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ORIGINAL ARTICLE

Short peptide constructs mimic agonist sites of AT_1R and BK receptors

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Abstract Extracellular peptide ligand binding sites, which bind the N-termini of angiotensin II (AngII) and bradykinin (BK) peptides, are located on the N-terminal and extracellular loop 3 regions of the AT_1R and $BKRB_1$ or $BKRB_2$ G-protein-coupled receptors (GPCRs). Here we synthesized peptides P15 and P13 corresponding to these receptor fragments and showed that only constructs in which these peptides were linked by S–S bond, and cyclized by closing the gap between them, could bind agonists. The formation of construct-agonist complexes was revealed by electron paramagnetic resonance spectra and fluorescence measurements of spin labeled biologically active analogs of AngII and BK ($Toac^1$-AngII and $Toac^0$-BK), where Toac is the amino acid-type paramagnetic and fluorescence quencher 2, 2, 6, 6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid. The inactive derivatives $Toac^3$-AngII and $Toac^3$-BK were used as controls. The interactions characterized by a significant immobilization of Toac and quenching of fluorescence in complexes between agonists and cyclic constructs were specific for each system of peptide-receptor construct assayed since no crossed reactions or reaction with inactive peptides could be detected. Similarities among AT, BKR, and chemokine receptors were identified, thus resulting in a configuration for AT_1R and BKRB cyclic constructs based on the structure of the $CXCR_4$, an α-chemokine GPCR-type receptor.

Keywords Angiotensin II · Bradykinin · Receptor · Toac · Peptide–peptide interaction

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Introduction

In globular proteins, amino acids are packed into folds by a general process (Wetlaufer 1973; Janin and Chothia 1985; Grishin 2001) but in membrane proteins some special rules need to be considered in this mechanism (Daley 2008). The lipid bilayer forms an interface with a membrane protein thus stabilizing segments of this macromolecule into bundles of transmembrane (TM) α-helices (Palczewski et al. 2000; Li et al. 2004; Cherezov et al. 2007) or β-strands (Marzin et al. 1994). Allowing the bundle assembly, each one of the TMs, after spanning the membrane, is connected by an extra-membrane segment to a neighbor TM. In contact with different environments, varying from the polar phospholipid head region to the adjacent aqueous medium, the extra-membrane linking groups are sometimes forming loci with ability for binding specific ligands.

This hypothesis was herein addressed by studying the extracellular portion of the agonist binding site in angiotensin II (AngII) AT_1 receptor (for a review see Oliveira et al. 2007). As a typical member of the rhodopsin-like family A of G-protein-coupled receptors (AGPCRs), the receptor AT_1R (see Vroling et al. 2011 for GPCRDB) has a basic fold of a seven-transmembrane-helix bundle (7TM) consisting of an extracellular N-terminal segment (N_t), a cytosolic C-terminal segment (C_t) including the helix VIII, three extracellular (EC) loops, and three cytosolic (IC) loops alternately connecting the seven helices (Fig. 1a).

Twenty years ago when most sequences of GPCRs were uncovered (see Vroling et al. 2011 for GPCRDB), it was verified that AngII, bradykinin (BK), endothelin, chemokine, purine, and Cys-leukotriene receptors, and some types of neuropeptide receptors, possessed a unique extracellular motif (Fig. 1a) (Correa et al. 2006), consisting of a 8–10 residue insertion in the middle of extracellular loop 3 (EC3 loop), including a conserved residue of Cys^{650} supposedly forming a disulfide bond with a second conserved Cys^{100} residue located at the N_t segment (Correa et al. 2006) (see Fig. 1b for normalized numbering of receptors and Fig. 1c for identification of motif residues).

In AT_1 receptors, this motif has previously been investigated for function. Assays searching for specific binding of AngII to mutated forms of receptor showed that the peptide affinity strongly depends on the binding of its C-terminal carboxylate to receptor helix V Lys^{512}, and specially on the binding of its N-terminal D^1 and R^2 residues to EC3 loop Asp^{709} and Asp^{712} and N_t Arg^{105} residues, respectively (Hjorth et al. 1994; Noda et al. 1995; Feng et al. 1995; Costa-Neto et al. 2000) (Fig. 1d). These findings indicated that the special disulfide bond residue insertion motif might constitute an extracellular site for binding of the N-terminal (D^1R^2) segment of the peptide (Fig. 1e).

A preliminary study to mimic the extracellular binding site of GPCRs, involving AT_1R constructs and $Toac^1$-AngII, was previously carried out by means of electron paramagnetic resonance (EPR) techniques leading to the definition of specific binding (Lopes et al. 2008). Currently, in this study on this receptor were complemented and a complete procedure was carried out with BK's $BKRB_1$ and $BKRB_2$ receptors. As before, all these proteins were investigated using amino acid constructs consisting of N_t and EC3 loop segments of extracellular sites known to form the core of agonist binding site (Fig. 1e). These segments were stabilized by covalently linking the segments into a pseudo-cyclic geometry either through the formation of peptide bond or through the insertion of a spacer disulfide bond and closing the gap between the C- and N-terminal ends of their N_t and EC3 loop segments through a peptide bond or by the insertion of an spacer (Fig. 1f). Results obtained show that the intended mimicry was apparently successful since, as shown by EPR and fluorescence data, the constructs could bind specifically but only to their original agonist-peptides. The strategy for cyclization drove an organization of the construct structures (site constitution), as revealed by CD analysis, thus suggesting that the procedure utilized gave rise to a system that seems to be able to substitute the membrane role in the assembly of the receptor extracellular site.

Materials and methods

All reagents and solvents were analytical grade and were used from freshly opened containers without further purification. Protected amino acids and protected amino acid-resins were purchased from Bachem (Torrance, CA). Peptides were synthesized by the solid phase methodology using the tert-butyloxycarbonyl (Boc) group for N^{α}-amino acid protection (Barany and Merrifield 1980; Kates and Albericio 2000). Toac-labeled peptides were synthesized according to the synthesis strategy (Marchetto et al. 1993) which combines the Boc and Fmoc (Fields and Noble 1990)-N^{α}-temporary protecting groups. Peptide purification was carried out on a Waters 510 HPLC instrument using a Vydac C_{18} preparative column (22-mm internal diameter, 250-mm length, 70-Å pore size, 10-µm particle size). Peptides were dissolved in 1 % acetic acid solution and sonicated and centrifuged at 10,000*g*. After filtration, each solution was loaded onto the column and eluted with a linear gradient using the solvent systems A [H_2O containing 0.1 % TFA (v/v)] and B [60 % acetonitrile in H_2O containing 0.1 % TFA (v/v)]. Linear gradients were used for elution of peptides (25–55 % B) or (45–75 % B) in 90 min, with a flow rate of 10 mL/min and UV detection at 220 nm. The fractions were screened under an isocratic condition in a Chromolit C_{18} analytical column.

