Journal of Peptide Science
J. Peptide Sci. **7**: 495–501 (2001)
DOI: 10.1002/psc.344

Chemical Synthesis and Biological Activity of Rat INSL3

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Received 30 April 2001
Accepted 7 May 2001

Abstract: The recently identified protein, insulin 3 (INSL3), has structural features that make it a *bona fide* member of the insulin superfamily. Its predicted amino acid sequence contains the classic two-peptide chain (A- and B-) structure with conserved cysteine residues that results in a disulphide bond disposition identical to that of insulin. Recently, the generation of *insl3* knockout mice has demonstrated that testicular descent is blocked due to the failure of a specific ligament, the gubernaculum, to develop. The mechanism by which INSL3 exerts its action on the gubernaculum is currently unknown. The purpose of this study was to, for the first time, synthesize rat INSL3 and test its action on organ cultures of foetal rat gubernaculum. INSL3 also contains a cassette of residues Arg-X-X-X-Arg within the B-chain, a motif that is essential for characteristic activity of another related member of the superfamily, relaxin. Hence, the relaxin activity of rat INSL3 was also tested in two different relaxin bioassays. The primary structure of rat INSL3 was determined by deduction from its cDNA sequence and successfully prepared by solid phase peptide synthesis of the two constituent chains followed by their combination in solution. Following confirmation of its chemical integrity by a variety of analytical techniques, circular dichroism spectroscopy confirmed the presence of high β-turn and α-helical content, with a remarkable spectral similarity to the synthetic ovine INSL3 peptide and to synthetic rat relaxin. The synthetic rat INSL3 bound with very low affinity to rat relaxin receptors and had no activity in a relaxin bioassay. Furthermore, it did not augment or antagonize relaxin activity. The rat INSL3 did however induce growth of foetal rat gubernaculum in whole organ cultures demonstrating that INSL3 has a direct action on this structure. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: insulin 3; relaxin-like factor; testis descent; gubernaculum; solid phase peptide synthesis

INTRODUCTION

Insulin 3 (INSL3; also called the relaxin-like factor, RLF; or Leydig cell insulin-like peptide, Ley-I-L) is a recently identified member of the insulin-relaxin superfamily of peptide hormones. It is primarily expressed in the Leydig cells of the testis and thecal cells of the ovary [1]. Recently, two independent groups developed knockout mice for INSL3 [2,3]. The major phenotype in these animals was the lack of testicular descent in the male due to the failure to develop of a specific ligament, the genito-inguinal ligament or gubernaculum. During normal testicular descent, the caudal end of the gubernaculum, the gubernacular bulb, enlarges or 'swells' and this process is disrupted in the INSL3 knockout. The mechanism(s) by which INSL3 induces this 'swelling' of the gubernacular bulb is unknown.

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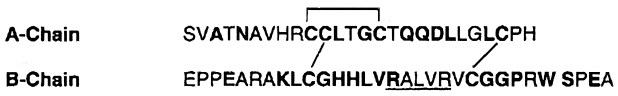

Figure 1 The predicted structure of the rat INSL3 peptide. Amino acid residues that are conserved between species are in bold. The relaxin-like receptor binding motif is underlined.

Although the INSL3 cDNA sequence has now been cloned from a number of species, the primary structure of the circulating INSL3 peptide is unknown. Nevertheless, the predicted protein sequences show a high degree of homology and are likely to be expressed as preprohormone precursors that undergo proteolytic processing to yield a mature two-chain product. More recently, the rat INSL3 cDNA has been cloned [4] and the predicted structure of the INSL3 prohormone shows high homology to that of predicted INSL3 structures from other species. Based on known relaxin and insulin prohormone processing patterns in various mammalian species [1], it is predicted that the mature rat INSL3 protein consists of a two chain peptide (A- and B-chains) containing two inter- and one intra-chain disulphide bonds (Figure 1). The disposition of the disulphides is identical to insulin and relaxin, making it a true member of the insulin super family.

The primary purpose of this study was to, for the first time, chemically synthesize rat INSL3 and test its ability to directly induce growth of the rat gubernaculum *in vitro*. Furthermore, as INSL3 shows high structural similarity to relaxin and it has a similar although displaced relaxin-like binding motif (Figure 1), its relaxin activity was tested in two characteristic relaxin bioassays.

