Journal of Peptide Science
J. Peptide Sci. **10**: 257–264 (2004)
Published online 10 November 2003 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.521

JOURNAL OF PEPTIDE SCIENCE

Synthetic Human Insulin 4 Does Not Activate the G-protein-coupled Receptors LGR7 or LGR8

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Received 8 May 2003
Accepted 12 June 2003

Abstract: In contrast to the cellular receptors for insulin and insulin-like growth factors that are known to be protein tyrosine kinases, those of both insulin 3 and relaxin have recently been identified as being members of the leucine-rich repeat-containing G-protein coupled receptor (LGR) family, LGR8 and LGR7, respectively. This has prompted an examination into the possibility that they might also be specific for another member of the insulin superfamily, namely, insulin 4. Towards this aim, a two-chain peptide corresponding to the predicted primary structure of insulin 4 was prepared by solid phase synthesis. As conventional aeration and combination of the two S-reduced chains in solution at high pH failed to produce target product, selectively S-protected A- and B-chains were prepared followed by stepwise, individual formation of each of the three disulfides, one intramolecular within the A-chain and two intermolecular. Chemical characterization confirmed the purity and identity of the synthetic insulin 4 analogue. However, secondary structural analysis indicated that the peptide was devoid of tertiary conformation suggesting that the native peptide may well be either significantly longer in length or is similar to insulin-like growth factor I or II in that it is a single chain product. Screening of the synthetic analogue for activation of transfected cells bearing LGR7, and LGR7 splice variant or LGR8 failed to identify a specific interaction. Thus, the *in vivo* structural identity of insulin 4 and its receptor (if any) as well as its potential function remains unknown.

Keywords: insulin 4; leucine-rich G-protein coupled receptor; solid phase synthesis; transfected cells

INTRODUCTION

The determination 30 years ago of the primary structure of the ovarian hormone, relaxin, and the finding that it consists of a two-chain peptide bearing three disulfide bonds in the exact disposition as in insulin established the concept of the insulin superfamily [1]. A further two members of the family were identified soon afterwards and named insulin-like growth factors (IGFs) I and II [2]. These were followed by insulin 3 (also known as Leydig cell insulin-like peptide or relaxin-like factor) and insulin 4 (placentin, early pregnancy insulin-like peptide) [3,4]. More recently, the existence of two further insulin-like peptides, insulin 5 and 6, has been predicted from cDNA sequences [5,6]. Finally, a novel relaxin sequence, relaxin 3, was discovered from the Celera

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Contract/grant sponsor: Howard Florey Institute; Contract/grant number: 983001.

genome database [7]. Subsequent analysis of both the Celera and public domain genome databases failed to reveal the presence of additional insulin-like peptides [8] thus showing that membership of the human insulin superfamily is restricted to ten.

The *insulin 4* gene was recently identified by screening a human cytotrophoblast-subtracted cDNA library [4]. It is highly tissue specific and is expressed predominantly in the placenta and most strongly in the first trimester of pregnancy [9,10]. *Insulin 4* encodes a 139-residue peptide that was originally named early placenta insulin-like peptide (EPIL, placentin) but is now more commonly called insulin 4 (INSL4). The predicted peptide structure bears the characteristics of a preprohormone containing a signal peptide, B-chain, C-peptide and A-chain. It shows greatest homology to human Genes 1 (H1) and 2 (H2) relaxin (44% and 43%, respectively) compared with insulin 6 (15% homology) suggesting that INSL4 likely arose from duplication of the common relaxin gene. In fact, recent data have demonstrated that no INSL4 equivalents exist in the rodent genome [8] and INSL4 and H1 relaxin were the results of a gene duplication that has only occurred in higher primates [8,11]. Use of an immunoassay specific for the C-peptide and A-chain indicated that INSL4 forms were present in both amniotic fluid and maternal serum of pregnant women [12]. This suggests that the peptide may play a crucial role in embryonic and fetal development. However, two peptides corresponding to a predicted mature insulin-like structure consisting of a 25 residue A-chain and either a 36 or 41-residue B-chain were chemically synthesized and shown to be devoid of secondary structure suggesting that INSL4 might well possess an insulin-like growth factor-like single-chain structure instead [13].