Pure fractions were pooled, lyophilized, and characterized for homogeneity by analytical HPLC (Waters Associates, Milford, MA, USA). Unless otherwise stated, peptides were analyzed on a Phenomenex® C_{18} column (4.6 × 150 mm), 300-Å pore size and a 5-µm particle size using the solvent systems: A [H_2O containing 0.1 % TFA (v/v)] and B [60 % (condition A) or 90 % (condition B) acetonitrile in H_2O containing 0.1 % TFA (v/v)]. A linear gradient of 10–90 % B in 30 min was applied at a flow rate of 1.5 mL/min and detection at 220 nm. Mass spectrometry was performed on LC/ESI–MS equipment (Micromass, Manchester, UK) using a Compaq AP200 workstation. The samples were automatically injected on a Waters narrow bore Nova-Pak C_{18} column ((2.1 × 150 mm), 60 Å pore size and 3.5 µm particle size). The elution was carried out

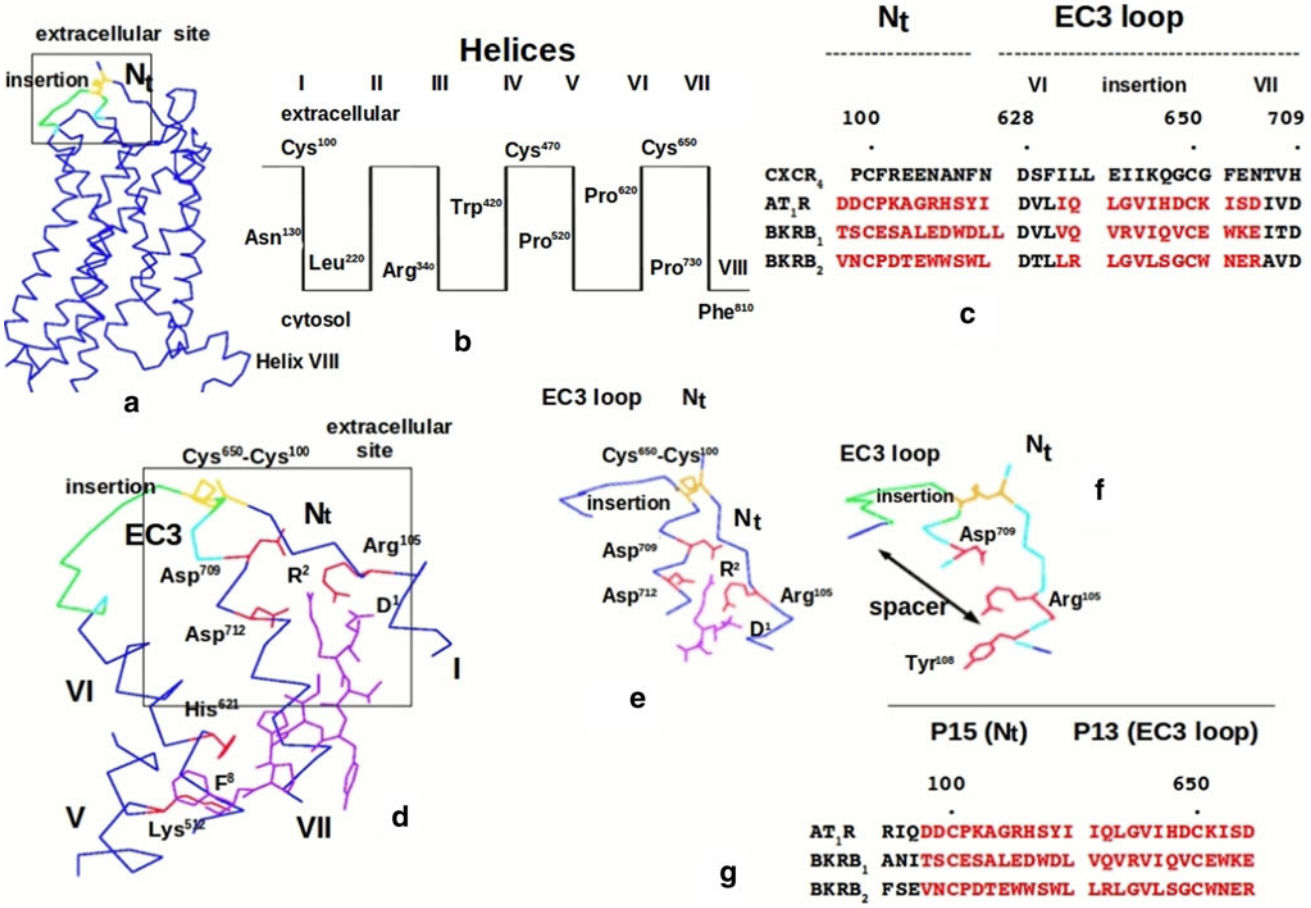

Fig. 1 **a** *AGPCR 7TM bundle* showing an insert of the extracellular site with the EC3 loop and N_t segments in *light blue* and second disulfide bond in *gold*. Note the insertion of residues in the N-terminus of EC3 loop (*green*) and the cytosolic helix VIII at the bottom of the figure. **b** *Normalized numbering system for residue positions of AGPCRs*. For each segment of the secondary structure, the most conserved position is the reference for numbering the other positions of the same segment. The numbers have three digits: the first is the helix (1–8 and loops between them), the second and the third ones are the relative location to the reference. References: Cys^{100}: N_t segment; Asn^{130}: hel I; Leu^{220}: hel II); Arg^{340}: hel III; Trp^{420}: hel IV; Cys^{470}: EC2 loop; Pro520: hel V; Pro^{620}: hel VI; Cys^{650}: EC3 insertion; Pro^{730}: hel VII; Phe^{810}: hel VIII. **c** *Sequence alignment of EC3 loop and N_t segments* used to model the extracellular site structure of AT_1R and BKRB receptors to the respective structure of $CXCR_4$ receptor by homology (Wu et al. 2010). Note the insertion of residues in the middle of EC3 loop and the two residues of Cys (100 and 650) which form the second disulfide bond. **d** *AT_1 receptor agonist binding site*. AngII C-terminal carboxylate and N-terminal residues (*magenta*) interact with receptor helix V Lys^{512} and the extracellular site (in *red* at the other side of the structure), respectively. **e** *AT_1 receptor extracellular site*. The AngII N-terminal segment D^1 and R^2 residues (*magenta*) interact with receptor Asp^{709} and Asp^{712} in EC3 segment and Arg^{105} in the N_t segment (*red*), respectively. **f** *Extracellular site of the AT_1 receptor* with the C-terminal carboxyl of the N_t segment (peptide P15) linked by a spacer or a peptide bond to the N-terminal amino group of the EC3 loop segment (peptide P13) and the disulfide bond between Cys^{100} and Cys^{650} set up, thus forming a closed or cyclized state of receptor. **g** *Sequence alignment of* N_t (peptide P15) and EC3 loop (peptide P13) segments of receptors used to build the extracellular site constructs. The residues shown in *red* correspond to the residues in red of Fig. 1c. Positions of Cys^{100} and Cys^{650} are shown as reference. All over the text, peptide residues are expressed as a single capital letter thus differing from the three-letter code used for protein residues