MATERIALS AND METHODS

Solid Phase Synthesis

Both A- and B-chains were synthesized by the continuous flow Fmoc solid-phase method on a 0.2-mol scale as previously described [5]. Briefly, the A-chain assembly was carried out using an automated MilliGen 9050 synthesizer (Bedford, MA, USA) and Fmoc-His(Trt)-Novasyn PA 500 (Novabiochem, Läufelfingen, Switzerland) as a solid support. For the B-chain, PAC-PEG-PS resin (Applied BioSystems, Scoresby, Australia) was manually derivatized by anchoring the first residue, alanine, using the symmetrical anhydride in the presence of dimethylaminopyridine. Fmoc deprotection was with 20% piperidine in DMF. All subsequent residues were activated with HBTU and DIEA in DMF, with the exception of Ala^{27} and $His^{19,20}$ (B-chain) which were acylated using HOBt-catalysed Fmoc-amino acid pentafluorophenyl esters (Auspep, Melbourne, Australia). All couplings were of 30-min duration. Side-chain protection was afforded by *tert*-butyl esters and ethers for Asp, Glu, Thr and Ser, Trt for Cys, Gln and His, Boc for Lys and Pmc for Arg.

On completion of the syntheses, both the protected A-chain and B-chains were separately treated at room temperature for 2.5 h with 82.5% TFA/5% phenol/5% H_2O/5% thioanisole/2.5% ethanedithiol plus five drops of triethylsilane to aid the quenching of thiols. TFA was removed to a minimal volume under a stream of nitrogen and the residue was precipitated in two stages using chilled diethyl ether. The precipitates were then separately dissolved in 0.1% aq. TFA and freeze-dried.

Purification

The separate crude chains and the chain-combined peptide were purified by preparative reversed phase high-performance liquid chromatography (RP-HPLC), using a Waters 600 multisolvent delivery system connected to a model 996 photodiode array detector. A 10×250 mm Vydac 218 TP column packed with C18 silica gel (330 Å pore size, 10 μm particle size; Hesperia, USA) was used. The peptides were eluted with a solvent system of (A) 0.1% aq. TFA (v/v) and (B) 0.1% TFA in acetonitrile (v/v) in a linear gradient mode (20–50% B over 30 min). The target fractions were collected and identified by matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF MS) performed in the linear mode at 19.5 kv on a Bruker Biflex instrument (Bremen, Germany) equipped with delayed ion extraction and freeze-dried.

Chain Combination

Purified A- and B-chains (4:1 w/w) were each dissolved separately in a concentration of 1 and 0.27 mol/mL, respectively in 0.15 M 3-(cyclohexylamino) propanesulphonic acid (CAPS)/1 M guanidine hydrochloride buffer pH 10.5, which had been degassed and cooled to 4°C [5]. The two solutions were combined slowly in an open beaker and then stirred vigorously at 4°C. Aliquots were removed during the course of the combination and analysed by RP-HPLC, the reaction being complete as evidenced by the absence of B-chain. The solution was acidified

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to pH ≈ 2.5 with neat TFA and then subjected to RP-HPLC as described earlier to isolate the chain-combined product.

Peptide Characterization

Peptide quantitation was by mean of duplicate amino acid analyses of 24-h acid hydrolysates on a GBC automatic analyser (Melbourne, Australia). Analytical RP-HPLC was carried out on a Waters system using a Vydac 218TP C18 column (Hesperia, USA) using 0.1% TFA-based buffer systems described earlier.

Circular Dichroism (CD) Spectrosopy

For CD spectroscopy, the peptide was dissolved in doubly distilled water at a concentration of 0.42 mg/mL (approximately 40 nM), determined by quantitative RP-HPLC [6]. The spectra were taken on a Jasco J-720 instrument at room temperature in a 0.2-mm pathlength cell. The raw spectra were converted into mean residue ellipticity ($[\theta]_{MR}$) format, were smoothed, and compared with the published spectra of synthetic rat relaxin [7]. Because the secondary structures of the peptides provided by the current computer-assisted curve analysing algorithms show a high error rate, the CD spectra evaluations were based on comparison with known peptide conformations [8].

