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Novel Strategy for the Synthesis of Template-assembled Analogues of Rat Relaxin[1]

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Abstract: The 'template-assembled synthetic protein' (TASP) concept provides a simple and elegant approach for the preparation of analogues that retain key structural elements. We have synthesized TASP molecules containing the putative active site of relaxin, a peptide that has similar structural features to insulin but a markedly different biological role. Two types of chemoselective thiol ligation strategies (thioether and thiazolidine) were used and compared. The synthetic pendant peptides contain an essential region for bioactivity that is located in the α-helical region of the relaxin B-chain. Depending on whether the thioether or the thiazolidine chemistry was used to attach the peptides to the template, the reacting amino acid was placed either at the *C*-terminus or *N*-terminus, respectively, thus allowing the choice of orientation relative to the carrier molecule. The template molecule consists of a decapeptide with two proline–glycine turns and four evenly spaced lysine residues that were functionalized with the appropriate chemical moiety. This allowed reaction with the appropriately derivatized peptides in solution. To improve the template ligation step using the thioether approach, a pendant peptide *C*-terminal cysteamine residue was used to reduce potential steric hindrance during conjugation. The design of the peptides as well as the synthetic strategy resulted in the acquisition of mimetics showing weak non-competitive and weak competitive antagonist properties. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antagonist; chemoselective ligation; rat isolated atrial bioassay; rat relaxin; template-assembled peptide

INTRODUCTION

Relaxin is a two-chain peptide member of the insulin family that is produced principally by the ovaries during pregnancy and has a primary role in facilitating parturition in many mammalian species [1]. All relaxins share several common features such as size (6 kDa), chain structure and the insulin cystine cross-linking pattern. Interestingly, up to 60% sequence variation exists between species. The only invariant residues are the six cysteines, the glycine residues adjacent to three of these, and the two B-chain arginine residues. According to both the crystal structure of human gene 2 relaxin [2] and previous

Abbreviations: Boc, *tert*-butoxycarbonyl; BrAc, bromoacetyl; DIEA, diisopropylethylamine; calc, calculated; DMF, dimethylformamide; EDTA, ethylenediaminetetraacetic acid; Fmoc, 9-fluorenylmethoxycarbonyl; Gua.HCl, guanidine hydrochloride; HATU, *O*-(7-azabenzotriazol-1-yl)–*N*,*N*,*N*′,*N*′-tetraethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PAM, 4-hydroxymethylphenylacetamidomethyl; PEG, polyethyleneglycol; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; PS, polystyrene; RP-HPLC, reversed phase high-pressure liquid chromatography; SPPS, solid phase peptide synthesis; TFA, trifluoroacetic acid; TIS, triisopropylsilane.

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structure–function studies, these two latter residues which are located in the α-helical region of the B-chain, interact selectively with the relaxin receptor [3]. The recent identification of specific relaxin binding sites in the heart and brain of both male and female rats suggests a wider physiological role for the hormone [4–6].

In the field of protein design it is now known that peptide domains can provide the basis for the development of shortened, conformationally restrained mimetics that possess either antagonist or agonist effects [7]. One approach for the preparation of peptide motifs adopting secondary and tertiary structures is the 'template-assembled synthetic protein' (TASP) concept. The peptidic template serves as a device for enforcing the intramolecular folding of covalently attached amphipathic peptides to a monomeric, compact and branched structure [8,9]. Our aim was to prepare relaxin mimetics based on this approach using the most conserved and important region for the binding of the hormone to the receptor, and to produce analogues in which the pendant chains have one of two different orientations. We developed a synthetic strategy to readily produce TASP molecules containing partial relaxin sequences based on chemoselective ligation strategies to bind the peptides to the template [11,16]. The thioether linkage approach was used to bind the peptides via their *C*-terminal end and the thiazolidine linkage approach to bind the peptides via their *N*-terminus. Linkages of the former type have been used successfully to generate a number of cyclic peptide analogues as well as linear and branched oligomers [10]. In our latter approach, the reaction of a 1,2-aminothiol, such as a cysteine residue (located at the *N*-terminus of the peptide) together with a carbonyl group, giving the thiazolidine linkage, is a method which has been successfully employed to synthesize glycoproteins and branched structures such as 'multiple antigenic peptides' (MAPs) [11]. For our purpose, the only requirement for both approaches was for the pendant peptide to have either a *C*-terminal or *N*-terminal cysteine amino acid, depending on which ligation method is employed. Here, we present the synthetic approach to produce two different analogues and report their respective effects in competitive binding assays together with human relaxin in the rat atrial bioassay [12].