Elucidation of a definitive biological role for INSL4 would be facilitated by isolation and localization of its receptor. Although the actions of both insulin and insulin-like growth factor I and II are well known to be mediated via a protein tyrosine kinase receptor [14,15], those of relaxin and insulin 3 (INSL3) are, surprisingly, mediated via two members of the leucine-rich repeat-containing G-protein coupled receptor family (LGRs), LGR 7 and 8 respectively [16,17]. Given the similarity of INSL4 to both relaxin and INSL3, and the recent evolution of the *insulin 4* gene, it is plausible that INSL4 may also be a ligand of LGR7 or LGR8, or a LGR7 splice variant. Therefore, a regioselective disulfide chemical synthesis of INSL4 was undertaken and it was screened for its ability to bind to, and activate, cells that express these receptors.

MATERIALS AND METHODS

Peptide Synthesis

Both the selectively S-protected A- and B-chains were chemically prepared by manual Boc-solid phase synthesis using a custom-made wrist shaker instrument. The scale of assembly was 0.20 mmol and the synthetic schedule was as previously reported [18]. Briefly, Boc-amino acids (4 equiv.) were pre-activated for 5 min with 2-(1H-benzotrazole-1-yl)-1,1,3,3-tetramethylronium hexaflurophosphate (HBTU) (4 equiv.) and DIEA (6 equiv.) in DMF prior to addition to the reaction vessel and agitation for 10 min. Each acylation was monitored by the quantitative ninhydrin test [19] and, if necessary, a repeat acylation was performed. A-chain assembly was carried out on Boc-Thr(OBzl)-PAM-polystyrene (loading 0.68 mmol/g, ABI) and the B-chain on Boc-Leu-PAM-polystyrene (loading 0.75 mmol/g, ABI). The side chain protecting groups used were as follows: Arg, tosyl; Asp and Glu, *O*-cyclohexyl; His, benzyloxymethyl; Lys, 2-chlorocarbobenzoxy; Ser and Thr, benzyl; Trp, For and Tyr, 2-bromobenzyloxycarbonyl. Selective S-protection was afforded as follows: A10 and 15, 4-methylbenzyl; A11, acetamidomethyl; A24, *tert*-butyl; B6, acetamidomethyl; B18, 4-methylbenzyl.

Cleavage and Purification

Protected A- and B-chain resins were each treated with 90% hydrogen fluoride in the presence of 5% *p*-cresol at 0 °C for 1.5 h. The HF was evaporated under vacuum and the resulting sludges washed thrice with diethyl ether and the resins then extracted twice with 0.1% aqueous TFA containing 20% CH_3CN. Crude peptides were purified using a Waters HPLC system using a Vydac C_{18} reverse-phase column (10 × 250 mm 218TP), with a solvent system of 0.1% aqueous TFA (buffer A) and 0.1%TFA in acetonitrile (buffer B) in linear gradient mode. Fractions were collected and lyophilized.

Regioselective Disulfide Bond Formation

Two major peaks were obtained from the purification of the crude A-chain. Mass spectroscopic identification showed these to be target peptide, [$Cys^{10,15}$

S-thiol, Cys^{11}(Acm), Cys^{24}(*t*Bu)], and the other to be peptide missing Cys^{24}(*t*Bu) protection, [$Cys^{10,15,24}$ S-thiol, Cys^{11}(Acm)].

A-chain Intramolecular Disulfide Oxidation

Purified [$Cys^{10,15,24}$ S-thiol, Cys^{11}(Acm)] A-chain (42.1 mg, 14.7 μmol) was dissolved in 0.1M Gly·NaOH, pH 8.5, (2.81 ml) and to this was added 1 mM 2-dipyridyl disulfide (DPDS) in MeOH (45 ml, 45 μmol) [20]. Oxidation was complete after 2 h as monitored by analytical RP-HPLC. The solution was acidified by addition of neat TFA and then the peptide was isolated by preparative RP-HPLC and subsequently freeze dried to give 22.0 mg (7.4 μmol) of purified [Cys^{11}(Acm), Cys(Pyr)]-A-chain.