Relaxin Receptor Assays

The ability of rat INSL3 to bind to rat cortical relaxin receptors was tested in a relaxin radio-receptor assay. H2 relaxin (B33) was labelled with [^{33}P] using the catalytic subunit of cyclic AMP-dependent protein kinase as previously described [9]. Rat cerebral cortex was collected from killed Sprague–Dawley rats, immediately frozen in liquid nitrogen and used within 1 week for assay. The tissue was homogenized in 20 (v:w) ice-cold binding buffer (20 mM Hepes, pH = 7.5, 0.1 mg/mL lysine, 1.5 mM $CaCl_2$, 50 M NaCl, 0.01% NaN_3) and the membrane fractions isolated by centrifugation at 1000 × *g* for 15 min followed by centrifugation of the supernatant at 50000 × *g* for 60 min (Sorvall RC5C plus). The resulting pellet containing the membrane fraction was resuspended in binding buffer for assay. Competitive binding to relaxin receptors was performed as described in Parsell *et al.* [10] with the following modifications. Increasing concentrations of recombinant H2 relaxin (B29), native rat relaxin [7] and rat INSL3 in 50 μL of binding buffer plus 1% BSA, were mixed with 100 pM [^{33}P]-labelled H2 relaxin (B33) in 50 μL of binding buffer plus 1% BSA and 100 μL of membrane solution. Non-specific binding was determined by addition of 500 nM of H2 relaxin (B29). Reactions were incubated for 90 min at room temperature and then terminated by the addition of ice cold PBS and centrifugation at 15000 rpm/20000 × *g* for 5 min in an Eppendorf centrifuge. The pellets were washed once with 1 mL of PBS followed by centrifugation and then resuspended in 500 μL of 1 M NaOH. They were then transferred to scintillation vials, mixed with 3 mL of liquid scintillation cocktail (Ultima Gold, Packard, Australia) and counted on a liquid scintillation analyser (Packard 1900 TR). Data are expressed as percent specific binding ± SEM of triplicate determinations from two independent experiments and are plotted using the single site competition function in PRISM (Graphpad Inc., San Diego, USA). Binding affinities (K_i) of H2 (B29), rat relaxin and rat INSL3 were calculated using the built-in Cheng and Prusoff [11] equation.

Relaxin Bioassay

Rat INSL3 was assayed for its ability to induce cAMP production in a relaxin expressing cell line (THP-1) following the procedure of Parsell *et al.* [10] with the following modifications. THP-1 cells which had been viability tested using Trypan Blue were resuspended in media and transferred to a 96-well plate at a density of 40000 cells/well. Peptides were added to the wells together with 1 μM forskolin and 50 μM isobutylmethylxanthine (IBMX) in media and incubated at 37°C for 30 min. The plate was then briefly centrifuged, the media were removed and the cells resuspended in lysis buffer for cAMP measurements using the cAMP Biotrak EIA system (Amersham Corp., USA). Repeat experiments showed that maximum stimulation of cAMP was achieved with 2.5 nM H2 relaxin (data not shown) and this concentration was therefore used alone or in combination with rat INSL3 to test the ability of rat INSL3 to augment or antagonize relaxin action.

Whole Organ Culture of Foetal Rat Gubernaculum

Gubernacula were harvested from male day 17 rat foetuses under sterile conditions and the gubernacular bulb immediately placed on agar in an organ culture dish. Gubernacula were cultured separately for 2 days under standard sterile conditions in either media alone or media supplemented with rat INSL3 (10^{-7} M), or separate rat INSL3 A and B

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chains (10^{-7} M) as a negative control. The culture medium used was Iscoves Modified Dulbecco's Medium (Gibco, Grand Island, NY) supplemented with 100 μg/mL streptomycin, 100 IU/mL penicillin, 1 mg/mL L-glutamine, 10% foetal calf serum, 10 μg/mL lipids, 10 μg/mL non-essential amino acids, 10 μg/mL nucleosides, 0.33 μg/mL transferrin and 10 μg/mL insulin. At the end of the culture period they were processed, blocked in paraffin and 5 μm sections cut for analysis. Sections of the gubernacular bulb were stained with haematoxylin and eosin and the area quantitated by projecting the section visualized under the microscope (100× magnification) and measuring it with a Leica (Deerfield, IL) calibrated slide and a Sigmaplot Digitizer (Jandel Scientific, Corte Madera, CA).