MATERIAL AND METHODS

Peptide Synthesis

Synthesis of peptides 1 and 4: H-G R G Y A R A L I E V C-OH (1) and H-C G R G Y A R A-OH (4). Both peptides **1** and **4** were synthesized automatically via continuous flow methodology on a MilliGen 9050 synthesizer using the 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase method. The solid support used was PEG-PS resin containing the first residue (PerSeptive Biosystems, USA). Amino acid acylation was carried out with HOBt-catalysed Fmoc-amino acid *O*-pentafluorophenyl esters (Auspep, Melbourne, Australia) (4 equivalents), except the arginine residues which were activated with HATU (3 equivalents) and diisopropylethylamine (DIEA) (6 equivalents) in dimethylformamide (DMF). All the coupling steps were of 30-min duration. The cleavage and deprotection of both peptides was carried out by treating the peptides for 2 h with trifluoroacetic acid (TFA)–TIS–H_2O (95:2.5:2.5). After removal of the TFA under a steam of nitrogen, the free peptides were taken up into 20% aqueous acetic acid and extracted twice with diethyl ether. The combined acid solution, diluted to 5% acetic acid with doubly distilled water, and the peptides were lyophilized to remove the acetic acid and any remaining scavengers. Crude peptide **1** was purified by preparative reversed phase high-pressure liquid chromatography (RP-HPLC) on a Vydac C_4 column (15% A–65% buffer B over 30 min at 10 ml/min) and characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (measured MW 1265.4; calc 1264.3). Buffer A was 0.1% aqueous TFA and B was 0.1% TFA in acetonitrile. Peptide **4** was purified by preparative RP-HPLC on a Vydac C_{18} column (0–60% buffer B over 30 min at 10 ml/min) and characterized by MALDI-TOF MS (measured MW 845.5; calc 844.5). Amino acid analysis of a 24 h acid hydrolysis gave the following ratio (expected/found): G (2/3.62); A (2/2.59); Y (1/0.79); R (2/1.82); C (n.d.).

Synthesis of peptide 2: H-G R G Y A R A L I E V-(NH-CH_2-CH_2-SH). Peptide **2** was synthesized manually on a 4-hydroxymethylphenylacetamidomethyl (PAM)-resin (Auspep, Melbourne, Australia) functionalized with a thioether phenoxy acetic acid linker (kindly provided by Dr P. Alewood, 3D Center, Brisbane, Australia) by N^{α}-Boc chemistry solid phase peptide synthesis (SPPS), according to published procedures [13]. All couplings were monitored by the ninhydrin colour test. The peptide was cleaved and

simultaneously deprotected from the resin by treatment with 90% HF/10% *m*-cresol for 1 h at 0°C. The crude peptide was taken up in a 20% aqueous acetic acid solution, diluted to a 5% acetic acid solution with doubly distilled water and the remaining *m*-cresol was extracted by a diethyl ether wash. The solution containing the peptide was then lyophilized to remove the acetic acid. Peptide **2** was purified by preparative RP-HPLC on a Vydac C_4 column (15–65% buffer B over 30 min at 10 ml/min) and characterized by MALDI-TOF MS (measured MW 1265.4; calc 1264.3). Amino acid analysis of a 24 h acid hydrolysis gave the following ratio (expected/found): E (1/1.23); G (2/2.83); A (2/2.42); Y (1/0.75); R (2/1.81); V (1/0.88); I (1/0.77); L (1/0.85).

Synthesis of peptide 3: H-C G R G Y A R A L I E V-NH_2. Peptide **3** was synthesized automatically via continuous flow Fmoc solid-phase methodology, as described earlier for peptides **1** and **4** on a Perseptive Biosystems Pioneer synthesizer. The peptide was purified by semipreparative RP-HPLC on a Vydac C_4 column (5–80% buffer B over 30 min at 10 ml/min) and characterized by MALDI-TOF MS (measured MW 1307.2; calc 1306.6).