with solvents A [0.1 % TFA/H_2O (v/v)] and B [60 % acetonitrile/0.1 % TFA/H_2O (v/v)] at a flow rate of 0.4 mL/min using a linear gradient from 5 to 95 % B in 30 min. The condition used for mass spectrometry measurements was a positive ESI. Amino acid analysis was performed on a Biochrom 20 Plus amino acid analyzer (Pharmacia LKB Biochrom Ltd., Cambridge, England) equipped with an analytical cation-exchange column. Analytical characterization (HPLC and LC/ESI–MS spectra) of synthesized receptors' constructs are displayed in Supplementary Materials section.

Synthesis of peptide constructs

Sequence constructs mimicking the extracellular sites of AT_1R, $BKRB_1$, and $BKRB_2$ receptors, consisting of N-terminal (N_t) and EC3 segments (Fig. 1g) were synthesized. As mentioned, AngII and BK were labeled with the

spin probe Toac (Rassat and Rey 1967) according to a synthesis strategy which makes use of two combined protocols (Nakaie et al. 1981; Marchetto et al. 1993). This probe was the first spin label incorporated in a peptide backbone directly via peptide bond and has been applied for a great variety of purposes (Toniolo et al. 1995; Smythe et al. 1995; Barbosa et al. 1999; Victor and Cafiso 2001; Karim et al. 2004; Marsh et al. 2007; Shafer et al. 2008; Van Eps et al. 2010), including physico-chemical solvation study of peptide-polymer beads targeting the improvement of the peptide synthesis methodology (Cilli et al. 1999; Oliveira et al. 2002; Marchetto et al. 2005; Zhang et al. 2007).

The observed increasing trend in the use of this cyclic paramagnetic amino acid-type probe is likely due to some advantageous properties such as the great sensitivity: (1) to detect motion and orientation of coupled macromolecules as a consequence of its linking via a peptide bond and rigid binding induced by its constrained $C^{\alpha\alpha}$-tetrasubstituted cyclic structure, where the rotation about side chain bonds is hampered by incorporation of the nitroxide nitrogen and C^{α}, C^{β}, and C^{γ} atoms into the same heterocyclic moiety; and (2) to polarity of medium (Dupeyre et al. 1964; Malavolta et al. 2008). Moreover, Toac is also a strong fluorescence quencher molecule (Pispisa et al. 2003; Venanzi et al. 2004) thus allowing the assessment of intermolecular interactions involving fluorophore groups. The potential of Toac for applications in different biochemical and chemical fields were described earlier (Wilson 2000) and also in a recent review report (Schreier et al. 2012). In this context, the present work relied upon the study of binding properties of AT_1R, $BKRB_1$, and $BKRB_2$ constructs with active $Toac^1$-AngII and $Toac^0$-BK analogs and with inactives $Toac^3$-AngII and $Toac^3$-BK (Nakaie et al. 2002), used as controls.

Monitoring of agonist-receptor constructs interactions

EPR spectra were obtained in a Bruker ER 200D-SRC spectrometer at room temperature. Samples were prepared at concentration of 5×10^{-5} M (0.02 M phosphate buffer, pH 7 at 22 ± 2 °C) of Toac peptides and receptor constructs and were placed in flat quartz cells for aqueous solutions (Wilmad). The rotational correlation time (τ_C) values (Kivelson 1964) were calculated as described elsewhere (Cannon et al. 1975). Static fluorescence spectra were obtained at room temperature in a Hitachi F2500 spectrofluorimeter. For AngII, Toac-AngII and AT_1R constructs, the excitation wavelength was 275 nm. For BK, Toac-BK derivatives and Trp-bearing $BKRB_1$ and $BKRB_2$ constructs, the excitation wavelength was 295 nm. In solutions containing a single peptide, the concentration was 10^{-4} M (0.02 M phosphate buffer, pH 7) and when mixed, at 1:1 (v/v) proportion. CD spectra were obtained on a Jasco J-810 spectropolarimeter at room temperature continually flushed with ultra-pure nitrogen. Peptide concentration was: 10^{-4} M in 0.02 M phosphate buffer, pH 7.0 or with addition of TFE (up to 90 %, v/v). Equivalent results were found in triplicate EPR, fluorescence, and CD experiments.

Models of receptor extracellular sites

The $CXCR_4$ chemokine receptor is the first AGPCR presenting the extracellular disulfide bond residue insertion motif for which a high-resolution structure has been determined (Wu et al. 2010). Thus, more accurate models of the extracellular site of AT_1R and BKRB receptors were built by WHAT IF program (Vriend 1990) in homology to the chemokine receptor according to the aligned sequences in Fig. 1c. This procedure consisted in transferring the 3D coordinates for main-chain residues of $CXCR_4$ receptor to the corresponding residues of other receptors. Final rearrangement of residue side chains and refinement of the structures are automatically carried out in a final step.

Results

Receptor constructs

The peptides P15 and P13 (N_t and EC3 loop segments, respectively) taken from the extracellular site of AT_1R and BKRB receptors (Fig. 1g) were used to build constructs as follows: (1) open state (P15–P13)o, where only the disulfide bond between Cys^{100} and Cys^{650} linking P15 to P13 was set allowing free rotation of the structure; and (2) cyclic state (P15–X–P13) where the C-terminus of P15 was connected to the amino group of P13 and cyclized by S–S bond as the constructs shown in Fig. 1e, f. In the latter derivatives, preliminary EPR experiments with Toac-labeled AngII (Lopes et al. 2008) showed that the best results were found using the [–$(CH_2)_6$–] or (C_6) instead of a single peptide bond or the C^{α}-aminoundecanoic acid (C_{11}) as the X spacer. Thus, this spacer was selected to build all BKRB receptors cyclic structures. EPR and fluorescence experiments were performed to examine construct-agonist interaction. CD spectroscopy was used to analyze peptide secondary structure.

