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ORIGINAL ARTICLE

Insights into cardiovascular effects of proline-rich oligopeptide (*Bj*-PRO-10c) revealed by structure–activity analyses: dissociation of antihypertensive and bradycardic effects

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Abstract We have previously reported that the proline-rich decapeptide from *Bothrops jararaca* (*Bj*-PRO-10c) causes potent and sustained antihypertensive and bradycardic effects in SHR. These activities are independent of ACE inhibition. In the present study, we used the Ala-scan approach to evaluate the importance of each amino acid within the sequence of *Bj*-PRO-10c (Pyr^1-Asn^2-Trp^3-Pro^4-His^5-Pro^6-Gln^7-Ile^8-Pro^9-Pro^{10}). The antihypertensive and bradycardic effects of the analogues *Bj*-PRO-10c Ala^3, *Bj*-PRO-10c Ala^7, *Bj*-PRO-10c Ala^8 were similar to those of *Bj*-PRO-10c, whereas the analogues *Bj*-PRO-10c Ala^2, *Bj*-PRO-10c Ala^4, *Bj*-PRO-10c Ala^5, *Bj*-PRO-10c Ala^9, and *Bj*-PRO-10c Ala^{10} kept the antihypertensive activity and lost bradycardic activity considerably. In contrast, *Bj*-PRO-10c Ala^1 and *Bj*-PRO-10c Ala^6 were unable to provoke any cardiovascular activity. In summary, we demonstrated that (1) the Pyr^1 and Pro^6 residues are essential for both, the antihypertensive and bradycardic effects of *Bj*-PRO-10c; (2) Ala-scan approach allowed dissociating blood pressure reduction and bradycardic effects. Conformational properties of the peptides were examined by means of circular dichroism (CD) spectroscopy. The different Ala-scan analogues caused either an increase or decrease in the type II polyproline helix content compared to *Bj*-PRO-10c. The complete loss of activity of the Pro^6 → Ala^6 mutant is probably due to the fact that in the parent peptide the His^5-Pro^6 bond can exist in the *cis* configuration, which could correspond to the conformation of this bond in the bound state. Current data support the *Bj*-PRO-10c as a promising leader prototype to develop new agents to treat cardiovascular diseases and its co-morbidities.

Keywords Proline-rich oligopeptide · Hypertension · Ala-scan approach · Arterial pressure · Heart rate · Circular dicroism

Abbreviations

ACE	Angiotensin-I converting enzyme
Ala-scan	Alanine scan
AsS	Argininosuccinate synthetase
Bj	*Bothrops jararaca*
Bj-PRO	*Bothrops jararaca*-proline-rich oligopeptides
CD	Circular dichroism
HR	Heart rate

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i.v.	Intravenous
MAP	Mean arterial pressure
MDLA	Alpha-methyl-DL-aspartic acid
NO	Nitric oxide
PAP	Pulsatile arterial pressure
Pyr	Pyroglutamic acid
SHR	Spontaneously hypertensive rat

Introduction

Bothrops jararaca (*Bj*) venom contains a family of proline-rich oligopeptides (PROs) that are able to potentiate the activity of bradykinin (Ianzer et al. 2004). Besides being proline-rich peptides, composed of 5–17 amino acid residues, these toxins present other structural similarities, such as pyroglutamic acid and proline residues at N- and C-terminal positions, respectively (Ianzer et al. 2004; Zelanis et al. 2010).

It has been previously shown that *Bj*-PRO-10c is part of the structure of the C-type natriuretic peptide precursor of the brain and the venom gland of *B. jararaca* (Hayashi et al. 2003; Murayama et al. 1997). Following i.v. injection, its biological effect includes a potent and long-lasting reduction of the arterial pressure, accompanied by bradycardia. Surprisingly, the antihypertensive doses of *Bj*-PRO-10c in the spontaneously hypertensive rats (SHRs) do not affect the angiotensin I-converting enzyme (ACE) activity in vivo (Ianzer et al. 2007), thus ruling out the classical hypothesis that the inhibition of ACE by snake venom peptides is responsible for its antihypertensive activity (Cushman et al. 1973). Recent results from our laboratory suggest that the cardiovascular effects of the *Bj*-PROs go far beyond the inhibition of ACE (Camargo et al. 2012b).

The cardiovascular effects of *Bj*-PRO-10c in SHR involve synergistic actions on the enzyme argininosuccinate synthetase (AsS) (Guerreiro et al. 2009) and other unknown receptor(s) (Guerreiro et al. 2009; Ianzer et al. 2007; Camargo et al. 2012b). In the citrulline–nitric oxide (NO) cycle, AsS is the rate-limiting enzyme to provide L-arginine, a substrate for the NO synthesis through its oxidation by the nitric oxide synthase (Flam et al. 2001; Shen et al. 2005). In vitro, the *Bj*-PRO-10c activates AsS and induces NO production in endothelial cells, whereas in vivo it increases L-arginine plasmatic levels. The partial reversion of the antihypertensive effect of *Bj*-PRO-10c by the alpha-methyl-DL-aspartic acid (MDLA), a specific AsS inhibitor, reinforces the NO-related mechanism (Guerreiro et al. 2009). Biodistribution studies showed that *Bj*-PRO-10c is also able to cross the blood brain barrier (Silva et al. 2008b), suggesting possible central actions, once it was observed reduction in the locomotor activity (Ianzer et al. 2007, 2010).

The present work aimed at assessing structure–activity relationships of *Bj*-PRO-10c, using a complete alanine scan (Ala-scan) approach (Cunningham and Wells 1989). In particular, we studied the cardiovascular parameters of SHR treated with *Bj*-PRO-10c and *Bj*-PRO-10c Ala-scan analogues. Conformational properties of the peptides were examined by means of circular dichroism (CD) spectroscopy.

Materials and methods

Synthesis and purification of peptides

The syntheses of *Bj*-PRO-10c (Pyr^1-Asn^2-Trp^3-Pro^4-His^5-Pro^6-Gln^7-Ile^8-Pro^9-Pro^{10}) and its analogues (Table 1) were performed on an automated PSSM-8 peptide synthesizer (Shimadzu Corp., Kyoto, Japan) by a stepwise solid-phase method using *N*-9-fluorenylmethoxycarbonyl (Fmoc) chemistry (Carmona and Juliano 1996). All Fmoc-L-amino acids and resins were purchased from Merck KGaA (Darmstadt, Germany). The following amino acids were used: Fmoc-L-Ala-OH, Fmoc-L-Asn(Trt)-OH, Fmoc-L-Gln(Trt)-OH, Fmoc-L-His(Trt)-OH, Fmoc-L-Ile-OH, Fmoc-L-Pro-OH, Fmoc-L-Trp(Boc)-OH, L-Pyr-OH; the resins employed were Pro-2-Cl-Trt resin (0.45 mmol/g) and Fmoc-Ala-Wang resin (0.67 mmol/g). Resins were first conditioned for 20 min in DMF. The Fmoc-amino acids were then added by a succession of deprotection and coupling steps. Fmoc group removal was performed through two consecutive treatments of 10 min each using piperidine 20 % in DMF. Activation of Fmoc-protected amino acids (fivefold of excess) was carried out in DMF in the presence of five equiv of *N*,*N*,*N*′,*N*′-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate and six equiv 4-Methylmorpholine for 5 min following addition for coupling the deprotected resin for 30 min. After each step, the resin was washed five times with DMF. Final cleavage of peptides from the resin and side chain deprotections were achieved by treatment with a mixture of trifluoroacetic acid (TFA) 87.5 %, 1,2-ethanedithiol 2.5 %, water 5 %, phenol 2.5 % and thioanisole 2.5 % for 2 h at room temperature. After removal of the resin by filtration the crude synthetic peptides were precipitated with diethyl ether and recovered after centrifugation. The crude synthetic peptides were purified by preparative reversed-phase high-performance liquid chromatography (HPLC) (Shimadzu Corp., Japan) on a YMC-Pack ODS column 20 × 150 mm (YMC, Kyoto, Japan), using a linear gradient from 5 to 40 % acetonitrile in 0.1 % TFA, at a flow rate of 8 ml/min. Buffer elution was removed by freeze drying, followed by confirmation of identity and purity of each synthetic peptide fraction by MALDI-TOF mass spectrometry an Ettan MALDI-TOF/Pro instrument