Synthesis of template peptide T_1 (5): H-K(BrAc) P G K(BrAc) A K(BrAc) P G K(BrAc) A-OH. The linear decapeptide was synthesized using N^{α}-Boc chemistry SPPS as described for **2** together with N^{ε}-Fmoc lysine side-chain protection on a Ala-OCH_2-PAM-resin (ABI, San Francisco, USA). Following the nine synthetic cycles, the N^{ε}-Fmoc protecting groups were removed by two 5-min treatments with 50% piperidine/DMF. The free ε-NH_2 groups were then bromoacetylated using bromoacetic acid/DIC coupling [14]. All couplings were monitored by the ninhydrin colour test. The peptide was cleaved and deprotected simultaneously from the resin by treatment with HF/10% *m*-cresol for 1 h at 0°C. The crude peptide was taken up in a 20% aqueous acetic acid solution, diluted to a 5% acetic acid solution with doubly distilled water and the remaining *m*-cresol was extracted by a diethyl ether wash. The solution containing the peptide was then freeze-dried to remove the acetic acid. Template peptide **5** was purified by preparative RP-HPLC on a Vydac C_4 column (5–50% buffer B over 30 min at 10 ml/min) and characterized by MALDI-TOF MS (measured MW 1453.03; calc 1452.30).

Synthesis of template peptide T_2 (6): H-K(S) P G K(S) A K(S) P G K(S) A-OH. The assembly of the linear decapeptide was as for peptide **5**. Following synthesis and removal of the N^{ε}-Fmoc protecting groups, Boc-Ser(Bzl)-OH was then coupled to the free ε-amino groups with benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) (3 equivalents) and DIEA (6 equivalents) in DMF. The peptide was cleaved and deprotected simultaneously from the resin and isolated as described above. Template peptide **6** was purified by preparative RP-HPLC on a Vydac C_{18} column (0–50% buffer B over 30 min at 10 ml/min) and characterized by MALDI-TOF MS (measured MW 1329.76; calc 1328.6).

Periodate oxidation of template peptide T_2 (7). The template peptide **6** (10 mg, 0.008 mmol) was dissolved in 1.5 ml of 0.2 M sodium acetate buffer (pH 5.6) and treated with 200 µl of 20 mM $NaIO_4$ at 25°C for 10 min. The reaction was terminated and the product purified by injecting the reaction mixture directly onto a preparative RP-HPLC with a Vydac C_{18} column (0–50% buffer B over 30 min at 10 ml/min) and characterized by MALDI-TOF MS (measured MW 1214.76; calc 1214.6).

Synthesis of TASP T_1 $(2)_4$. The conjugation of the peptide **2** (17.4 µmol, sixfold excess compared to the template peptide) via the *C*-terminal thiol group to the $[BrAc]_4$-template **5** (29 µmol) was carried out in 50 mM degassed Tris–HCl buffer pH 8.5 at 25°C. The solution was stirred under N_2 at room temperature and the reaction monitored by analytical RP-HPLC with a Vydac C_{18} column (10–90% buffer B over 30 min at 1.5 ml/min). After 2.5 h, the reaction was quenched by adding dithiothreitol (10 µmol) and the mixture then separated by size-exclusion chromatography using a TSK HW-40 fine column (25 × 290 mm). The column was eluted with 20% aqueous acetic acid at a flow rate of 1.2 ml/min. Absorbance was measured at 279 nm. Fractions containing the TASP molecule were pooled and diluted to a 5% acetic acid solution and freeze-dried. The products were characterized by MALDI-TOF MS (measured MW 6186.5; calc 6204.6) and by analytical RP-HPLC on a Vydac C_{18} column (10–90% buffer B over 30 min at 1.5 ml/min). Amino acid analysis of a 24 h acid hydrolysis gave the following ratio (expected/found): E (4/3.95); G (10/10.95); A (10/10.83); P (2/1.14); Y (4/2.99); R (8/8.37); V (4/3.42); I (4/4.12); L (4/4.22); K (4/3.39).

Synthesis of TASP T_2 $(4)_4$. A sixfold excess of peptide **4** (200 µl) (3 mM in 0.2 M sodium acetate buffer containing 0.01 M ethylenediaminetetraacetic acid (EDTA), pH 5.25) was mixed together with the

tetra-N$^{\alpha}$-glyoxylyl template **7** (100 μl) (1 mM in 0.2 M sodium acetate buffer containing 0.01 M EDTA, pH 5.25). The conjugation was carried out at room temperature and monitored by analytical RP-HPLC with a Vydac C_{18} column (5–50% buffer B over 30 min at 1.5 ml/min) over 24 h. The tetrameric product was then separated from non-further reacting trimer and excess peptide **4** by semipreparative RP-HPLC with a Vydac C_{18} column (10–45% buffer B over 30 min at 4 ml/min) and characterized by MALDI-TOF MS (measured MW 4550.60; calc 4550.68). Amino acid analysis of a 24 h acid hydrolysis gave the following ratio (expected/found): G (10/13.20); A (10/11.48); P (2/1.49); Y (4/3.87); R (8/7.35); K (4/3.61).