RESULTS AND DISCUSSION

The preparation of two-chain, multi-disulphide bond peptide members of the insulin superfamily present a special challenge in solid phase synthesis. Three general approaches are available for the synthesis of such peptides [12], *viz*. (i) separate assembly of the two chains followed by their combination and simultaneous disulphide generation in solution; (ii) regioselective disulphide bond formation of selectively *S*-protected peptides; and (iii) folding and oxidation of single-chain prohormone intermediates followed by excision of the connecting *C*-peptide linker between the A- and B-chains. Each has its advantages and disadvantages but our experience has been that native peptide chain combination generally proceeds well and in acceptable yields. This is not usually the case when preparing analogues that have significantly modified secondary structures. For the preparation of rat INSL3, we chose to use this simple approach; that of separate assemblies of the A- and B-chains followed by their combination in solution at high pH. This approach was recently successfully employed to produce ovine INSL3 [5]. No difficulties were experienced with either the assembly or cleavage of the separate rat INSL3 chains. However, modest recovery of each of the peptides after RP-HPLC resulted in overall yields of 8.4% for the A-chain and 8.9% for the B-chain, based on crude cleaved peptides. Confirmation of the high purity of the two synthetic chains was by chemical characterization by analytical RP-HPLC (data not shown) and MALDI-MS (A-chain: theory, 2741; found, 2741.98; B-chain: theory: 3375.89, found, 3376).

The combination of the two chains was monitored by analytical RP-HPLC and found to be complete after 21 h. The principal by-products were oxidized monomeric A- and B-chains but these could be collected and re-reduced and used in further combination experiments. RP-HPLC purification yielded the synthetic INSL3 in overall recovery of approximately 5% based on starting B-chain. Analytical RP-HPLC showed a single product (data not shown) with a MH^+ value of 6114.06 (calc. MH^+ 6113.87) by MALDI-TOF MS (Figure 2).

To examine further the structure of the synthetic rat INSL3 peptide and to compare its conformation with that of rat relaxin, ovine INSL3 as well as various other relaxin family members, CD spectra were collected in water (Figure 3). The rat INSL3 peptide exhibited a positive CD band at 190 nm ($[\theta]_{MR} = 21000$), a negative band at 207 nm ($[\theta]_{MR} = 14000$), and a negative shoulder around 222 nm. These spectral features corresponded to mixtures of β-turns and α-helices, and were remarkably similar to those of the ovine INSL3 variant [5] and to a series of relaxin analogues. While no spectral differences to the ovine peptide were detected, the rat INSL3 bands were slightly blueshifted compared with those representing rat relaxin [7], indicating that the helical conformation of relaxin is somewhat stabilized compared to INSL3. The calculated α-helicity content for rat INSL3 based on the ellipticity value at 208 nm [13] reached as high as 30–35%, a level still significant considering the purely aqueous conditions and the size of this peptide. Thus, taken together, CD spectroscopy confirmed the proper folding of the synthetic rat INSL3 peptide, and its structural similarity, but not identical homology, to other peptides in the relaxin family.

The ability of rat INSL3 to bind to rat relaxin receptors was tested using rat cerebral cortex cell membranes and [^{33}P]-labelled H2 relaxin. Competition binding to rat cortex (Figure 4) was best fitted by a one-site competition function for rat and H2

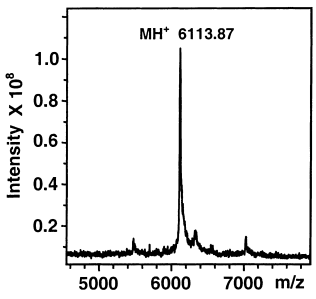

Figure 2 MALDI-TOF MS of purified synthetic rat INSL3.