Combination of (Cys^{11}(Acm), Cys^{24}(Pyr))A-chain with (Cys^{6}(Acm), Cys^{18}(S-thiol))B-chain

A-chain peptide (16.81 mg, 5.7 μmol) was dissolved in 50 mM NH_4HCO_3 (17 ml) and added to B-chain (12.4 mg, 3.3 μmol) in H_2O (6 ml). The mixture was stirred vigorously at room temperature and the reaction was monitored by analytical RP-HPLC. After 1 h, the reaction was terminated by addition of neat TFA, and the target product was isolated by preparative RP-HPLC to give 6.3 mg (1.0 μmol, 29.1%).

Insulin 4

The [Cys^{11}(Acm)]A-chain/[Cys^{6}(Acm)]B-chain (8.3 mg, 1.3 μmol) was dissolved in glacial acetic acid (9.3 ml) and to this was added 3.7 ml of 20 mM iodine/acetic acid (75.4 μmol) and 0.7 ml of 60 mM HCl. After 1 h, the reaction was stopped by addition of 3.8 ml of 20 mM ascorbic acid and the reaction mixture diluted with 120 ml of H_2O and freeze dried. Preparative RP-HPLC, as described above, was then used to isolate and purify the product (1.3 mg, 0.2 μmol, 4.7% overall).

Characterization

The purity of the peptides was analysed using MALDITOF MS, performed in the linear mode on a Bruker BIFLEX instrument (Bremen, Germany), and by both analytical RP-HPLC and capillary zone electrophoresis. Peptide quantitation was performed by amino acid analysis on a GBC automatic amino acid analyser (Melbourne, Australia). For tryptic mapping, about 1 μg peptide was enzymatically digested for 4 h in 10 μl 10 mM NH_4HCO_3 at 37 °C using a trypsin : substrate ratio of 1 : 50 (w/w). Prior to mass analysis, 0.5 μl of digest solution was mixed 'on target' with 0.5 μl of matrix (saturated α-cyanocinnamic acid in 30% aqueous acetonitrile) and left to dry.

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy was performed using a Jasco J-720 spectropolarimeter and a 0.2 mm path length cell. The peptide was dissolved in water (0.5 mg/ml, approximately 80 nM) and spectra were taken in the 180 to 260 nm wavelength range. Curves were smoothed by an algorithm provided by Jasco. Mean residue ellipticity ($[\theta]_{MR}$) was expressed in degrees·cm^2/dmole by using a mean residue mass of 110 Da. Because the secondary structures of the peptides provided by the current computer-assisted curve analysing algorithms show a high error rate, evaluations of the CD spectra were based on comparison with known peptide conformations [21].

Transfection of Cells and cAMP Assays

Human 293T cells derived from human embryonic kidney fibroblasts were maintained and transfected with LGR7, LGR7 short or LGR8 expression plasmids as previously described [22]. Cells were preincubated in the presence of 0.25 mM 3-isobutyl-1-methyl xanthine (IBMX, Sigma) before various treatments for 16 h. At the end of the incubation cells and media were collected for measurement of cAMP using a well characterized radioimmunoassay [23]. All experiments were repeated at least three times using cells from independent transfections. The results were plotted as pmol cAMP/ml/5×10^5 cells.

Ligand Binding Analysis

The method for binding of ^{33}P-labelled relaxin to 293T cells transiently transfected with either LGR7 or LGR8 has been described previously [22]. Data are expressed as mean ± SEM of the % specific binding of triplicate determinations performed on at least three independent plates of transfected cells. Data were plotted using the one-site competition functions of the PRISM program (Graphpad Inc., San Diego, USA).

RESULTS AND DISCUSSION

The structure of the native INSL4 peptide (if it exists *in vivo*) is unknown. Hence the sequence of the synthetic INSL4 peptide was predicted from the cDNA sequence. The derived preprohormone sequence contains a typical signal sequence after the ATG start codon that is predicted to be cleaved between Ala^{25} and Ala^{26} [24]. The C/A chain junction contains a furin consensus sequence (Arg-Lys-Lys-Arg) [25] strongly indicating cleavage should occur at this point and hence the A-chain sequence begins from the Ser^{115} residue. However, there are no typical enzymic cleavage signals at the B/C chain junction hence the length of the B chain has been predicted based on the length of other relaxin family members. Consequently the synthetic INSL4 peptide comprised a B-chain of 33 amino acids and an A-chain of 25 amino acids (Figure 1).