Circular Dichroism (CD) Studies

CD measurements were performed on an Aviv circular dichroism spectrometer, model 62S (Lakewood, USA), using rectangular quartz window cells of 1 mm optical path length. Peptides were dissolved in 10 mM phosphate buffer, pH 7.2 at a concentration of 0.18–0.25 mg/ml total peptide. The concentration of the peptide solutions was determined by amino acid analysis. All measurements were made at 25°C from 190 to 250 nm, taken in intervals of 0.5 nm for 1 s. The data points were converted to mean residual ellipticity using a mean residue weight of 108.27, in deg cm^2/dmol.

Pharmacology

The rat isolated atria bioassay was set up as previously described [12]. After the tissues were primed with 0.1 μM (−)-isoprenaline, washed, and the chronotropic and inotropic responses had returned to baseline, they were exposed to 1 μM of either T_1 $(\mathbf{2})_4$ or T_2 $(\mathbf{4})_4$ for 20min. This was followed by construction of cumulative concentration–response (CR) curves to 1, 10 and 100 nM human gene 2 (B29) relaxin. At the end of the experiment, 0.1 μM (−)-isoprenaline was added to the baths to determine the maximum response of the tissues. Results were normalized and expressed as a percentage of the maximum response produced by 0.1 μM (−)-isoprenaline.

RESULTS AND DISCUSSION

Design and Synthesis

The central region of relaxin B-chain consists of an α-helix that contains two important arginines residues. These have their side chains fully extended, and face out of the α-helix in a nearly parallel manner. As they are among the very few conserved residues between species and clearly possess a vital role for bioactivity, it was of interest to develop relaxin peptide mimetics based on this central α-helical region of the B-chain. The assembly of peptides on template molecules according to the TASP concept has been shown to induce or stabilize specific conformations of peptides and therefore to modify their biological and pharmacokinetic properties [15]. Here, molecules were composed of truncated rat relaxin B-chain α-helix peptide fragments (region B15–B26) in 'long' (**1–3**) and 'short' (**4**) forms (Figure 1). In peptides **1–3** a leucine residue was introduced instead of the native tryptophan residue (B22) to prevent any side reactions during the synthesis. Peptides were then selectively attached via their *N*- or *C*-termini at four sites to a semi-cyclic peptidic carrier molecule. For the binding of the peptides to the carrier molecule, two chemoselective ligation methodologies were used, giving a thioether or a thiazolidine linkage. For this purpose, both approaches used thiol groups from cysteine residues as the reacting functionality on the peptides. For the first ligation strategy, the template molecule was functionalized with either bromoacetyl groups on the four lysine side chains [16], or aldehyde groups after oxidation of the serine residues (Figure 2). The template peptide **5** was synthesized using a combination of acid- and base-labile protecting groups in order to introduce the bromoacetyl moieties to the ε-amino groups of the four lysine residues while the peptide was still attached to the resin. After cleavage and deprotection, the crude peptide containing four bromoacetyl groups was purified by RP-HPLC. The template peptide **6** was prepared similarly but instead of the bromoacetyl groups, protected serine residues were

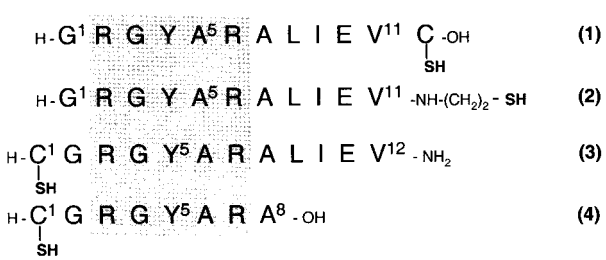

Figure 1 Rat relaxin B-chain α-helix peptide fragments in 'long' (**1–3**) and 'short' (**4**) versions. The highlighted region represents the 'arginine cassette' essential for receptor binding.