Table 1 Maximal antihypertensive and bradycardic effects following i.v. injection of *Bj*-PRO-10c and *Bj*-PRO-10c Ala-scan analogues (71 nmol/kg) in SHR and PPII helix content of *Bj*-PRO-10c and its Ala-scan analogues

Treatment	Amino acid sequence	Basal MAP (mmHg)	Δ MAP (mmHg)	Basal HR (bpm)	Δ HR (bpm)	PPII (%)
Vehicle	–	175 ± 4	−13 ± 1	361 ± 8	−14 ± 4	–
Bj-PRO-10c	Pyr-Asn-Trp-Pro-His-Pro-Gln-Ile-Pro-Pro	176 ± 3	−30 ± 3*	367 ± 10	−71 ± 12*	46
Bj-PRO-10c Ala^{1}	**Ala**-Asn-Trp-Pro-His-Pro-Gln-Ile-Pro-Pro	168 ± 4	−13 ± 2$^{\#}$	354 ± 10	−18 ± 5$^{\#}$	47
Bj-PRO-10c Ala^{2}	Pyr-**Ala**-Trp-Pro-His-Pro-Gln-Ile-Pro-Pro	165 ± 4	−26 ± 3*	360 ± 10	−32 ± 3*, $^{\#}$	48
Bj-PRO-10c Ala^{3}	Pyr-Asn-**Ala**-Pro-His-Pro-Gln-Ile-Pro-Pro	178 ± 6	−24 ± 2*	367 ± 11	−52 ± 12*	33
Bj-PRO-10c Ala^{4}	Pyr-Asn-Trp-**Ala**-His-Pro-Gln-Ile-Pro-Pro	167 ± 5	−24 ± 4*	357 ± 8	−36 ± 3*, $^{\#}$	32
Bj-PRO-10c Ala^{5}	Pyr-Asn-Trp-Pro-**Ala**-Pro-Gln-Ile-Pro-Pro	178 ± 6	−23 ± 4*	360 ± 10	−44 ± 7*	55
Bj-PRO-10c Ala^{6}	Pyr-Asn-Trp-Pro-His-**Ala**-Gln-Ile-Pro-Pro	160 ± 6	−15 ± 2$^{\#}$	358 ± 15	−27 ± 15$^{\#}$	58
Bj-PRO-10c Ala^{7}	Pyr-Asn-Trp-Pro-His-Pro-**Ala**-Ile-Pro-Pro	162 ± 5	−24 ± 2*	356 ± 9	−43 ± 7*, $^{\#}$	49
Bj-PRO-10c Ala^{8}	Pyr-Asn-Trp-Pro-His-Pro-Gln-**Ala**-Pro-Pro	177 ± 6	−27 ± 4*	369 ± 17	−57 ± 11*	60
Bj-PRO-10c Ala^{9}	Pyr-Asn-Trp-Pro-His-Pro-Gln-Ile-**Ala**-Pro	170 ± 5	−25 ± 4*	369 ± 26	−40 ± 7*, $^{\#}$	47
Bj-PRO-10c Ala^{10}	Pyr-Asn-Trp-Pro-His-Pro-Gln-Ile-Pro-**Ala**	169 ± 7	−30 ± 1*	357 ± 13	−34 ± 9*, $^{\#}$	42
Bj-PRO-10c Glu^{1}	**Glu**-Asn-Trp-Pro-His-Pro-Gln-Ile-Pro-Pro	175 ± 4	−10 ± 6$^{\#}$	353 ± 5	−39 ± 8 *, $^{\#}$	nd

The maximal changes in MAP and HR were sampled after i.v. injection in each experiment, at the time point they occurred. Data are expressed as mean ± SEM, $n = 5$–7. Bold represents the replacement of the original amino acid residue by Ala

Pyr pyroglutamic acid, *nd* not determined

* $p < 0.05$ compared with vehicle

$^{\#}$ $p < 0.05$ compared with *Bj*-PRO-10c

(Amersham Biosciences, Uppsala, Sweden) and by analytical, Finnigan Surveyor MSQ Plus Single Quadrupole LC/MS (Thermo Finnigan, San Jose, CA) and reversed-phase HPLC in two different solvent systems. Samples were frozen and then freeze dried (Edwards Freeze Dryer Super Modulyo Pirani 1001, Thermo Fisher Scientific, Waltham, MA) for 48 h at −50 °C under vacuum. The *Bj*-PRO-10c and its Ala-scan analogues were used with purity higher than 95 % and dissolved in saline (0.9 % NaCl) just before use.

Circular dichroism (CD) spectroscopy

CD spectra were acquired at room temperature in a Jasco J-720 spectropolarimeter. Samples were placed in 0.1 mm optical length quartz cells. The final spectra resulted from the average of six scans, after subtracting the spectrum obtained under the same conditions of a sample without peptide. The peptides, whose concentration ranged between 160 and 205 μM, were dissolved in isotonic saline. Except for *Bj*-PRO-10c Ala^{3}, whose concentration was determined by weighing, the peptides concentration was determined spectrophotometrically by measuring the absorption of tryptophan ($\varepsilon_{280} = 5{,}690\ M^{-1} \cdot cm^{-1}$). CD spectra were scanned from 190 to 260 nm at 50 nm $\cdot$ s^{-1} using a 2-nm slit. The CD spectra are reported as mean residue ellipticity ([θ]) in degrees $cm^2 \cdot dmol^{-1}$.

In vivo assays: blood pressure recording in rats

Animals

Experiments were carried out in male SHRs (280–350 g) from CEBIO, University of São Paulo. The animals were bred at the animal facility of the Special Laboratory of Applied Toxinology, Butantan Institute. The animals had free access to food and water and were submitted to a light–dark cycle (12 h each) before the preparation for the experiments. All animals were handled under ethical conditions according to international rules of animal care, stated by the International Animal Welfare Recommendations, and in accordance with the guidelines established by (1) our local institutional animal welfare committee (CEUAIB/IBu, protocol 520/08 and CEP/UNIFESP, protocol 0934/09); (2) EU Directive 2010/63/EU for animal experiments and (3) Uniform requirements for manuscripts submitted to biomedical journals.

Arterial pressure measurements

The cardiovascular parameters, pulsatile arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR) were monitored by a solid-state strain gauge transducer connected to a computer through a data acquisition system (MP 100; BIOPAC Systems Inc., USA). The PAP, MAP

and HR were monitored jointly during experiments in different monitor channels and recorded in the computer hard disk for later analysis.