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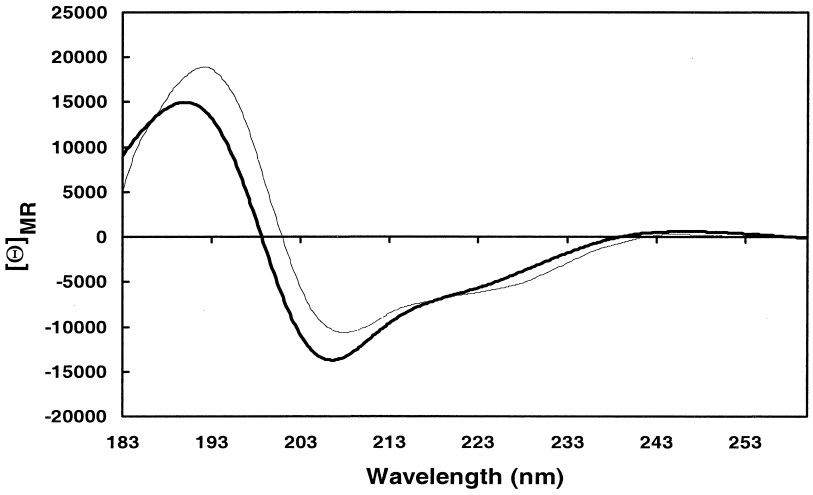

Figure 3 CD spectra of synthetic rat INSL3 (–) and native rat relaxin (—) in water

relaxin as previously described [9]. Unlabelled H2 relaxin (B29) displaced [^{33}P]-H2 relaxin (B33) binding with high affinity ($K_i = 0.49$ nM), whereas native rat relaxin competed for the binding sites with slightly lower affinity ($K_i = 2.54$ nM). Rat INSL3 competed very poorly for the rat cortical binding site (estimated $K_i = 3.32$ μM) indicating its low cross-reactivity with the rat relaxin receptor. This is the first time that an INSL3 ligand has been tested for binding activity on a relaxin receptor from the same species. Previously, sheep INSL3 was shown to bind with very low affinity to the rat relaxin receptor [5] and synthetic human INSL3 was shown to compete poorly with ^{125}I-labelled porcine relaxin binding sites in cell membrane extracts of mouse uterus and brain [14,15]. In the same study specific, high affinity INSL3 binding sites were demonstrated in the mouse uterus and brain, which had a very low affinity for relaxin [14,15].

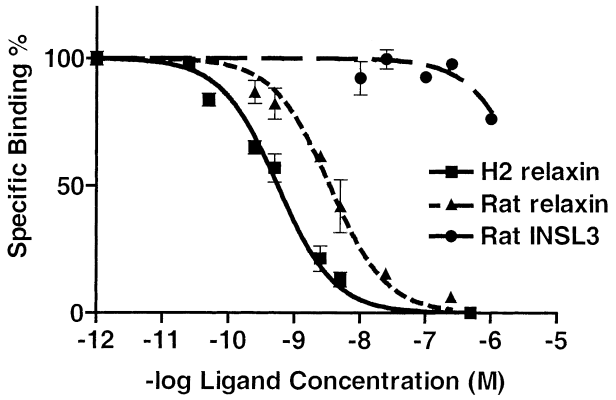

Figure 4 Competitive displacement curves of H2 relaxin, rat relaxin and rat INSL3 competing with [^{33}P]-labelled H2 (B33) relaxin for relaxin receptors in rat cortex crude membranes.

The ability of rat INSL3 to augment or antagonize relaxin activity was also tested in a relaxin expressing cell line (THP-1), which exhibits an increase in cAMP production in response to H2 relaxin [10]. As can be seen in Figure 5 treatment of the cells with 2.5 nM relaxin resulted in significant cAMP production whereas treatment with rat INSL3 alone at 100 or 500 nM did not stimulate cAMP production and furthermore did not augment or antagonize the response to 2.5 nM relaxin.

Similar results have been obtained previously demonstrating that sheep INSL3 at concentrations up to 1 μM also did not augment or antagonize relaxin activity in a rat atrial relaxin bioassay [5]. In contrast, previous experiments have suggested that human INSL3 will augment the action of relaxin on the mouse pubic symphysis [14]. As INSL3 will bind to relaxin receptors, albeit with very low affinity, it is therefore possible that it may have relaxin bioactivity at supraphysiological concentrations. The limited quantities of rat INSL3 peptide precluded its testing at higher concentrations in this study.