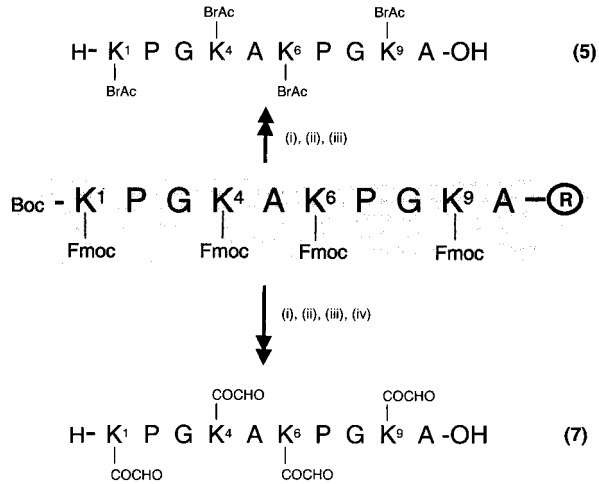

Figure 2 Synthesis of template molecules T_1 (**5**) and T_2 (**7**). Steps: (i) 50% piperidine/DMF; (ii) BrAc/DIC for **5** and Boc-Ser(Bzl)-OH/PyBOP/DIEA for **7**; (iii) HF/10% *m*-cresol; and (iv) $NaIO_4$ in NaOAc, pH 5.6 for **7**.

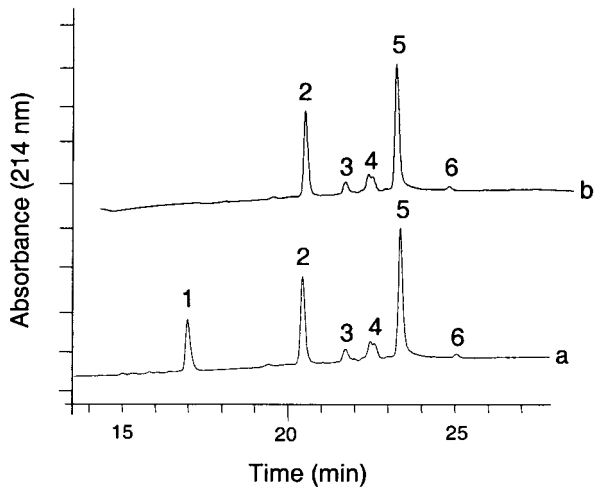

Figure 3 Analytical RP-HPLC of TASP T_1 $(\mathbf{2})_4$ synthesis at (a) 5 min and (b) 3 h, after reducing the reaction mixture. Gradient 10–90% buffer B, Vydac C_{18} column. Peaks: 1, template T_1 (**5**); 2, peptide **2**; 3 and 4, intermediates; 5, tetrameric TASP T_1 $(\mathbf{2})_4$; and 6, dimer of peptide **2**.

coupled to the ε-amino groups of the four lysine residues. A purification step was required after cleavage and deprotection and prior to the mild oxidation step which produced the corresponding tetrameric aldehyde template **7.** Peptides **1**, **3** and **4** were synthesized using standard Fmoc continuous-flow methodology whereas peptide **2** was assembled by Boc chemistry due to its regiospecific *C*-terminal thiol being bound to a TFA resistant linker [17]. In preliminary ligation experiments, the condensation of peptide **1** onto template **5** via the thioether linkage (*C*-terminal cysteine residue) did not go to completion as determined by analytical RP-HPLC and MALDI-TOF MS. No tetrameric bundle could be detected; instead a mixture of monomer, dimer and trimer were the only species present. Because this may be due to a steric hindrance problem, peptide **2** was synthesized with a cysteamine at its *C*-terminus in order to have a more readily accessible thiol group (aligned with the peptidic backbone). In comparison, peptide **1** had the thiol group on the cysteine side chain. The condensation of the latter peptide to the template proceeded very rapidly (few intermediates could be observed on RP-HPLC) and gave the tetrameric product in good yield (70%) (Figure 3). As the condensation reaction is carried out at pH 8.5, a major side-product was observed, that of the dimer of peptide **2**, despite the reaction being carried out under inert atmosphere. For this reason, a fivefold excess of peptide **2** relative to the template **5** was used. Unreacted peptide **2** could be retrieved by reducing the reaction mixture with dithiothreitol once all sites on the template were occupied. The tetrameric product was then separated from the excess reduced peptide present in the reaction mixture by gel-filtration chromatography and both were purified by RP-HPLC.