Other insulin-like peptides contain atypical B/C junction cleavage sites. The native bovine INSL3 peptide also contains a furin sequence at the C/A junction and has an atypical cleavage signal at the B/C junction [26]. Some relaxin peptides also contain atypical cleavage sites at the B/C junction but are still cleaved *in vivo* [27]. Mock *et al.* [12] demonstrated immunoreactive INSL4 protein in human plasma using a sandwich ELISA with antibodies directed against the C- and A-chains. Although this would suggest that the native INSL4 peptide is unprocessed, the identity of the immunoreactive protein was not confirmed. Other data suggest that the INSL4 peptide should be cleaved *in vivo*. INSL4 transfected human kidney 293 cells produce an immunoreactive 6 kDa peptide together with pro-hormone [9]. As INSL4 is produced by the placenta that also produces mature relaxin peptides *in vivo* [28], it is therefore likely that the INSL4 peptide is also processed *in vivo*.

Previous efforts to synthesize chemically two-chain insulin-like peptides such as relaxin and INSL3 have been achieved successfully by the simplest of experimental approaches, that of random combination of the individual A- and B-chains in solution at high pH [29,30]. The overall

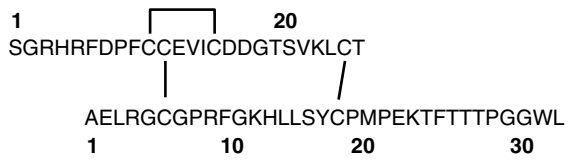

Figure 1 Predicted primary structure of human INSL4.

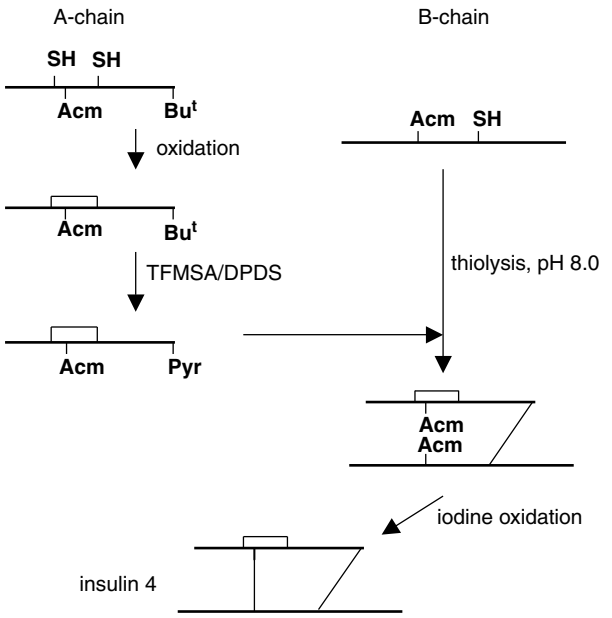

Figure 2 Scheme for regioselective disulfide bond formation of INSL4 following solid phase synthesis of individual A- and B-chains. Abbreviations: Acm, acetamidomethyl; Bu*t*, *tert*-butyl; DPDS, 2-dipyridyl disulfide; Pyr; 2-pyridinesulfenyl; TFMSA, trifluoromethanesulfonic acid.

recovery of peptide generally can be as high as 10% relative to the starting B-chain using these methods. However, similar approaches to prepare INSL4 were surprisingly unsuccessful with the primary products being polymers of each of the two individual chains. For this reason, the chemical synthesis of the predicted sequence of the INSL4 was undertaken using regioselective disulfide bond formation methods that have been successfully employed for other insulin-like peptides including insulin itself [31,32]. By careful selection of appropriate specific cysteine S-thiol protecting groups that can be removed in an orthogonal manner, it is possible to form each of the three disulfide bonds sequentially and in good yield. There are now several different permutations of protecting groups and disulfide bond formation reactions but a variation (Figure 2) was chosen of the protocol of Maruyama *et al.* that was employed for the synthesis of the insect brain hormone bombyxin [33]. Each of the two selectively S-protected peptide chains was readily prepared by routine Boc-solid phase peptide synthesis. However, two major products were obtained following HF cleavage of the selectively S-protected A-chain with MALDITOF mass spectral analysis identifying one as being the target product and the other having