The assays for evaluate the effects of *Bj*-PRO-10c analogues on cardiovascular parameters of SHR were performed as described by Ianzer et al. (Ianzer et al. 2007). Briefly, 20 h before the experiment, under anesthesia with 0.05 ml of 10 % ketamine and 2 % xylazine (1:1) intraperitoneally, a polyethylene catheter (PE-10 connected to PE-50) was introduced into the abdominal aorta through a femoral artery for measurements of cardiovascular parameters and into a femoral vein for intravenous (i.v.) injection. After recovery from anesthesia, the rats were kept in individual cages with free access to water and chow until the end of the experiments.

The cardiovascular parameters were monitored for 1 h before drug administration (baseline period). After this period, i.v. *bolus* injection of *Bj*-PRO-10c and its Ala-scan analogues (71 nmol/kg) or vehicle (NaCl 0.9 %) in a total volume of 0.5 ml was made ($n = 5$–7). The cardiovascular parameters were monitored continuously for 6 h after drug administration. MAP and HR values were sampled for 30-s periods every 5 min during the entire recording period. The choice of dose (71 nmol/kg) was based on the effects of *Bj*-PRO-10c found in a dose–response curve (0.47–71 nmol/kg) in SHR (Ianzer et al. 2007).

Statistical analysis

The time-course changes were calculated from the difference between basal average value (5 min average sampled immediately before i.v. injection) and value of every 5 min post injection time. The maximal changes in MAP and HR were sampled after i.v. injection in each experiment, at the time point they occurred.

Comparisons were made by Student's unpaired *t* test or two-way ANOVA with Bonferroni post-test when appropriate. GraphPad Prism 5.0, GraphPad Software, Incorporation software program (USA) was used in all statistical analysis. The criterion for statistical significance was set at $p < 0.05$.

Results

Evaluation of cardiovascular parameters

In order to investigate the role and the importance of each amino acid residue of the *Bj*-PRO-10c sequence for the cardiovascular effects in SHR, we performed an Ala-scan strategy, in which each residue was systematically replaced by the small and neutral amino acid, alanine (Alana et al. 2006; Quartara et al. 2000; Corzo et al. 2007).

Figures 1 and 2 present the changes in arterial pressure and heart rate, respectively, produced by the i.v. *bolus* injection of *Bj*-PRO-10c and Ala-scan analogues (71 nmol/kg). There were slight changes in MAP and HR following injection of vehicle which can be attributed to the fact that the experimental procedure was performed in free-behaving animals (Figs. 1a, 2a). In these cases, it is not unlike to observe discrete variations in the cardiovascular parameters during similar experimental conditions (Ianzer et al. 2007).

As expected and previously described (Ianzer et al. 2007), *Bj*-PRO-10c caused potent and long-lasting antihypertensive and bradycardic effects in SHR. Changes in MAP were evident after 15 min, achieving amplitudes that ranged between −20 and −27 mmHg after 180 min of administration. These changes were maintained until the end of the observation period (360 min) (Fig. 1b). HR falls were seen from 25 min past injection and reached a sustained bradycardia until the end of the experiment, achieving −52 bpm 270 min following i.v. injection (Fig. 1b). The monitoring of cardiovascular parameters of SHR following i.v. injection of the *Bj*-PRO-10c Ala-scan analogues (71 nmol/kg) showed that most of these peptides were able to reduce MAP and HR. However, the amplitudes of the antihypertensive and bradycardic effects were different for each peptide (Figs. 1, 2).

Comparisons with the group injected with vehicle revealed that the *Bj*-PRO-10c Ala^1 did not significantly alter the MAP and HR of SHR (Figs. 1c, 2c). The *Bj*-PRO-10c Ala^2 caused gradual reduction in MAP, reaching −25 mmHg after 200 min of administration, which was sustained until the end of the recording (Fig. 1d). *Bj*-PRO-10c Ala^2 caused a slight reduction in HR. The bradycardic effect (about −20 bpm) was clear 75 min following i.v. injection, remaining stable until the end of the experimental period (Fig. 2d).

The time-course of MAP changes evoked by *Bj*-PRO-10c Ala^3 was significantly different from the one elicited by the vehicle (Fig. 1e). In addition, *Bj*-PRO-10c Ala^3 slightly reduced the heart rate. The average reduction reached −40 bpm, close to end of experimental period (Fig. 2e).

The antihypertensive effect of the *Bj*-PRO-10c Ala^4 followed a different time course. In general, the changes caused by *Bj*-PRO-10c were evident after 15 min. Otherwise, the reduction in MAP caused by *Bj*-PRO-10c Ala^4 was late, starting around 180 min after injection. The amplitude of the *Bj*-PRO-10c Ala^4 effects on MAP reached approximately −17 mmHg 260 min following i.v. injection, which was kept until the end of the record (Fig. 1f). Despite causing a negative chronotropy smaller than that evoked by *Bj*-PRO-10c, the *Bj*-PRO-10c Ala^4 caused a sustained bradycardia (−25 bpm), also starting at 180 min (Fig. 2f).