In order to prepare template-assembled relaxin mimetics with the peptide attached via the *N*-terminus, we chose the thiazolidine bond formation approach and synthesized peptide **3** having a *N*-terminal cysteine residue. The conjugation was carried out at pH 5.25 and required an equal volume of 6 M guanidine hydrochloride (Gua.HCl) to improve the poor solubility of the peptide. The reaction proceeded very slowly; after 24 h, the peptide-dimer (side-product) was the predominant product besides monomeric, dimeric and trimeric template-assembled products in the ratio 1:1:0.3. No tetrameric product could be detected. A shorter peptide was then synthesized with increased solubility properties compared to peptide **3** and limited tendency to form secondary structures. Conjugation of **4** with the template was completed after 24 h and the major product was the tetramer (Figure 4). Some unreacted trimer and the peptidic-dimer were also present but these could easily be separated from the target product by RP-HPLC. The identity and purity of TASP T_1 $(\mathbf{2})_4$ and TASP T_2 $(\mathbf{4})_4$ molecules were confirmed by analytical RP-HPLC and MALDI-TOF MS.

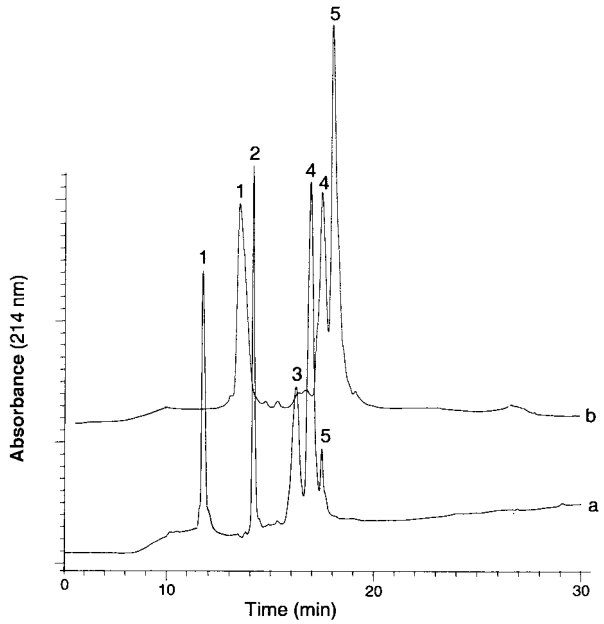

Figure 4 Analytical RP-HPLC of TASP T_2 $(\mathbf{4})_4$ synthesis at (a) 5 h and (b) 24 h. Gradient 10–45% buffer B, Vydac C_{18} column. Peaks: 1, peptide **4**; 2, dimer of peptide **4**; 3, dimeric TASP; 4, trimeric TASP; and 5, tetrameric TASP.

Conformational Studies

It is well established that both natural and synthetic B-chains of rat relaxin are particularly difficult to dissolve in aqueous solution and exhibit unusual adsorptive properties [18]. Conformational analysis of the individual B-chain by circular dichroism spectroscopy has shown that it contains at least 90% β-sheet and 10% unordered structure [19]. These properties have made purification of synthetic relaxin B-chain peptides and combination with A-chain particularly difficult. Interestingly, the β-structure changes to a largely unordered conformation if the peptide chain is shortened at the carboxyl terminus.

The use of templates to carry organic molecules, in particular peptides, is a versatile tool in peptide mimicry [20]. Although the template used in these studies is not cyclic, it nevertheless contains two β-turns bringing the lysine residues in a near environment. This facilitates interactions between the four peptides as well as providing them with maximum flexibility. In this approach, the emphasis is on different types of ligation chemistries and the *N*- and *C*-terminal amino acids involved in the ligations have no function in inducing some secondary structure. These mimics are based on self-association of amphiphilic peptides in solution, the major driving force for formation of characteristic packing topologies in proteins. Both peptides **2** and **4** showed a fully unordered structure under physiological conditions as can be expected for free peptides of this length in solution. Attachment to the template induced a β-sheet type structure for T_1 $(\mathbf{2})_4$ and no ordered structure was observed for T_2 $(\mathbf{4})_4$ (Figure 5). Considering the sequence of peptide **2** and individual amino acid propensities, both conformations, namely α-helix or β-sheet, are likely to form. This peptide shows a conformational switch that is dependent on the peptidic environment; this could explain its CD spectra when bound to the template. Furthermore, as mentioned earlier, the relaxin B-chain on its own has no tendency to form a α-helical structure. Only upon stabilization by the A-chain it will adopt the native $\alpha\beta$ type structure.