The ability of rat INSL3 to induce growth of the gubernacular bulb was tested in whole organ culture of foetal rat gubernaculum. Figure 6 shows haematoxylin and eosin stained sections of representative gubernacular bulbs, where the culture medium was supplemented with rat INSL3 (10^{-7} M) or with separate rat INSL3 A-and B-chains (10^{-7} M) as a control. There is a clear increase in the size of the gubernacular bulb in the sample supplemented

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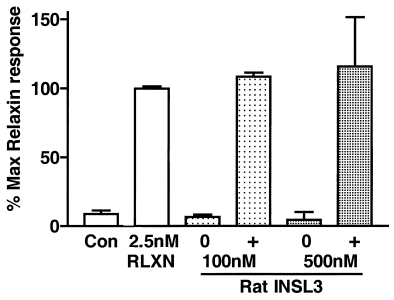

Figure 5 cAMP production from THP-1 cells in the absence of peptide (Con), in response to H2 relaxin (RLXN), rat INSL3 alone (0) and rat INSL3 in combination with 2.5 nM H2 relaxin (+). Data are expressed as percentages of the maximum cAMP response, which occur in response to 2.5 nM relaxin.

with INSL3. Experiments where medium was not supplemented with peptides showed no difference in size to those supplemented with separate rat INSL3 A- and B-chains (data not shown). These experiments, therefore, demonstrate that INSL3 directly influences growth of the gubernacular bulb. A recent study using foetal rat gubernaculum co-cultured with the testis from normal or *insl3* knockout mice has demonstrated that co-culture with normal testis results in growth of the gubernaculum whereas co-culture with *insl3* knockout testis resulted in much less growth [16]. This provides indirect evidence for an action of INSL3 on gubernacular growth. However the current study is the first to show a direct action of INSL3 on growth of the gubernacular bulb. Future experiments will involve clearly defining the role of INSL3 in gubernacular growth including the role of possible co-factors.

Although the native structure of the native INSL3 peptide is currently unknown, the current study is the first to show a biological action for an INSL3 peptide of any form. The ability of the synthetic rat INSL3 to influence growth of the foetal rat gubernaculum, together with the ability of synthetic human INSL3 to bind in the nanomolar range to mouse tissues [14,15], is strong evidence that the native structure of the INSL3 peptide is likely to be in a processed A/B heterodimer form.

CONCLUSIONS

The two-chain, three disulphide-bonded peptide, rat INSL3, was chemically synthesized successfully and shown to possess high homogeneity by a number of criteria including MALDI-TOF MS. Despite its close similarity to another member of the insulin superfamily, relaxin, it was shown to be devoid of characteristic relaxin-like activity. Experiments using whole organ cultures of foetal rat gubernaculum *in vitro* show that rat INSL3 will induce growth of this structure. This is the first demonstration of a direct action of INSL3 on gubernacular growth as well as of a biological activity for a synthetic INSL3 peptide. Synthetic rat INSL3 will be a critical tool for determining the mechanism of action of INSL3 in gubernacular growth.

Acknowledgements

Work carried out at the Howard Florey Institute was supported by an Institute Block grant (reg key #983001) from the NHMRC. We thank Istvan Varga (Wistar Institute) for the CD spectroscopy.

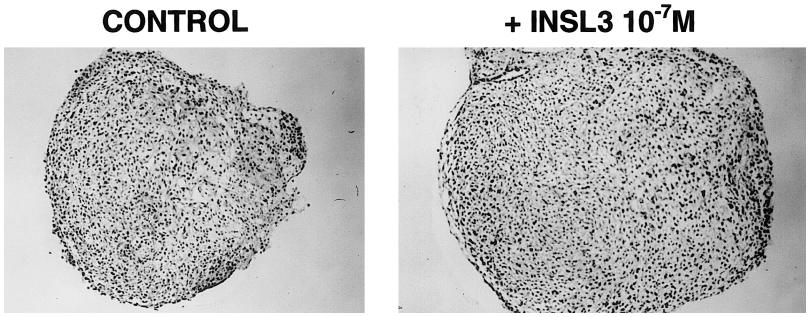

Figure 6 Representative cultures of foetal rat gubernaculum showing increased growth of the gubernacular bulb only in the culture supplemented with rat INSL3 (10^{-7} M). Culturing with INSL3 A- and B-chains alone (control) did not result in growth. Magnification 100 × for both.

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