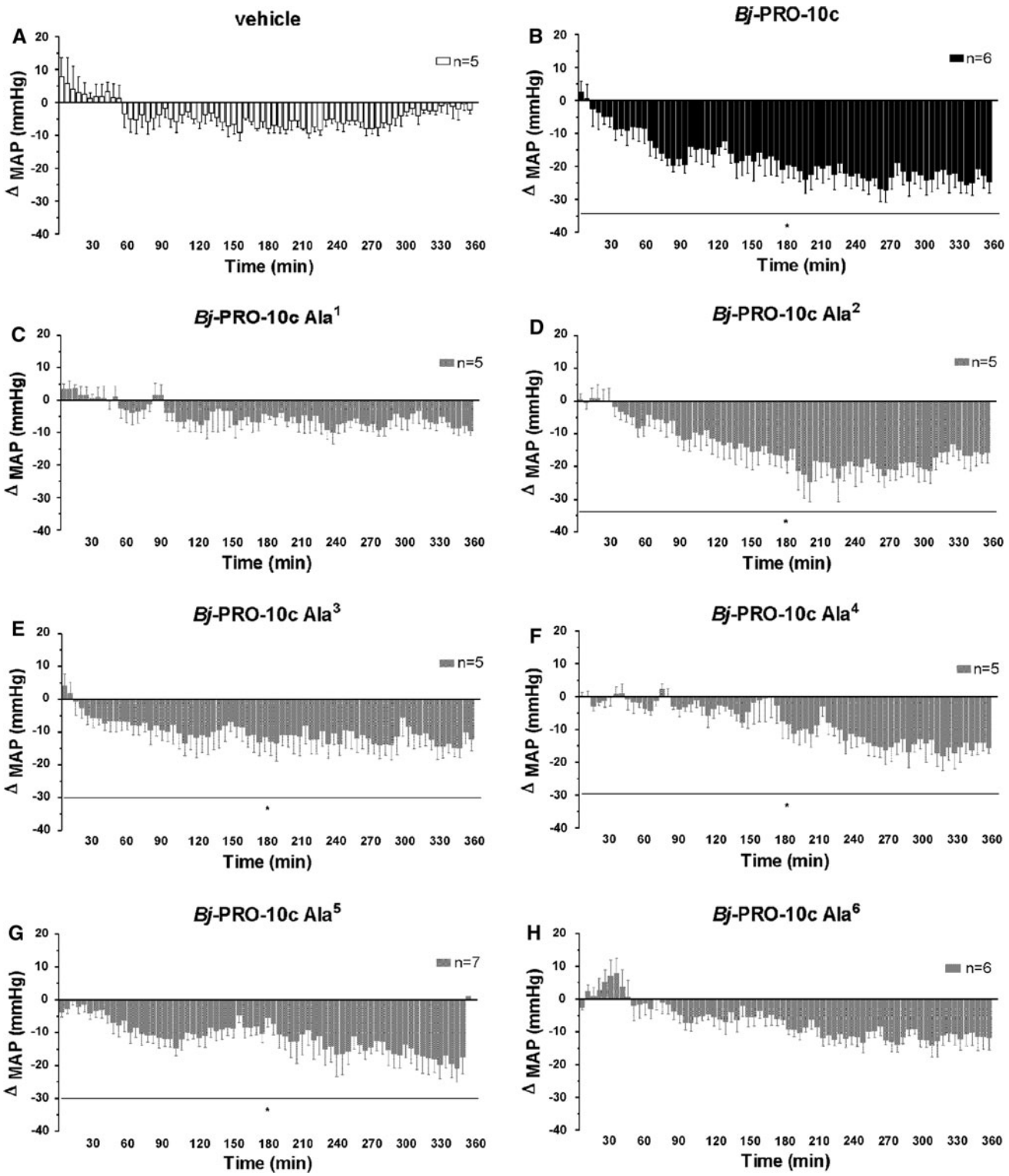

Fig. 1 Time course of MAP changes after administration of *Bj*-PRO-10c and *Bj*-PRO-10c Ala-scan analogues (71 nmol/kg) in SHR. The data are presented as mean ± SEM, $n = 5–7$

As shown in Fig. 1g, the antihypertensive effect of *Bj*-PRO-10c Ala^5 was gradual after 5 min, decreasing to about −20 mmHg close to the end of experimental period. The *Bj*-PRO-10c Ala^5 caused slight and transient bidirectional chronotropic effects during the entire experimental period (Fig. 2g).

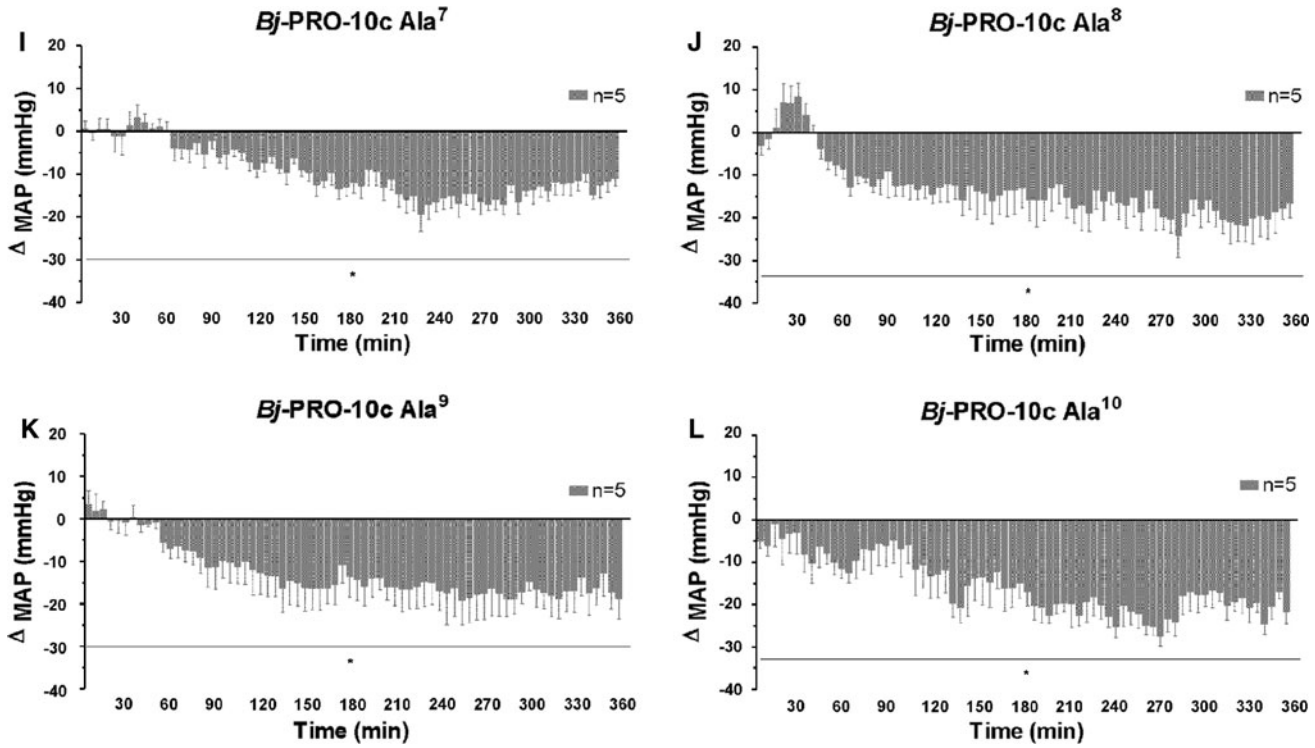

Fig. 1 continued

Similar to *Bj*-PRO-10c Ala[1], the *Bj*-PRO-10c Ala[6] was not able to cause important changes in MAP and in HR when compared with vehicle (Figs. 1h, 2h).

The *Bj*-PRO-10c Ala[7] caused a gradual decrease in MAP reaching approximately −20 mmHg at 235 min, which was kept until 360 min (Fig. 1i). This peptide also promoted a bradycardic effect. The HR reductions were evident immediately after injection and reached −35 bpm at 325 min (Fig. 2i).

The antihypertensive effect evoked by *Bj*-PRO-10c Ala[8] was gradual and an average of −22 mmHg was reached at 290 min (Fig. 1j). The *Bj*-PRO-10c Ala[8] caused marked bradycardia (about −44 bpm), likely initiated 5 min following injection. This effect was observed throughout the experimental period (Fig. 2j).

The changes in MAP after *Bj*-PRO-10c Ala[9] injection reached peak values at about 150 min and lasted until the end of the experimental period (Fig. 2k). The bradycardia caused by the *Bj*-PRO-10c Ala[9] was discrete and oscillatory, as that observed for *Bj*-PRO-10c Ala[5] (Fig. 2k).

The antihypertensive effect observed after *Bj*-PRO-10c Ala[10] injection displayed a gradual and stable reduction in MAP until the end of experimental period, reaching maximal averages of −27 mmHg at 270 min (Fig. 1l). In contrast to other *Bj*-PRO-10c Ala-scan peptides, *Bj*-PRO-10c Ala[10] caused transient bidirecional chronotropic effects. Figure 2l shows the time-course of the effects evoked by *Bj*-PRO-10c Ala[10] on HR. The fast positive chronotropy is noteworthy from the fifth minute, showing a variable profile until 245 min after injection. However, from the 250-min time point, a small bradycardic effect was predominant and persisted until the end of the experimental period.