EPR studies

Figure 2 shows the EPR spectra of Toac-bearing AngII or BK alone and mixed with C_6– spacer containing cyclic constructs of AT_1R, $BKRB_2$, and $BKRB_1$ receptors. Line broadening was observed when agonists were mixed with

their corresponding cyclic receptor constructs (Fig. 2b, d, e). Such spectral changes indicate a decrease in the mobility of spin labeled molecules and, in the present case, can be ascribed to the occurrence of agonist-construct interaction. Figure 3 presents rotational correlation times (τ_C) calculated from the spectra as well as measured hyperfine splittings (a_N). This figure shows that the values of τ_C increased in the spectra of the Toac-containing agonists only in the presence of their corresponding P15–X–P13 cyclic constructs (Fig. 3a, b), indicating a significant decrease in the labeled peptides tumbling rates upon interaction with their respective constructs. In the case of Toac^1-AngII (Fig. 3a), a nearly threefold increase of τ_C was observed (from about 2×10^{-10} s to 6×10^{-10} s). Interestingly, Toac^0-BK interacted with both $BKRB_1$ and $BKRB_2$ cyclic constructs, the increase in τ_C being larger for the latter fragment (Fig. 3b). It is noteworthy that complex formation was not observed for Toac^1-AngII in the presence of cyclic $BKRB_1$ or $BKRB_2$ fragments and, conversely, Toac^0-BK was not responsive to the addition of AT_1R constructs. These results point to a specific agonist-receptor binding for the Toac-labeled AngII and BK peptides.

These conclusions are also in agreement with variations of a_N (Fig. 3c, d), a parameter sensitive to the polarity of the medium, increasing with increasing polarity. In the current study, significant decreases in a_N were detected in the EPR spectra of Toac^1-AngII and Toac^0-BK only upon addition of their corresponding cyclic receptor constructs (Fig. 3c, d). The a_N value in the spectra of Toac^1-AngII varied, for instance, from 16.5 G to 15.9 G upon addition of the AT_1 receptor P13–C_6–P15 construct (Fig. 3d). Similar changes were observed for Toac^0-BK (Fig. 3d) in the presence of its respective $BKRB_1$ and $BKRB_2$ constructs. This finding suggests that the Toac moiety in the labeled agonists is located in a less polar environment, provided by the intermolecular peptide-construct complex. As observed for τ_C values (Fig. 3a, b), no variation in a_N was detected when non-specific constructs were added to Toac^1-AngII or Toac^0-BK (Fig. 3c, d). In addition, the biologically inactive Toac^3-AngII and Toac^3-BK analogs did not display intermolecular interactions with any of the receptor constructs, including those of cyclic structure (see Supplementary Figure 1).

Fluorescence studies

Fluorescence spectra (Fig. 4) indicated that when both native and N-terminally Toac-labeled agonists were mixed with their respective cyclic constructs, a considerable decrease in fluorescence intensity was observed. Fluorescence reduction upon complex formation might be caused by quenching promoted by close contacts of groups present in the interacting peptides and by the exposure of fluorophores to a different environment due to rearrangements in peptide conformation. In addition, in the case of Toac-labeled

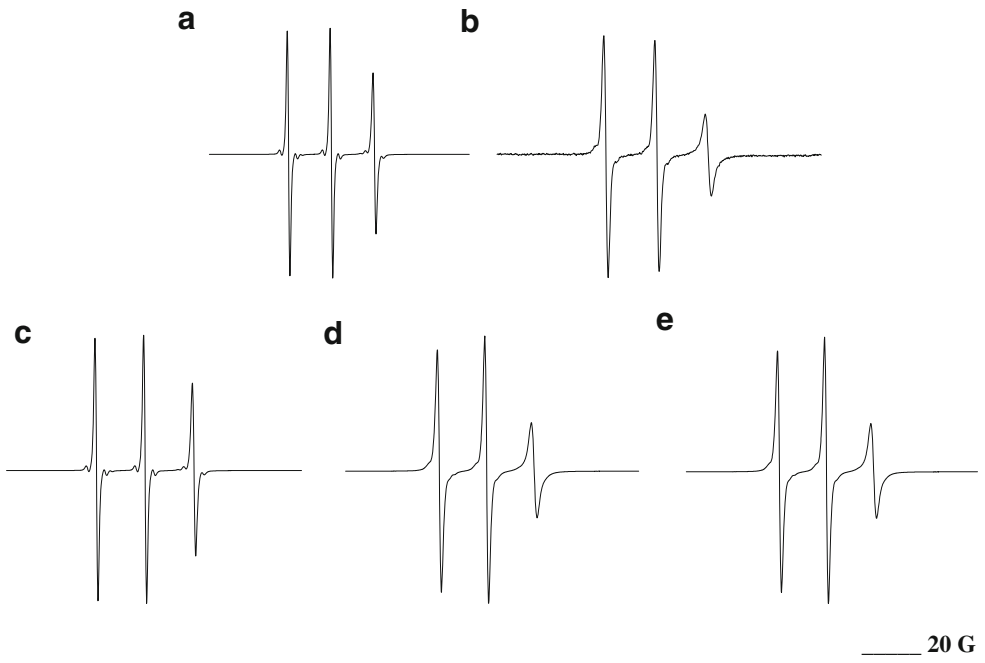

Fig. 2 EPR spectra of aqueous solutions of: *top*, Toac^1-AngII in the absence (**a**) and the presence (**b**) of the AT_1 receptor construct containing the –C_6– spacer, P15–C_6–P13; *bottom row*, Toac^0-BK in the absence (**c**) and the presence of the $BKRB_1$ (**d**) and $BKRB_2$ (**e**) receptor constructs containing the –C_6– spacer, P15–C_6–P13. The scan width was 100 G