the S-*t*Bu protecting group missing from the *C*-terminal cysteine residue. This finding, surprising given that the S-But group is stable to HF, suggested that the HF cleavage temperature was erroneously allowed to rise from the recommended 4 °C with a corresponding loss of S-protection. In the course of subsequent preliminary investigation including tryptic mapping (data not shown), it was observed that aqueous DPDS-mediated oxidation [20] led, remarkably, to efficient formation of the intramolecular disulfide bond and simultaneous selective S-pyridinylation of the *C*-terminal cysteine resulting in a product that was suitable for direct combination with the corresponding B-chain via thiolysis. Final intermolecular disulfide bond formation was achieved by iodinolysis of the S-Acm groups. Overall yield of synthetic INSL4 was approximately 4.7% relative to the starting B-chain. Comprehensive chemical characterization confirmed the high purity of the synthetic product. Analytical RP-HPLC produced a single eluting peak (Figure 3) and MALDITOF-MS showed a single species with MH^+ 6439.3 (theory: MH^+ 6436.5).

The preferred conformation of the INSL4 analogue was estimated by CD spectroscopy. In water, the peptide exhibited a single negative band at 203 nm, and spectral features of type C spectra characteristic for type I (III) beta turns (Figure 4) [34]. The slight redshift of the band in 2% octyl-beta-D-glucoside solution indicated the potential to assume a more ordered structure in a membrane-like environment. This structure stabilization was

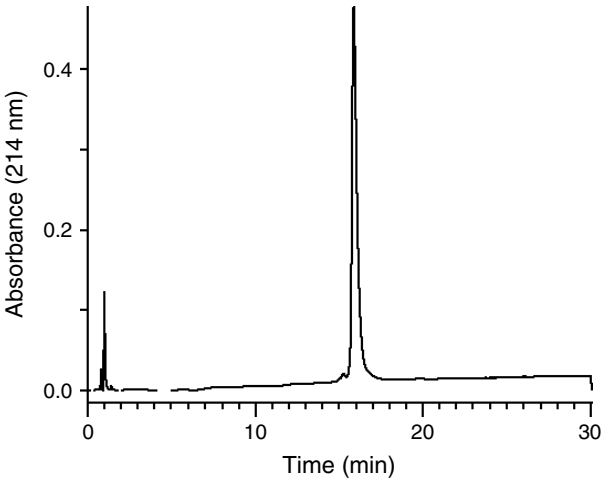

Figure 3 Analytical RP-HPLC on a Waters C18 column (3.9 × 150 mm). Buffer A: 0.1% aqueous TFA; Buffer B: 0.1% TFA in CH_3CN. Gradient: 20%–35%B in 30 min. Flow rate: 1.5 ml/min.

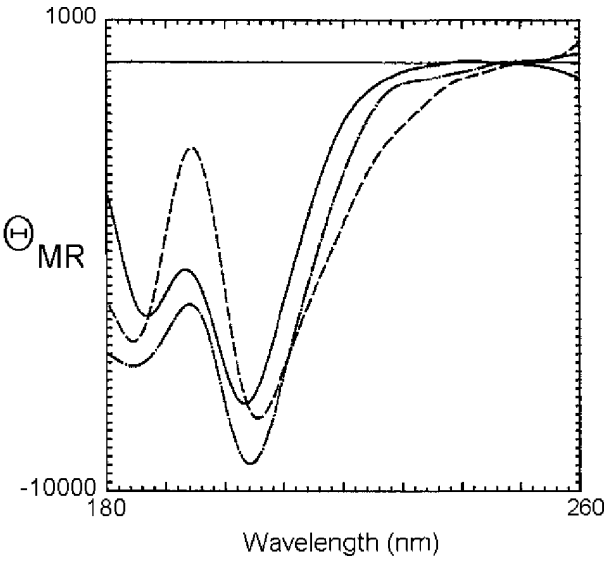

Figure 4 Circular dichroism spectra of synthetic INSL4 recorded in water (solid line), 2% aqueous octyl-β-D-glucoside (OG) (dots and dashes), and 50% aqueous trifluoroethanol (TFE) (dashes) solutions.

more prominent in 25% aqueous trifluoroethanol with the negative band further redshifted to 206 nm and the appearance of negative shoulders at higher wavelengths. However, the dominant conformation remained a series of reverse-turns even in 25% trifluoroethanol. This lack of structure is in great contrast to other members of the insulin superfamily including relaxin [18] and insulin 3 [30] and provides an explanation for the early inability to produce INSL4 by simple combination of the individual A- and B-chains in solution. Successful combination of other members of the insulin superfamily, namely relaxin and insulin, occurs via a hierarchic process along a redox-sensitive series of energy landscapes that is crucially dependent on the formation of structured chain intermediates [35,36].