Table 1 presents the mean maximal changes in cardiovascular parameters (MAP and HR) caused by the administration of *Bj*-PRO-10c and its Ala-scan analogues. Besides the *Bj*-PRO-10c Ala-scan analogues, Glu replacement at position 1 (*Bj*-PRO-10c Glu[1]) was assessed to verify whether Pyr is a key residue for *Bj*-PRO-10c. *Bj*-PRO-10c Glu[1] did not cause any antihypertensive effect. Compared to *Bj*-PRO-10c, the analogue *Bj*-PRO-10c Glu[1] evoked a smaller effect upon HR.

The maximum changes following i.v. administration of *Bj*-PRO-10c and analogues at the dose of 71 nmol/Kg revealed the following: (1) the replacement of pyroglutamic acid and proline residues at positions 1 and 6 (*Bj*-PRO-10c Ala[1] and *Bj*-PRO-10c Ala[6]), respectively, determined the lack of both antihypertensive and bradycardic effects; (2) maximal changes in blood pressure caused by the analogues *Bj*-PRO-10c Ala[2], *Bj*-PRO-10c Ala[3], *Bj*-PRO-10c Ala[4], *Bj*-PRO-10c Ala[5], *Bj*-PRO-10c Ala[8], *Bj*-PRO-10c Ala[9] and *Bj*-PRO-10c Ala[10] were statistically similar than the positive control, *Bj*-PRO-10c; (3) *Bj*-PRO-10c Ala[3], *Bj*-PRO-10c Ala[7] and *Bj*-PRO-10c Ala[8] accomplished a maximal bradycardia statistically similar to that found for *Bj*-PRO-10c, whereas *Bj*-PRO-10c Ala[2], *Bj*-

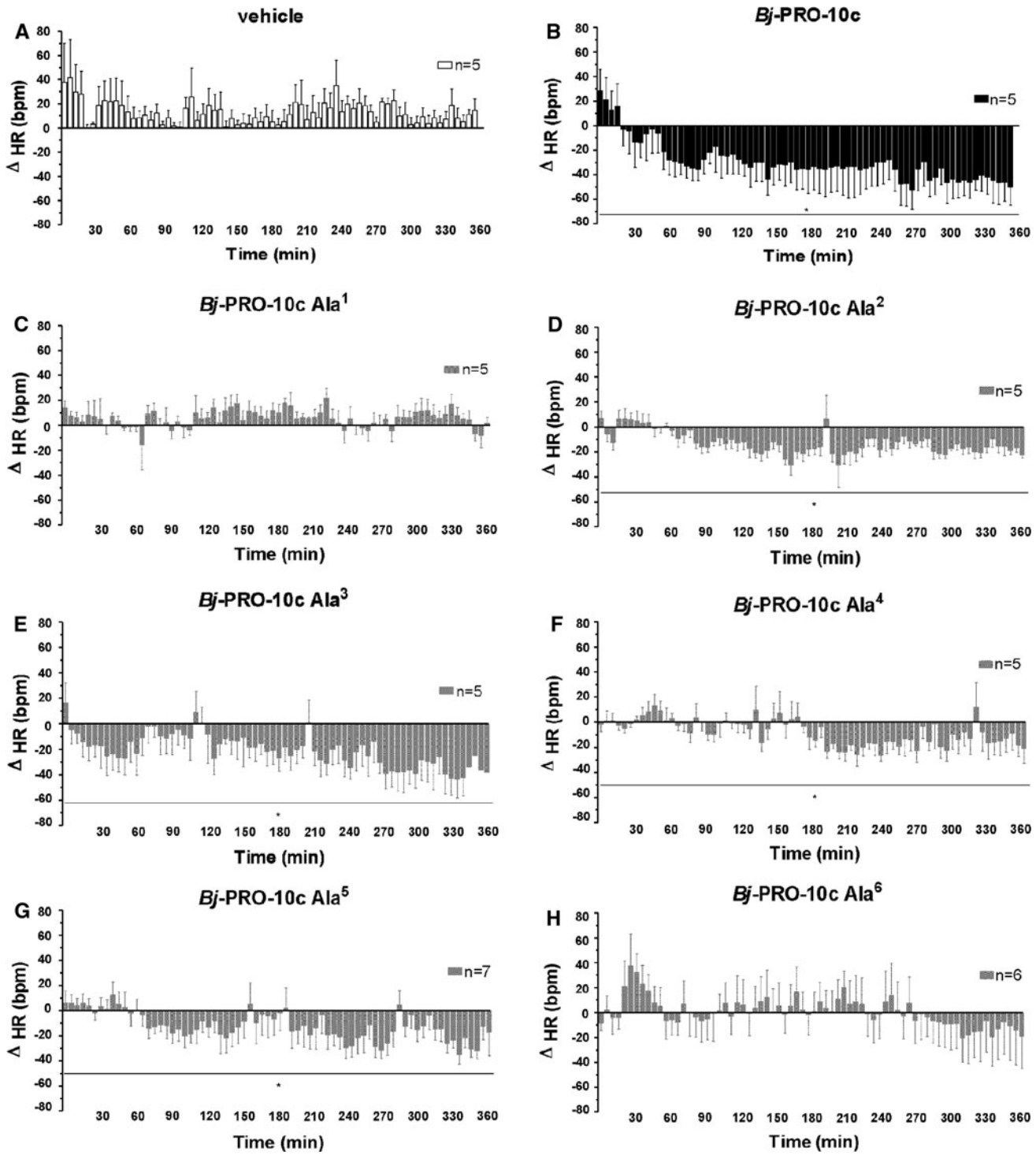

Fig. 2 Time course of HR changes after administration of *Bj*-PRO-10c and *Bj*-PRO-10c Ala-scan analogues (71 nmol/kg) in SHR. The data are presented as mean ± SEM, $n = 5–7$

PRO-10c Ala^4, *Bj*-PRO-10c Ala^5, *Bj*-PRO-10c Ala^9 and *Bj*-PRO-10c Ala^{10} presented statistical difference in the range and direction of HR changes when compared with *Bj*-PRO-10c; and (4) none of Ala-scan analogues was more effective than the *Bj*-PRO-10c for both antihypertensive and bradycardic effects.

Some Ala-scan analogues modified the standard response evoked by *Bj*-PRO-10c (Table 1). Compared with

