Agonist Activity at the Kinin B1 Receptor: Structural Requirements of the Central Tetrapeptide

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A series of analogues of desArg^9-Lys-bradykinin (BK), Lys-Arg-X-Ac$_n$c-X-Ser-Pro-Phe, in which the spacer X-Ac$_n$c-X replaces the central tetrapeptide Pro-Pro-Gly-Phe of BK, have been synthesized and functionally characterized at the B1 receptor. The 1-aminocycloalkane-1-carboxylic acids (Ac$_6$c, Ac$_7$c, Ac$_8$c, Ac$_9$c, Ac$_{12}$c) were incorporated to impart conformational constraint and probe the importance of the hydrophobicity of the residue in the central position. The linker is varied in length (X = Gly, βAla, γAbu) to examine the optimal distance between the biologically important residues at the N- and C-termini. The biological assays indicate that the optimal length is obtained with X = Gly, with reduced activities for the longer linkers. Although the size of the central cyclic amino acid does not significantly alter the biological activity, the hydrophobic residue Ac$_n$c which may tether the peptide in the membrane environment is required (Lys-Arg-Gly-Gly-Gly-Ser-Pro-Phe is inactive). Two of the analogues, Lys-Arg-Gly-Ac$_7$c-Gly-Ser-Pro-Phe and Lys-Arg-γAbu-Ac$_7$c-γAbu-Ser-Pro-Phe, have been structurally characterized in the presence of a zwitterionic lipid environment by high-resolution NMR. Both compounds have similar structural features, differing greatest in the distance between the termini (9 and 15 Å for the Gly- and γAbu-containing analogues, respectively). The correlation of the smaller distance with activity at the B1 receptor is in complete accord with the results from our previous examination of Lys-Arg-NH-(CH$_2$)$_{11}$-CO-Ser-Pro-Phe. With the results from this series of compounds we are beginning to define some of the molecular descriptors important for activity at the B1 BK receptor.

Introduction

We have previously described a series of desArg^9-Lys-bradykinin (BK) (Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe) analogues where the central tetrapeptide Pro-Pro-Gly-Phe is replaced by alkyl spacers.[1−3] These analogues displayed significant agonistic activity, selective for the kinin B1 receptor. The most active compound of this series (**1** in Table 1) contains 12-aminododecanoic acid (Ado) in place of the central portion and has a pA_2 of 6.0 in the rat ileal longitudinal smooth muscle, a preparation selective for the kinin B1 receptor.[1]

Subsequent conformational studies addressed the relative topological arrangement of the N- and C-terminal residues of the Ado-BK peptide, as well as the role of the hydrophobicity of its central portion.[3] These studies, carried out in the presence of zwitterionic lipid micelles made up of dodecylphosphocholine (DPC) to mimic a membrane environment, indicated that Ado is embedded into the hydrophobic environment dictating the topological display of the biologically important residues of the N- and C-termini.[3]

Table 1. Structure and Biological Activity of desArg^9-Lys-BK Analogues

		HPLC[b]	MW (ES/MS[b])		
no.	structure[a]	t_R (min)	calcd[c]	found[d]	RI[e] (pD_2[f])
1	H-Lys-Arg-Ado-Ser-Pro-Phe-OH	10.1			6.0 ± 0.12
2	H-Lys-Arg-Ac$_7$c-Ser-Pro-Phe-OH	9.5	772.4	772.3	na[g]
3	H-Lys-Arg-Gly-Ac$_7$c-Gly-Ser-Pro-Phe-OH	8.8	886.5	886.7	5.4 ± 0.21
4	H-Lys-Arg-βAla-Ac$_7$c-βAla-Ser-Pro-Phe-OH	8.9	914.5	914.6	4.8 ± 0.20
5	H-Lys-Arg-γAbu-Ac$_7$c-γAbu-Ser-Pro-Phe-OH	9.1	942.6	942.3	4.3 ± 0.17
6	H-Lys-Arg-Gly-Ac$_6$c-Gly-Ser-Pro-Phe-OH	8.7	872.5	872.5	5.1 ± 0.19
7	H-Lys-Arg-Gly-Ac$_8$c-Gly-Ser-Pro-Phe-OH	10.0	900.5	900.4	5.7 ± 0.11
8	H-Lys-Arg-Gly-Ac$_9$c-Gly-Ser-Pro-Phe-OH	10.9	914.5	914.7	5.4 ± 0.23
9	H-Lys-Arg-Gly-Ac$_{12}$c-Gly-Ser-Pro-Phe-OH	12.1	956.6	956.4	5.5 ± 0.18
10	H-Lys-Arg-Gly-Gly-Gly-Ser-Pro-Phe-OH	8.0	804.4	804.2	na[g]