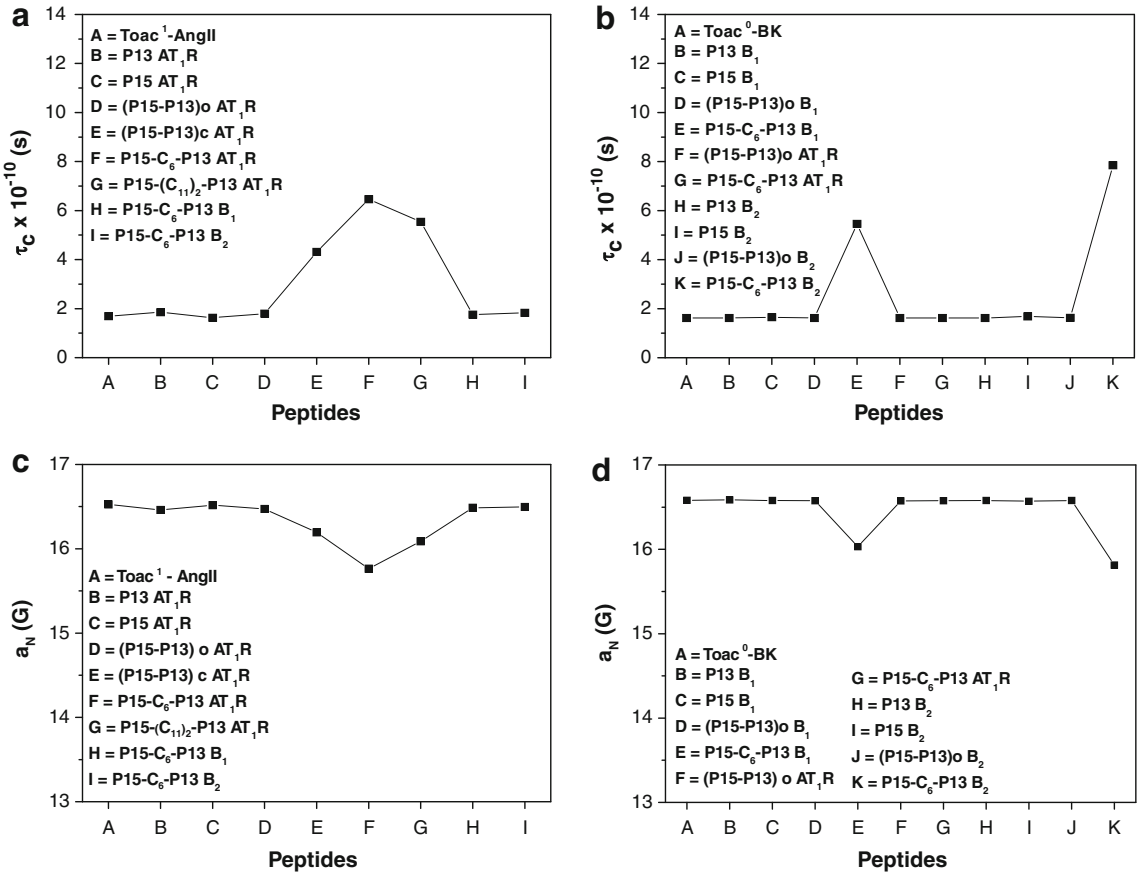

Fig. 3 Values of τ_C (**a**, **b**) and a_N (**c**, **d**) calculated from the spectra of Toac[1]-AngII and Toac[0]-BK in the absence (*A*) and in the presence of several AT_1R, $BKRB_1$, and $BKRB_2$ receptors constructs (*B–K*). All constructs containing both P15 and P13 are linked by a disulfide bond. (P15–P13)o, open peptide; (P15–P13)c, cyclic peptide, linked by a peptide bond between P15 C-terminus and P13 N-terminus; P15–C_6–P13 and P15–$(C_{11})_2$–P13 cyclic peptides, with a $(–CH_2)_6–$ and $[(–CH_2)_{11}]_2$*—spacers, respectively, linking P15 C-terminus to P13 N-terminus. *[α-amino-undecanoic acid]$_2$. Compounds *A*: Toac agonist with no receptor peptide

peptides, fluorescence quenching could occur as a result of the proximity of the paramagnetic nitroxide moiety to a fluorescent group. This effect is known to often occur intramolecularly (Toniolo et al. 1998), as seen in the case of Toac[1]-AngII where the proximity of Toac[1] to Tyr[4] causes a decrease of approximately 40 % of the peptide's fluorescence (compare Fig. 4a, b), in agreement with previous work (Vieira et al. 2009).

A quantitative analysis of the data in Fig. 4 (Table 1) reveals that the loss of fluorescence intensity was similar for both native and Toac-labeled agonists upon complex formation with their respective receptor constructs, suggesting that the Toac moiety does not play an important role in the fluorescence quenching mechanism. Interestingly, a different pattern was, however, found in the values of emission wavelengths (Table 1): these values were reduced in the spectra of agonist-construct complexes formed by AngII, Toac[1]-AngII, and BK (shifts of ca. 4 and 10 nm, respectively) but were practically unchanged in the spectra of complexes formed by Toac[0]-BK. This difference could suggest that the complex formed between the latter peptide and the BK receptor constructs displays a different molecular organization. The possibility could be that the Toac group may be constraining the BK peptide and thus reducing its affinity and time of interaction.

Essentially no effects were observed for systems containing P13, P15, or open state (P15–P13)o peptides. Moreover, no change in fluorescence intensity was observed when inactive Toac[3]-AngII and Toac[3]-BK were mixed with any of their respective receptor constructs, including those containing the $–C_6–$ spacer (data collectively shown in Supplementary Figures 2–10). These results thus corroborate the EPR findings and point to the fact that interactions of AngII and BK and their active Toac-attaching derivatives occur in a specific mode only with cyclic structures containing P15 (N_t) and P13 (EC3) segments that seem to be able to mimic the native arrangement of these portions of the AT_1R and BKRB receptors.