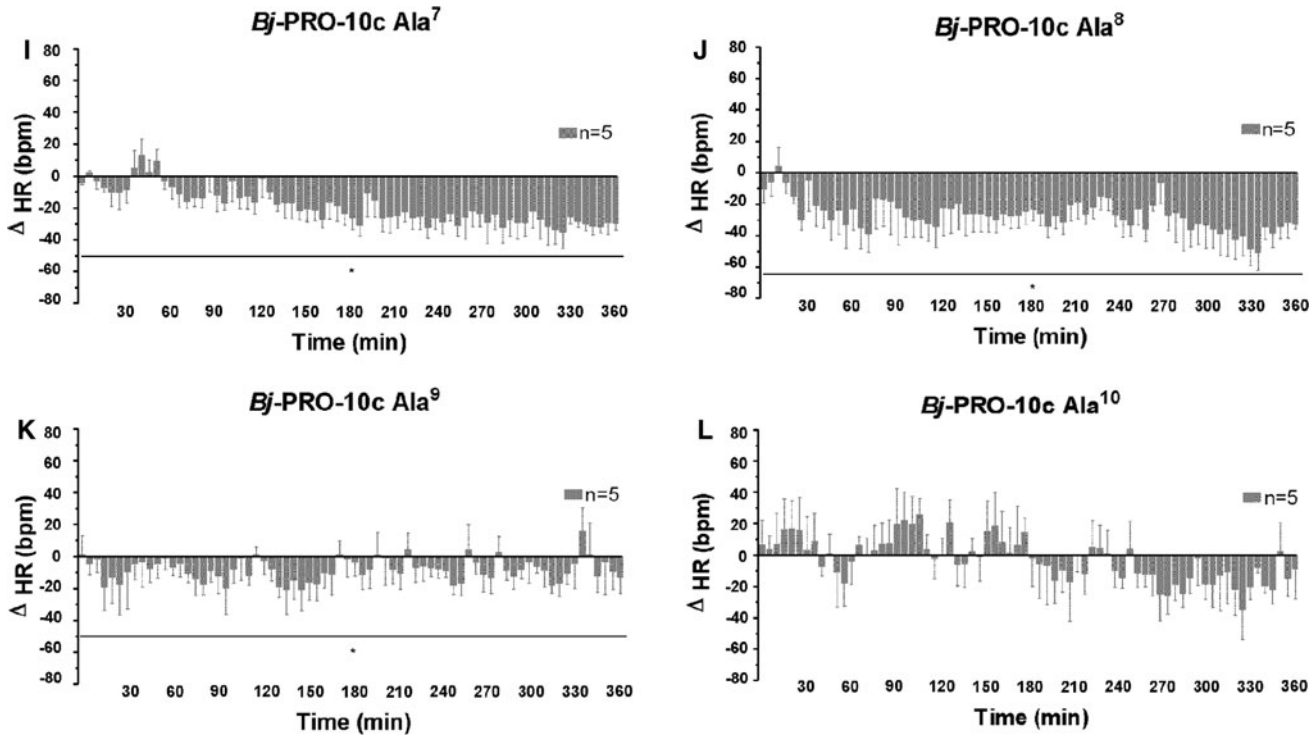

Fig. 2 continued

Bj-PRO-10c (100 %), the active analogues *Bj*-PRO-10c Ala^{10}, Ala^{8}, Ala^{2}, Ala^{9}, Ala^{3}, Ala^{7}, Ala^{4} and Ala^{5} reached, respectively, 100, 90.0, 86.7, 83.3, 80.0, 80.0, 80.0 and 76.7 % of the antihypertensive effect of *Bj*-PRO-10c. Comparisons of maximal HR reduction showed that the analogues *Bj*-PRO-10c Ala^{8}, Ala^{3}, Ala^{5}, Ala^{7}, Ala^{9}, Ala^{4}, Ala^{10} and Ala^{2} reached, respectively, 80.3, 73.2, 62.0, 60.6, 56.3, 50.7, 47.9 and 45.1 % of the bradycardic effect of *Bj*-PRO-10c. The time-course of the chronotropic effect was also substantially altered when compared with *Bj*-PRO-10c profile (Fig. 2).

Conformational studies

In order to investigate structure–activity relationships, the peptide conformation was examined in solution (saline) by means of CD spectroscopy. In solution, very likely, an equilibrium exists between different conformational states; it is usually assumed that, upon binding to a "receptor", this equilibrium will be shifted towards the conformation that corresponds to the bound state. As expected for proline-rich oligopeptides, the CD spectra of the native peptide and of several of its Ala-scan analogues (*Bj*-PRO-10c-Ala^{1}, *Bj*-PRO-10c-Ala^{2}, *Bj*-PRO-10c-Ala^{5}, *Bj*-PRO-10c-Ala^{6}, *Bj*-PRO-10c-Ala^{7}, *Bj*-PRO-10c-Ala^{8}, and *Bj*-PRO-10c-Ala^{9}) present features characteristic of type II polyproline helix (PPII). These spectra displayed more intense negative peak between 200 and 203 nm and a less intense positive peak between 226 and 228 nm (Fig. 3). In contrast, the spectra of *Bj*-PRO-10c-Ala^{3}, *Bj*-PRO-10c-Ala^{4}, and *Bj*-PRO-10c-Ala^{10} did not present a positive peak between 226 and 228 nm; instead, a "shoulder" was observed in this region (Fig. 4).

The intensity of the positive peak in the 226–228 nm region varied for the different peptides (Fig. 3). In order to compare their conformational properties, we used the equation given by (Kelly et al. 2001) to calculate the percentage of PPII helix content in the CD spectra: $\%\text{PPII} = 100\ ([\theta]_{\text{MÁX}} + 6{,}100)/13{,}700$, where $[\theta]_{\text{MÁX}}$ is the maximum value of the molar ellipticity in the 226–228 nm region.

To extend this analysis to all peptides, the equation was also applied to the spectra of the analogues *Bj*-PRO-10c-Ala^{3}, *Bj*-PRO-10c-Ala^{4}, and *Bj*-PRO-10c-Ala^{10}. Since the positive peak in the 226–228 nm region was absent in these spectra, the values of [θ] at 227 nm were used for the calculation.

As seen in Table 1, the spectrum of *Bj*-PRO-10c corresponds to 46 % PPII helix. Similar PPII helical contents were found for *Bj*-PRO-10cAla^{1} (47 %), *Bj*-PRO-10c-Ala^{2} (48 %), *Bj*-PRO-10c-Ala^{7} (49 %), and *Bj*-PRO-10c-Ala^{9} (47 %). For the analogues, *Bj*-PRO-10c-Ala^{5}, *Bj*-PRO-10c-Ala^{6}, and *Bj*-PRO-10c-Ala^{8} the PPII helix content was increasingly higher (55, 58, and 60 %, respectively). The

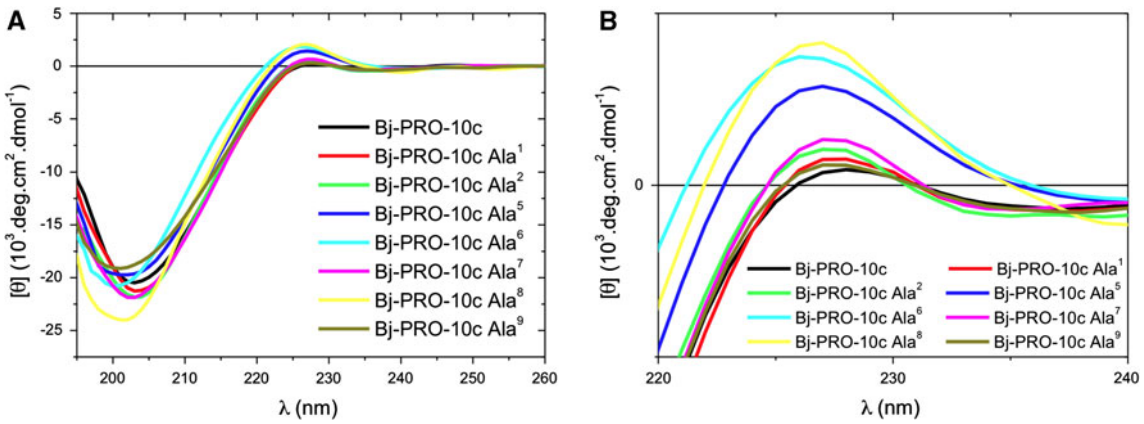

Fig. 3 CD spectra of *Bj*-PRO-10c and *Bj*-PRO-10c Ala-scan analogues *Bj*-PRO-10c Ala^1, *Bj*-PRO-10c Ala^2, *Bj*-PRO-10c Ala^5, *Bj*-PRO-10c Ala^6, *Bj*-PRO-10c Ala^7, *Bj*-PRO-10c Ala^8, and *Bj*-PRO-10c Ala^9 in saline solution. **a** full spectra, run between 195 and 260 nm; *right*, **b** expansion of the 220–240 nm region