Bioassays

The results from the rat atria bioassays showed that neither T_1 $(\mathbf{2})_4$ nor T_2 $(\mathbf{4})_4$ (1 μM) had relaxin-like bioactivity (Figure 6). However, both TASP analogues (1 μM) partially antagonized the effect of human gene 2 (B29) relaxin in producing inotropic responses but had no significant effect on the chronotropic responses. T_1 $(\mathbf{2})_4$ shifted relaxin CR curves without affecting the maximum response, while T_2 $(\mathbf{4})_4$ shifted the relaxin CR curves and significantly reduced the maximum response. Thus, T_1 $(\mathbf{2})_4$ appeared to be a competitive and T_2 $(\mathbf{4})_4$ a

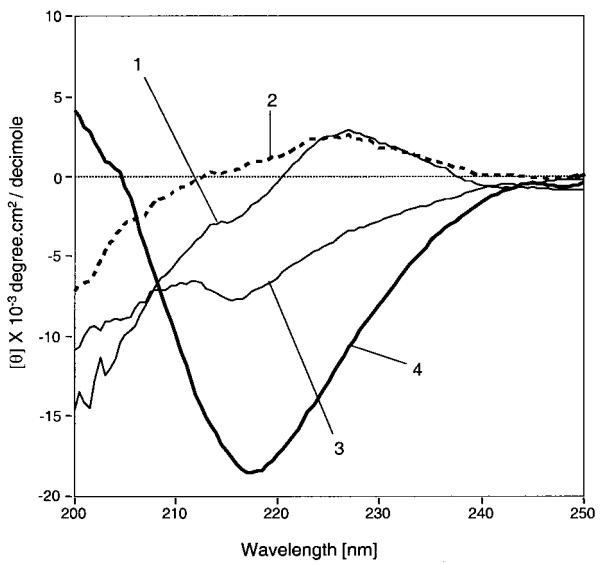

Figure 5 CD spectra of rat relaxin template-assembled mimetics and free peptidic B-chain analogues curve. 1, peptide **4**; 2, TASP T_2 $(\mathbf{4})_4$; 3, peptide **2**; 4, TASP T_1 $(\mathbf{2})_4$.

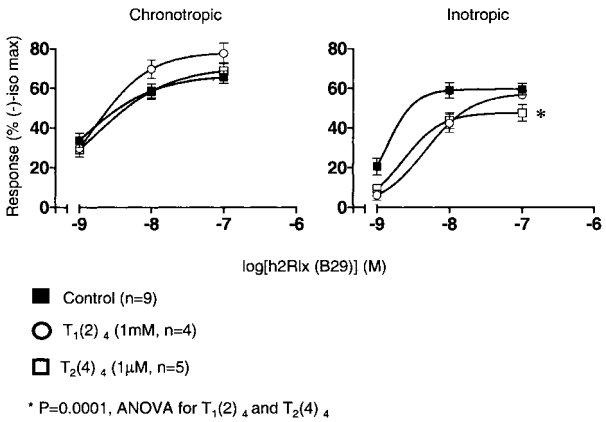

Figure 6 Effects of T_1 $(\mathbf{2})_4$, T_2 $(\mathbf{4})_4$ and human gene 2 (B29) in producing positive chronotropic and inotropic responses in rat isolated atria. Shown here are mean responses expressed as a percentage of the (−)-isoprenaline maximum.

non-competitive antagonist. However, both compounds failed to shift the CR curves for relaxin on the chronotropic response in the right atria. It is feasible therefore that the antagonist effects seen in the left atria may result from interference with relaxin signalling rather than an effect exerted directly on relaxin receptors. Thus, the results obtained in current studies do not indicate whether the TASP analogues bind to relaxin receptors in the rat atria, thereby reducing the number of receptors available for relaxin activation. We are currently investigating this hypothesis by performing competition binding studies [21].

Conclusion

The TASP concept together with thiol ligation chemistries provides a useful approach for producing antagonist mimetics of relaxin. Conformational analysis demonstrated a β-sheet type structure for TASP T_1 $(\mathbf{2})_4$, reflecting the environment-dependent structural nature of the B-chain α-helix. Future work will attempt the design of a helix-forming peptide following attachment to the template.

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