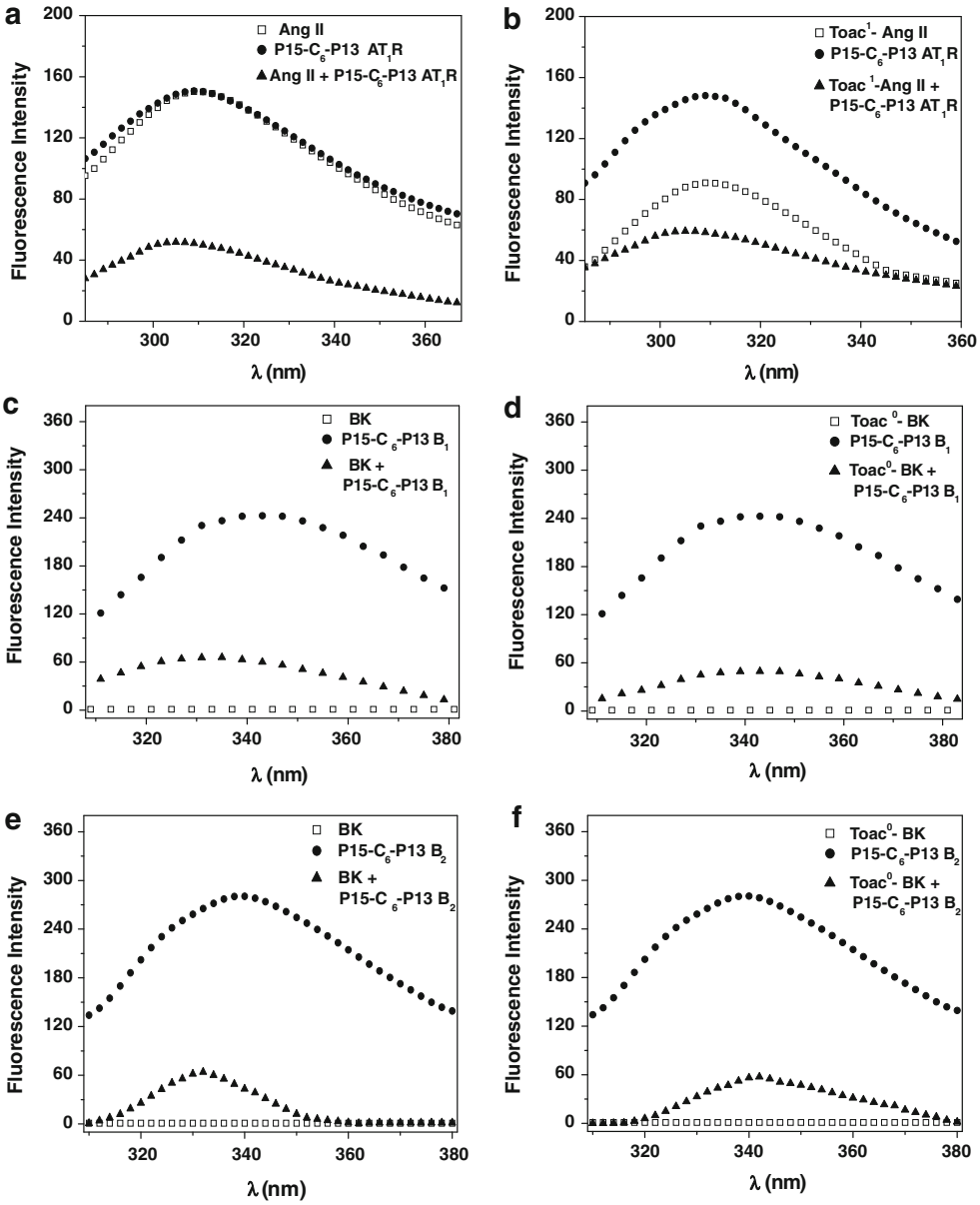

Fig. 4 Fluorescence intensity of solutions containing native AngII and BK (*left*) and their N-terminally labeled analogs Toac^1-Ang II and Toac^0-BK (*right*), their respective cyclic constructs containing the –C_6– spacer (P15–C_6–P13), and of solutions containing agonist plus construct. In the case of AngII and Toac^1-Ang II, the construct corresponds to the AT_1 receptor (**a**, **b**), and in the case of BK and Toac^0-BK, the constructs correspond to the $BKRB_1$ (**c**, **d**) and $BKRB_2$ (**e**, **f**) receptors

Table 1 Percent loss of fluorescence intensity in the spectra of agonists in the presence of their corresponding cyclic constructs and wavelengths of maximal emission (λ_{max}) of constructs alone and in the presence of agonists

Peptide solution	Loss of intensity (%)	Construct λ_{max} (nm)	Construct + Agonist λ_{max} (nm)
AngII + AT_1R (P15–C_6–P13)	65	310	306
Toac^1-AngII + AT_1R (P15–C_6–P13)	53	310	306
BK + BKB_1 (P15–C_6–P13)	47	342	332
Toac^0-BK + BKB_1 (P15–C_6–P13)	56	342	342
BK + BKB_2 (P15–C_6–P13)	56	340	332
Toac^0-BK + BKB_2 (P15–C_6–P13)	58	340	342

Excitation wavelengths of 275 and 295 nm were used for AT_1R and BKRB constructs, respectively

Structural features of receptor constructs

The CD spectroscopy (Fasman 1996) was selected for examining structural characteristics of all synthesized AT_1R, $BKRB_1$, and $BKRB_2$ constructs. Accordingly to the CD theory, the measured spectral θ (ellipticity, deg cm^2 $dmol^{-1}$) values vary according to the localization of different excitable chromophores (in the far-UV region) existing in the macromolecule structure. Thus, CD curves given by θ values as a function of the applied wavelength may give rise to typical spectum, depending upon the peptide conformation. In contrast to an extended structure where a minimum θ value is observed at about 195 nm, a more constrained conformation such as a typical α-helix is usually characterized by a positive (∼195 nm) and two negatives (∼208 and 220 nm) bands in the CD spectrum. In this context, the curves displayed in Fig. 5a revealed a propensity of AT_1R- and $BKRB_2$-P15–C_6–P13 cyclic constructs to acquire α-helical conformation in aqueous solution, although this trend seemed less pronounced for the corresponding $BKRB_1$ construct. Conversely, less organized structures were found for the open (P15–P13)o analogs (Fig. 5b).

In complement, CD curves of AT_1R, $BKRB_1$, and $BKRB_2$-P15–C_6–P13 constructs were also comparatively evaluated but in the presence of TFE, a known secondary structure inducing solvent that would in part mimic membrane environment. The Supplementary Figure 11 reinforced the observed trend of these cyclic peptides where they revealed more pronounced helicoidal conformation in TFE than in aqueous solutions (Fig. 5a). Contrariwise, only random coil-type structures were observed for all linear P13, P15, and (P15–P13)o constructs synthesized (Supplementary Figs. 12–14). These findings thus point to the existence of a characteristic structural feature for cyclic constructs, forming a specific cluster in the binding site of these GPCR-type receptors.

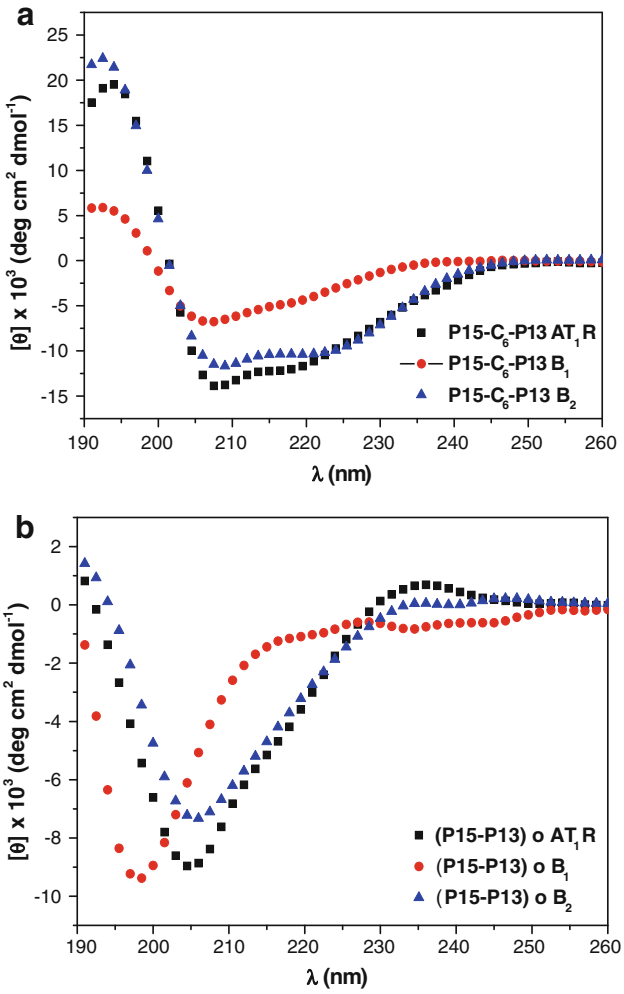

Fig. 5 CD spectra of P15–P13 constructs of AT_1R, $BKRB_1$, and $BKRB_2$ receptors in aqueous solution. **a** Cyclic constructs containing the –C_6– spacer; **b** open constructs

Discussion

In previous studies, we have examined the conformational properties of AngII and BK and their Toac-labeled analogs in solution and in membrane-mimetic environments (Schreier et al. 2004; Vieira et al. 2009). Concerning the structure of these receptors, the investigations carried out have been limited to physico-chemical studies of loop fragments through several experimental approaches (Franzoni et al. 1997; Pertinhez et al. 1997; Salinas et al. 2002). Here we introduce a multi-spectroscopic approach in combination with models of agonist-construct interaction to study the multiple events occurring at the interface of AT_1R and BKRB receptors during their activation. In the present work the spectroscopic data were analyzed in light of 3D structures, resulting in a more comprehensive framework of the mechanisms studied.