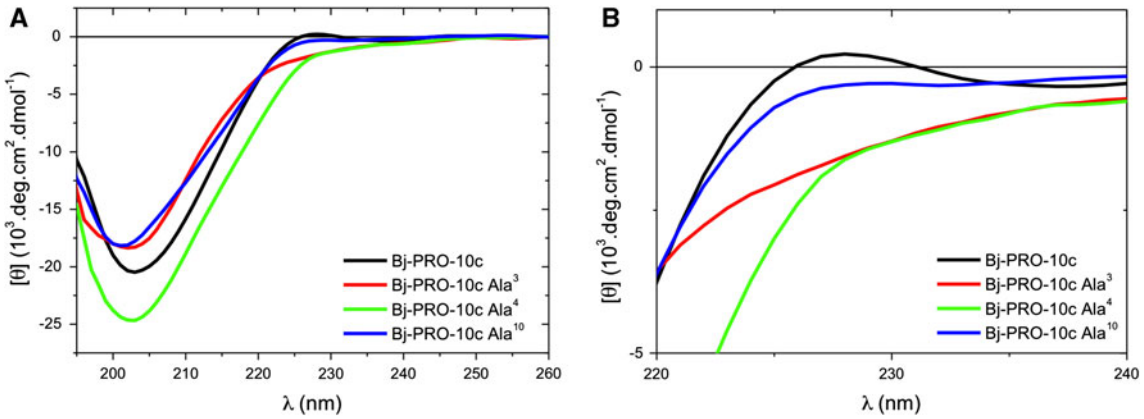

Fig. 4 CD spectra of *Bj*-PRO-10c and *Bj*-PRO-10c Ala-scan analogues *Bj*-PRO-10c Ala^3, *Bj*-PRO-10c Ala^4 and *Bj*-PRO-10c Ala^{10} in saline solution. **a** full spectra, run between 195 and 260 nm; **b** expansion of the 220–240 nm region

PPII helix contents for *Bj*-PRO-10c-Ala^3, *Bj*-PRO-10c-Ala^4, and *Bj*-PRO-10c-Ala^{10} were 33, 32, and 42 %, respectively.

Discussion

The primary structure of *Bj*-PRO-10c (Pyr^1-Asn^2-Trp^3-Pro^4-His^5-Pro^6-Gln^7-Ile^8-Pro^9-Pro^{10}) was found in the C-Type natriuretic peptide precursor in the venomous gland of *B. jararaca* (Murayama et al. 1997). A very similar protein precursor, containing the *Bj*-PRO-10c, was found in the neuroendocrine area of the *B.jararaca* brain, suggesting a possible role for this peptide in cardiovascular homeostasis and hydroelectrolytic balance of the animal (Hayashi et al. 2003).

The different cardiovascular effects evoked by *Bj*-PROs in normotensive and hypertensive rats, as a function of the administered doses (Ianzer et al. 2007, 2011), determined the choice of the strain (SHR) and dose (71 nmol/kg) used in the present study. In SHR, the biological activity of *Bj*-PRO-10c includes a sustained reduction in MAP (−30 %) and in HR (−17 %). In normotensive rats, only a slight reduction in blood pressure was detected after i.v. injection of *Bj*-PRO-10c (71 nmol/kg) (Ianzer et al. 2007).

The Ala-scan strategy (Cunningham and Wells 1989) applied in the present work does not intend to define the pharmacophore of *Bj*-PRO-10c as suggested by Peter Gund (Gund 1977), since this approach is usually applied when an identified target (receptor or enzyme) is involved in some biological effect (Alana et al. 2006; Corzo et al. 2007; Quartara et al. 2000). Obviously, the structure–activity relationship of *Bj*-PRO-10c depends upon multifactorial systems, which regulate the blood pressure and heart rate (Camargo et al. 2012b).

In general, for a specific biological activity, peptides must present a conformation that aligns essential functional groups in a required spatial orientation. It is important to

emphasize that proline (Pro) plays a distinct role in the structure of peptides/proteins (Biedermannova et al. 2008). The side-chain of proline is cyclized back on to the backbone amide position, which providing rigidity and a considerably restriction in its conformational freedom compared to other amino acids (Machado et al. 1993; Moradi et al. 2010). This gives support to data presented here where the structural changes resulted from the replacement of Pro residues by Ala at specific position of the *Bj*-PRO-10c sequence manifest either through the extent or the nature of biological effects are affected.

Comparisons of the time-course profile and peak responses showed that the original proline-rich decapeptide, *Bj*-PRO-10c, was more potent than all generated Ala-scan peptides in changing the cardiovascular parameters. The great majority of the individual replacements of amino acid residues in the *Bj*-PRO-10c sequence culminates in reduction or lack of the *Bj*-PRO-10c effects. To better understand the biological results, conformational studies were carried out. It is important to bear in mind that the conformational studies were performed in solution; therefore, the conformations at eventual binding sites were not trapped. Some points were considered when analyzing the effect of Ala substitution on peptide conformation: (1) the propensity of the different amino acids to favor PPII helix conformation, based on the scale established by Rucker et al. (Rucker et al. 2003); (2) the fact that aromatic amino acids tend to favor the *cis* conformer of proline, thereby being unfavorable to PPII helix conformation, which requires Pro in the *trans* conformation (Rucker et al. 2003). In this context, it is noteworthy that the aforementioned factors can account for the observed increase in the PPII helix content of *Bj*-PRO-10c-Ala^5, *Bj*-PRO-10c-Ala^6, and *Bj*-PRO-10c-Ala^8.

Regarding the maximal cardiovascular effects, we divided the Ala-scan peptides into three groups, as follows:

Group 1—Amino acid residues that poorly contribute to the cardiovascular effects: tryptophan at position 3, glutamine at position 7, and isoleucine at position 8 seem to provide minor contributions to the cardiovascular effects of *Bj*-PRO-10c. Despite slight reduction in HR evoked by *Bj*-PRO-10c Ala^3, *Bj*-PRO-10c Ala^7, and *Bj*-PRO-10c Ala^8, the time course profile of changes in cardiovascular parameters and the maximal peak changes in MAP and HR evoked by *Bj*-PRO-10c Ala^3, *Bj*-PRO-10c Ala^7, and *Bj*-PRO-10c Ala^8 were statistically similar to those evoked by *Bj*-PRO-10c ($p = 0.06$–0.28). Accordingly, conformational studies showed that the Ala-scan analogues from this group showed different PPII helix content when compared with native peptide. The replacement of Gln^7 did not alter very strongly the PPII helical content. The replacement of Ile^8 for Ala did not alter the bradycardic effect compared with the native peptide. Although in the native peptide the branched residue Ile^8 disfavors the PPII helix, the replacement of this residue by Ala considerably increased the PPII helix content since this amino acid strongly favors this conformation (Table 1).