Leucine-rich repeat-containing G-protein coupled receptors (LGR) are a subfamily of the rhodopsin-like G protein coupled receptor (GPCR) family, which currently includes eight separate receptors split into three sub-groups. Group one contains the glycoprotein hormone receptors (LHR, TSHR and FSHR) [37]. The second group incorporates the orphan receptors LGR4, LGR5 [38] and LGR6 [39] and the third group comprises LGR7 and LGR8, the receptors for relaxin [16] and INSL3 [17], respectively. These receptors were grouped together based on their large extracellular 'ectodomains' that

contain a characteristic leucine-rich repeat domain important for glycoprotein hormone binding [37]. A human LGR7 alternate splicing variant (LGR7 short) was also identified in the initial process of LGR7 gene identification [16]. The transcript encoding this variant is missing 34 amino acids from the NH_2-terminal region of its ectodomain. Although this receptor will couple to cAMP activation, it is not activated by relaxin [16], suggesting that it could be another orphan LGR. As the *insulin 4* gene is a result of a recent gene duplication and is only found in higher primates, it was postulated that it may be a ligand of LGR7, LGR7 short or LGR8. It is unlikely to be a ligand of the orphan receptors LGR4–6 as these receptors are also found in rodents [38,39] that lack an *insulin 4* gene.

LGR7, LGR7 short and LGR8 were tested for their ability to respond to synthetic INSL4 stimulation. The peptide did not induce cAMP production from LGR7 short transfected cells. This receptor is expressed on the cell surface as demonstrated by cell surface binding to an anti-FLAG antisera and will also couple to cAMP production [16,17]. However, as this receptor does not respond to relaxin or INSL3, and hence there is no positive control for cAMP accumulation, the data are not shown. As expected, LGR7 transfected cells responded to H2 relaxin stimulation and LGR8 transfected cells responded to INSL3 and H2 relaxin stimulation with cAMP release, however, neither receptor responded to INSL4 stimulation with cAMP release (Figure 5). It remains possible that INSL4 stimulation results in a different second messenger signal. Therefore synthetic INSL4 was tested for its ability to displace ^{33}P-labelled H2 relaxin binding from the LGR7 and LGR8 receptors (Figure 6). However, INSL4 demonstrated no ability to displace relaxin binding from LGR7 and LGR8. Hence it would seem that INSL4 is not a ligand of LGR7, LGR7 short or LGR8. It still remains possible that INSL4 binds to a different domain of these receptors resulting in alternate signal transduction. It is also possible, as discussed above, that INSL4 is produced *in vivo* as a pro-hormone and is only able to bind these