Toac^1-AngII binding to AT_1 receptor constructs

Both EPR and fluorescence experiments provide solid indication that the native and N-terminal Toac-derivative forms of AngII specifically bind their constructs but only when they are stabilized in a closed configuration (Fig. 6a–c). An immediate question may be asked regarding this finding. Would the detected binding be like the native binding between the peptides and respective receptors? As shown in Fig. 1d for the AT_1 receptor-AngII system, the peptide N-terminal D^1 and mainly the R^2 residue are crucial to the formation of a binding complex with receptor extracellular site (specially with EC3 loop Asp^{709} and Asp^{712} and N_t Arg^{105} residue) (Hjorth et al.

1994; Feng et al. 1995; Le et al. 2002). For this condition to be held, it would be required that the N_t and EC3 loop segments involved in binding were contiguous in the receptor structure, a feature that was observed in the 3D structure of a $CXCR_4$ chemokine receptor (Wu et al. 2010) and thus, due to strong sequence similarities (Fig. 1c, g), was transported to the current AngII and BK receptor models.

Interestingly, a modification of the AT_1R construct configuration (Fig. 6b) could be made by folding the C-terminal sequence of its P13 or EC3 loop segment (after the Cys^{650} residue) so that it would form a parallel arrangement with the C-terminal sequence of its P15 or N_t segment (after the Cys^{100} residue). The resulting configuration (Fig. 6c) is consistent with the receptor native structure (Fig. 1d–f) since it has in the core, the side chain

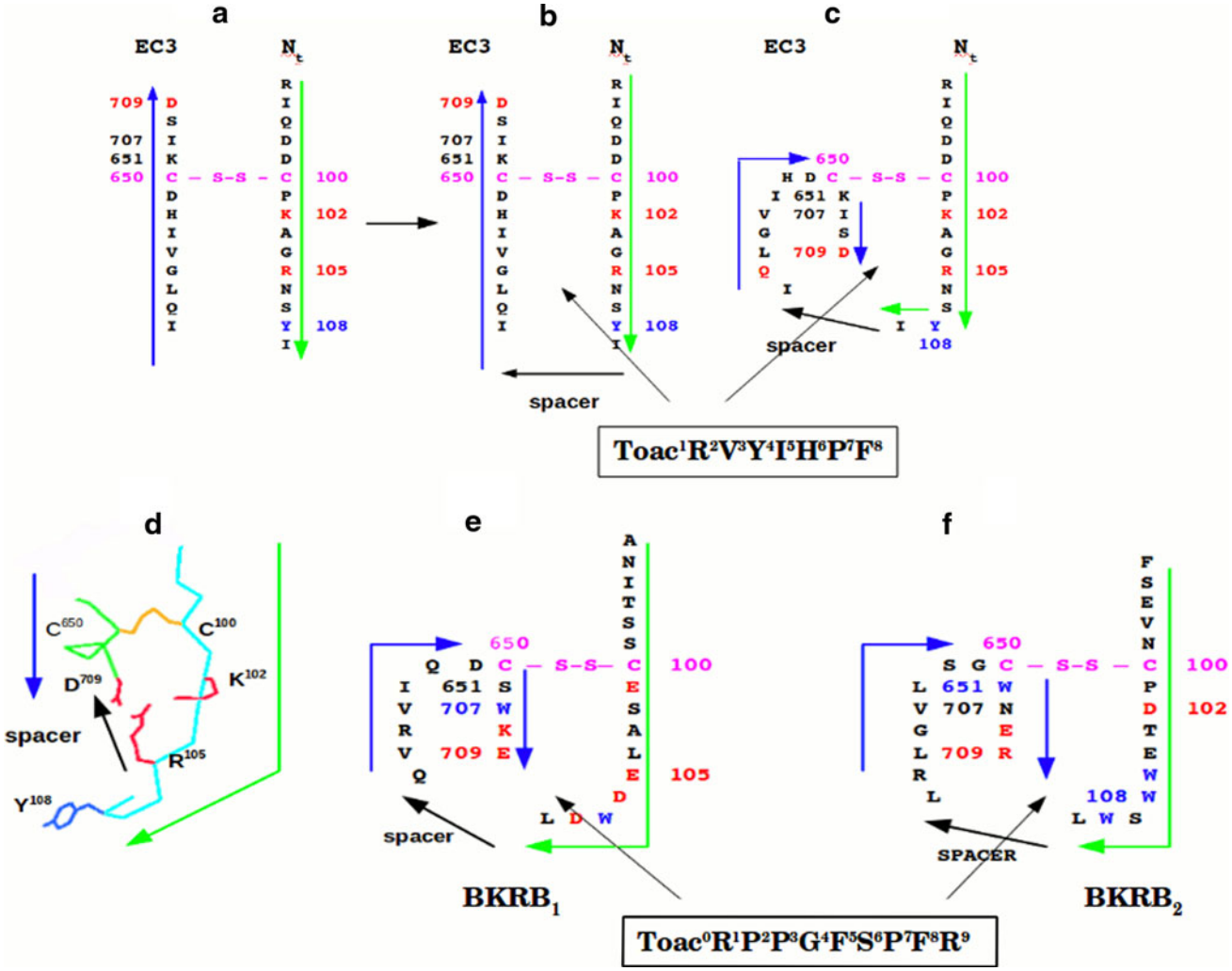

Fig. 6 *Constructs of the AT_1R and BKRB receptor extracellular site*. For clarity, the path followed by the sequences of N_t and EC3 loop segments along the constructs in the figures are demarked by *blue* and *green* traces, respectively. Cys^{100}-Cys^{650} disulfide bond is shown in gold, charged residues are red and aromatic residues, some of them fluorophores, are blue (**a**). An inactive form (no binding to AngII and random structure) of the receptor is shown in which the segments N_t and EC3, connected by a disulfide bond, can rotate around this bond and face each other in a random fashion. **b** A state in which the segments are linked in series (N_t segment preceding EC3 segment) by a spacer of methylene groups, or by a peptide bond, and thus have a cyclic and organized configuration. It has a high content of helicoidal structures and is active since it can bind $Toac^1$-AngII. **c** New form of active receptor showing the C-terminal end of the EC3 segment folded and near the N_t segment thus mimicking the native-receptor-like arrangement of the extracellular site sequences (Fig. 1d). Two arrows point to the site at which the N-terminal residues of $Toac^1$-AngII are supposed to bind. **d** The molecular model of the AT_1R extracellular site is shown with a same scheme. Note the limit of membrane in the upper half of the figure and the insertion of residues at the left top. For the $BKRB_1$ (**e**) and $BKRB_2$ (**f**) receptor extracellular sites, the same active and folded configuration of AT_1R constructs were built

of EC3 loop (P13 segment) Asp^{709} close to the N_t (P15 segment) Arg^{105}.