In spite of the similar cardiovascular effects, the conformation of *Bj*-PRO-10c Ala^3 differs from that of the native *Bj*-PRO-10c. Although tryptophan (Trp) at position 3 weakly contributes to reduce MAP and HR, this amino acid could be important for an orally active prototype formulation. In fact, it has been shown that the side chain of Trp is required for complex formation between the native decapeptide and β-cyclodextrin (De Sousa et al. 2010). Cyclodextrins are widely used as strategy for pharmaceutical formulations, because a peptide–cyclodextrin conjugate can survive passage through the stomach and the small intestine (Uekama et al. 1998; Goldberg and Gomez-Orellana 2003).

Group 2—Amino acid residues responsible for the bradycardic effect: *Bj*-PRO-10c Ala^2, *Bj*-PRO-10c Ala^4, *Bj*-PRO-10c Ala^5, *Bj*-PRO-10c Ala^9, and *Bj*-PRO-10c Ala^{10} are the peptides which lost part of the ability of *Bj*-PRO-10c to cause a remarkable bradycardia. Moreover, the time-course of the effects also revealed different features. For example, there was an oscillatory HR profile after i.v. injection of *Bj*-PRO-10c Ala^4 and *Bj*-PRO-10c Ala^5. Strikingly, *Bj*-PRO-10c Ala^{10} initially evoked slight tachycardia. Following, a reduction in heart rate was only observed at end of the experimental period (approximately 4 h after i.v. injection).

No straightforward relationship between structure and activity was evident for all Ala-scan analogues of this group. Weak evidence could be suggested, for instance, for the increase in PPII helix content. It could be related to the decrease in ΔHR *Bj*-PRO-10c Ala^2 and *Bj*-PRO-10c Ala^5. However, better evidence is likely to occur for the replacements of Pro^4 and Pro^{10} by Ala due to the diminishment of PPII helix content of the resulting peptides. This structural modification could explain why Ala^4 and *Bj*-PRO-10c Ala^{10} specifically affect the bradycardic effect observed for the native peptide. Thus, the presence of proline at positions 4 and 10 of *Bj*-PRO-10c seems to be crucial for the peptides activities, suggesting that the ability of these residues to undergo *cis–trans* isomerization plays a key role in the activity of *Bj*-PRO-10c. In the bound state, the result suggests that the prolines in the C-terminus of *Bj*-PRO-10c could be in the *trans* configuration. Current data are in agreement with a previous study that showed a significant conformational role for the C-terminal prolyl-proline sequence in the activity of *Bj*-PRO-9a (Marlborough et al. 1981).

Taken together, the results allow us to suggest dissociation between the effects on blood pressure and on HR elicited by *Bj*-PRO-10c. This dissociation was especially clear, for example, for *Bj*-PRO-10c Ala^{10}. Considering that

Bj-PRO-10c is able to cross the blood–brain barrier (Silva et al. 2008b), it is worth hypothesizing that the described heart rate changes caused by *Bj*-PRO-10c Ala^{10} and other analogues of this group may reflect their difficulty to achieve or to act on the CNS. If this hypothesis proves to be correct, we might support that the bradycardic effect of *Bj*-PRO-10c resulted from a specific central activity of this peptide. In fact, unloading baroreceptors during an antihypertensive effect, a tachycardia would be expected (Bunag et al. 1975). Altogether, the residues Asn^2, Pro^4, His^5, Pro^9, and Pro^{10} are part of an important set of residues related not only to the vascular but also to the cardiac effects of *Bj*-PRO-10c.

Group 3—Relevant amino acid residues: *Bj*-PRO-10c Ala^1 and *Bj*-PRO-10c Ala^6 were completely inactive. Since the CD spectrum of *Bj*-PRO-10c Ala^1 did not differ from *Bj*-PRO-10c, the inactivity of *Bj*-PRO-10c Ala^1 could be explained by its susceptibility to hydrolysis by vascular aminopeptidases. Peptides are susceptible to proteolytic degradation in the blood and/or some organs as liver, kidneys, and gastrointestinal tract (Camargo et al. 2012a). However, some *Bj*-PROs are not susceptible to enzymatic proteolysis (Silva et al. 2008a, b), which is due, at least in part, to the presence of pyroglutamate (Pyr) residue at N-terminal, which can provide metabolic protection against aminopeptidase action (Isaac et al. 2009). In this regard, besides the Ala scan approach, we tested Glu replacement (*Bj*-PRO-10c Glu^1) to ensure whether Pyr is a key residue for *Bj*-PRO-10c. The complete lack of antihypertensive effect as well as a weak bradycardia found in animals treated with *Bj*-PRO-10c Glu^1 (Table 1) confirmed that cyclization/blockade of N-terminal conferred by Pyr is essential for achieving cardiovascular effects of *Bj*-PRO-10c.

An entirely different reason might explain the absence of biological activity of *Bj*-PRO-10c Ala^6. It could be related to the critical changes in structural conformation from the native peptide. This is further confirmed by the conformational analysis of *Bj*-PRO-10c Ala^6, which differed from *Bj*-PRO-10c. The occurrence of the *cis*-Pro configuration has been mostly described for Trp-Pro and Tyr-Pro sequences; it seems that in *Bj*-PRO-10c the His^5-Pro^6 exists preferentially in this configuration. The replacement of Pro^6 by Ala leads to a great increase in the content of PPII helix. The energy barrier for *cis–trans* isomerization of peptide bonds is much lower for the imino acid Pro than for the other amino acids. Therefore, replacing Pro^6 by Ala would favor an increase in PPII content, supporting the notion that the His^5-Pro^6 sequence exists preferentially in the *cis* configuration. In such configuration, -X-Ar-Pro-X- sequences (where Ar = aromatic) give rise to type VI β-turns (Meng et al. 2006; Thomas et al. 2006). The data suggest that the *cis* configuration is required at the binding site(s) for both MAP and HR activities.

The present study allowed us to identify that *Bj*-PRO-10c displays two independent cardiovascular activities (MAP and HR), which was dissociated when distinct Ala-scan analogues were applied. A second important finding was that the Pyr^1 and Pro^6 residues are essential for both antihypertensive and bradycardic effects in SHR.

Bj-PROs are able to evoke a diverse spectrum of biological activities (Camargo et al. 2012b). In particular, the biological activities described for the *Bj*-PRO-10c include bradykinin potentiation (Ianzer et al. 2007), inhibition of ACE (Hayashi et al. 2003), activation, increase in NO and L-Arg production (Guerreiro et al. 2009), which may modulate the action at the central and the peripheral nervous system. The hypothesis that a number of signaling events could happen due to promiscuous interactions of these peptides with domain-binding sites of proteins [PDZ domains, for instance—PDZ domains are abundant protein interaction modules that often recognize short amino acid motifs at the C-terminal of target proteins (Lee and Zheng 2010)], should be considered. In fact, a large number of biological activities are likely to occur upon interaction between a particular region in one protein and a short peptide stretch (Neduva and Russell 2006).

Conclusion

The presented manipulations in the primary structure of the *Bj*-PRO-10c allowed improving the knowledge about this molecule. It showed that *Bj*-PRO-10c may be a leader prototype to develop at least two new agents, specifically driven to treat either specific targets, such as high blood pressure or tachycardia.

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Conflict of interest The authors declare that they have no conflict of interest.

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