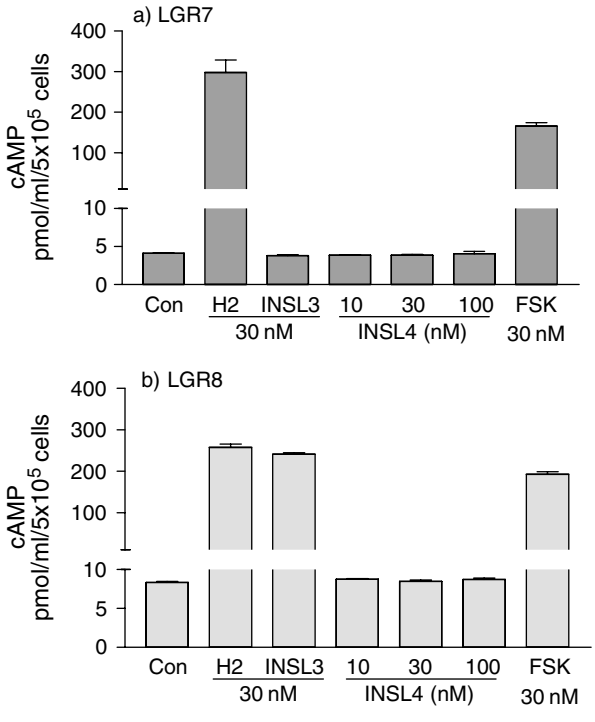

Figure 5 cAMP accumulation (pmol/ml/5 × 105 cells) in (a) LGR7 and (b) LGR8 transfected cells in response to various ligands. Data are mean ± SEM of at least three independent experiments performed in triplicate. Con: control; H2: human Gene 2 relaxin; INSL3: human insulin 3; INSL4: human insulin 4; FSK: forskolin.

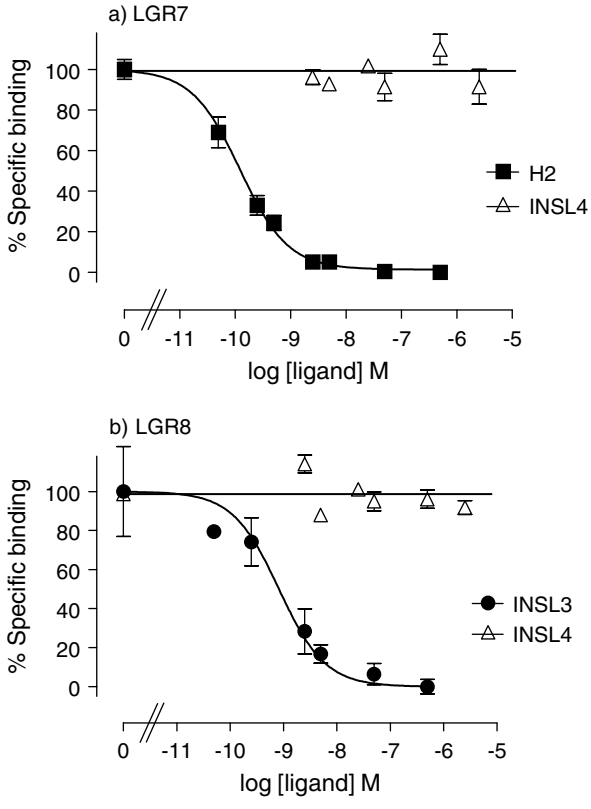

Figure 6 Competition experiments with human Gene 2 relaxin (H2), insulin 3 (INSL3) and insulin 4 (INSL4) displacing ^{33}P-labelled H2 relaxin from (a) LGR7 and (b) LGR8 transfected cells. Data are mean ± SEM of at least three independent experiments performed in triplicate.

receptors in this form. Experiments are underway in our laboratory to identify the molecular form of the peptide *in vivo* using an immobilized INSL4 antibody 'bait' and mass spectrometry analysis of the subsequent bound ligand. A more likely alternative is that the receptor for INSL4 is yet to be identified. Stimulation of placental cells with recombinant INSL4 results in tyrosine phosphorylation of a protein of a similar size to the insulin receptor ß subunit [9]. Although this protein was not the insulin receptor it is possible that it is another receptor tyrosine kinase which could potentially be the receptor for INSL4. Further work is presently underway to examine this possibility.

CONCLUSIONS

A predicted two-chain form of human INSL4, a hormone member of the insulin superfamily that is postulated to play a key role in embryonic and fetal development, was successfully chemically assembled in good overall yield by a combination of solid phase peptide synthesis and regioselective disulfide bond formation. The resulting product was shown to be devoid of significant secondary structure including in the structure-inducing solvent, TFE. The peptide did not bind to, nor activate, the G-protein coupled receptors, LGR7, its splice variant, LGR7 short, nor LGR8. Both the molecular composition of INSL4 and the nature of its receptor remain unidentified.

Acknowledgements

Work carried out at the Howard Florey Institute was supported by an Institute Block grant (reg key 983001) from the NHMRC. The authors wish to thank Sharon Layfield and Tania Ferraro (HFI) for assistance with cell transfections and binding assays. We also thank Dr Mare Cudic (Wistar Institute) for the CD spectroscopy. We gratefully acknowledge the interest and advice provided by Sheau Yu Hsu and Aaron J.W. Hseuh (Stanford University School of Medicine).

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