SC sécrètent un facteur qui a les caractéristiques physico-chimiques et biologiques de la Sm-C/IGF-I et cette sécrétion est stimulée par le FGF seul ou associé à la FSH. L'ensemble de ces résultats suggère fortement que la Sm-C/IGF-I peut jouer un rôle autocrine et paracrine dans l'expression et le maintien des fonctions différenciées des cellules somatiques testiculaires.

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