$Toac^0$-BK binding to BKRB receptors constructs

Similar to what was observed with $Toac^1$-AngII (EPR and fluorescence studies in Figs. 2, 3, 4), $Toac^0$-BK only interacted with its specific BKRB receptor cyclic constructs. These compounds were now built (Fig. 6e, f) following the same assumptions considered for building the AT_1R construct structure (Fig. 6a–c). In accordance with the specificity of $Toac^0$-BK to equally bind to $BKRB_1$ and $BKRB_2$ cyclic constructs, the N-terminal end of this agonist molecule may be placed in the construct loci as AngII was placed in the AT_1 receptor (Fig. 6e, f). A fact reinforcing this idea is that BK Arg^1 residue is pharmacologically for BKRB receptors while the AngII Arg^2 is for AT_1 receptor (Marceau and Regoli 2004). The importance of Arg residues for functioning of BKRB and even AT_1 receptors is consistent with two points: (1) the side chain of this residue is able to make hydrogen bonds by giving protons to carboxyl or carbonyl groups; and (2) BKRB receptors have the core of their extracellular site constructs displaying a large number of acidic Asp or Glu side chains (5 for the type 1 receptor and 3 for the type 2) (Fig. 6e, f).

Electrostatic interaction for peptide binding to construct

For both AT_1R and BKRB receptors, an electrostatic interaction involving peptide Arg residues (R^2 for AngII and R^1 for BK) and acidic side chains (Asp and Glu residues) of receptors' constructs has been the determinant of binding. However, there is discordance between this interpretation and other data presented in this work. Besides indicating peptide binding to constructs, EPR and fluorescence assays also revealed decrease of Toac a_N values for bound labeled AngII and BK (Fig. 3c,d) thus suggesting that in both bound peptides the coupled nitroxide group is located in a less polar environment. This means that Toac groups could not interact like Arg residues at a locus with charged residues but at a different place.

Toac is known to significantly quench the fluorescence of Y^4 residue when placed at the AngII position 1 (Vieira et al. 2009). Table 1 data reveal a same level of fluorescence quenching when labeled- or not labeled-peptide is bound to constructs thus attesting that quenching depends on the binding itself but not on the Toac presence. Thus, it is plausible to assume that upon formation of a binding complex, the nitroxide groups of $Toac^1$ or $Toac^0$ may be oriented apart from the R^2 side chain (in AngII) or from the R^1 side chain (in BK), and thus be positioned at a non-polar environment. On the other hand, Arg residues that are certainly involved in the binding itself, should be oriented toward the acidic residues thus interfering more or less with the fluorophores located at different positions of the site, Y^{108} in AT_1R construct (Fig. 6c, d) and different Trp residues in $BKRB_1$ and $BKRB_2$ constructs (Fig. 6e, f).

Other Table 1 data, as those expressing the wavelengths of maximal emission observed for free and peptide-bound constructs, show different patterns for AngII and BK systems. They might be useful to complement the present study but cannot be safely interpreted provided that numerous fluorescent aromatic rings were present in the constructs simultaneously participating in a same process of fluorescence emission (see Fig. 6a–d).

Specificity of constructs binding to the corresponding Toac peptides

A remarkable finding of the current work was to uncover by means of the EPR experiments (Fig. 3) that the binding of $Toac^1$-AngII and $Toac^0$-BK is specific, that is, it occurs only with the respective receptor constructs. This condition is intriguing if it is considered that the N-terminal segments of $Toac^1$-AngII and $Toac^0$-BK have the same sequence (Toac-Arg). Thus, it may be suggested that the structural requirements determining the specificity are located beyond the N-terminal end of the peptides.

In the $Toac^1$-AngII and $Toac^0$-BK peptides, aromatic rings (Y^4 in AngII and F^5 in BK) are probably sticking out from the main chain but at different distances from the common R residue located at positions 2 and 1, respectively. BK sequence carries P^2, P^3, and G^4 residues between the common R^1 and F^5 aromatic residue, thus differing from the shorter homologous sequence of AngII that contains only the V^3 residue. It is plausible to assume that these structural differences might be related to the specific binding specificities observed with the two types of BKRB receptors.

Concluding remarks and perspectives

Two major concerns in the present work were to verify: (1) if Ang II and BK can bind their respective receptor extracellular site constructs and if this binding when present is specific; and (2) if the interactions of AngII and BK with their constructs are ruled by the same factors observed in the original interactions between native receptors and agonists.

The first concern was surmounted when EPR and fluorescence techniques revealed Toac immobilization and intrinsic fluorescence quenching, respectively, (Figs. 2, 3, 4, 5) but only when pairs of peptides and constructs of respective receptors (such as AngII-AT_1R-construct and BK-BKRB-construct pairs) were mixed thus defining a type-specificity with discrimination between AngII and BK receptors.

The second question was hardly answered in this work despite the fact that some data (supplementary material) might be shedding light over the subject: (1) Toac peptides labeled at the positions containing binding- or function-involved residues in the native agonist-peptides, as R^2, V^3, and Y^4 in AngII or R^1 in BK, are unable to bind their constructs. Due to this fact, $Toac^0$-BK and $Toac^1$-AngII were the peptides used in the studies of BKRB and AT_1 receptor constructs, respectively; (2) BK is able to equally bind $BKRB_1$ and $BKRB_2$ constructs and this ability is supposedly due to interaction of its N-terminal R^1 residue (Fig. 1c, d). This fact is in concordance with the current knowledge that the C-terminal sequence of BK, but not the N-terminal one, is the motif responsible for discriminating the two receptor sub-types (Marceau and Regoli 2004).

As a whole, the doubt about the bindings of AngII and BK to their extracellular site constructs, whether or not they may represent the native peptide-receptor binding, remains to be clarified. The most straightforward way to solve this problem would be an extensive mutagenesis study aimed at verifying if the receptor residues found to be crucial for binding of native peptides, are also involved in binding of peptides and receptor constructs.

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

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