[a] Ado, 12-aminododecanoic acid; Ac$_n$c, 1-aminocycloalkane-1-carboxylic acids, n = number of carbons in ring; βAla, 3-aminopropionic acid; γAbu, 4-aminobutyric acid. [b] See Experimental Section for HPLC and MS methods. [c] Monoisotopic relative molecular mass. [d] Quasi-molecular ion minus 1 (m/z). [e] RI, rat ileum longitudinal smooth muscle. [f] pD_2, negative log of the agonist molar concentration which produces 50% of the maximal effect. Values are the mean ± SEM of 4−10 experiments. [g] na, no activity up to 10 μM concentration.

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One drawback of the Ado residue is that the long alkyl chain introduces conformational variability greater than that of a peptide backbone. To address this issue, we have designed a series of desArg^9-Lys-BK analogues containing C$^\alpha$-disubstituted α-amino acids of the family of 1-aminocycloalkane-1-carboxylic acids (Ac$_n$c). These cyclic amino acids maintain the hydrophobic character of the linear alkyl chains previously used,[1] while imparting greater conformational constraint. Indeed, the cyclic substituents may mimic folded conformations of the linear alkyl spacers. To optimize the distance between the termini of the peptide, the Ac$_n$c residue was flanked by amino acids of different length ranging from Gly to γAbu (see Table 1). Here, we describe the design, synthesis, pharmacological characterization, and conformational studies of this new series of desArg^9-Lys-BK analogues. (Following the residue numbering of BK, the Lys-BK analogues described here will be numbered as Lys^0-Arg^1-linker$^{2-4}$-Ser^6-Pro^7-Phe^8.)

Results

Design and Synthesis. C$^\alpha$-Disubstituted α-amino acids of the family of Ac$_n$c were used to structurally constrain the spacer by varying both the overall hydrophobicity and the distance between the N- and C-termini of the BK analogue. The overall length of the spacer was modulated by the utilization of residues flanking the Ac$_n$c residue. For the Ac$_7$c-containing analogues, the residue was used either alone (**2**) or flanked by two residues of Gly (**3**), βAla (**4**), or γAbu (**5**). Maintaining the Gly-based spacer which produced the most active analogue (**3**), the size of the cyclic alkane ring was modified (**6**−**9**) to examine the consequences of altering the hydrophobicity and ability of the central region of the analogue to tether into a membrane environment. The peptides were prepared by automated solid-phase synthesis as previously described[2] and purified to homogeneity by semipreparative HPLC.

Pharmacology. The pharmacological activity of the peptides was tested in the rat ileum longitudinal smooth muscle, a kinin B1 receptor assay.[4] As shown in Table 1, **2** was found to be inactive up to 10 μM concentration as an agonist at the kinin B1 receptor. On the contrary, the three analogues bearing the longer spacers behave as full agonists at the kinin B1 receptor, with pD_2s of 5.4, 4.8, and 4.3, respectively. Clearly the optimal length was obtained when the Ac$_7$c residue is flanked by two Gly residues. Accordingly, the Gly-based spacer was further modified with Ac$_n$c with smaller (Ac$_6$c) or larger (Ac$_8$c, Ac$_9$c, Ac$_{12}$c) rings. The effect of these modifications on the agonistic activity is less pronounced, although a clear trend can be detected. In fact, **6** (Ac$_6$c) is slightly less active than **3** (Ac$_7$c), while **7** (Ac$_8$c) is slightly more active. With the two largest members of this series, Ac$_9$c (**8**) and Ac$_{12}$c (**9**), a plateau of activity is apparently reached, with pD_2 values around 5.5. Finally, the important role of the hydrophobic and conformationally constrained character of the spacer was confirmed by the lack of agonist activity of **10**, bearing the simple Gly-Gly-Gly spacer.

Structural Characterization. The activities of **3**−**5** clearly indicate the importance of an optimal spacer controlling the distance between the N- and C-termini. On the basis of these results we chose to structurally characterize **3** and **5**, to explore if indeed the lengthening of the linker leads to increased distances between the termini or induces other structural differences which may account for the pharmacological profiles.

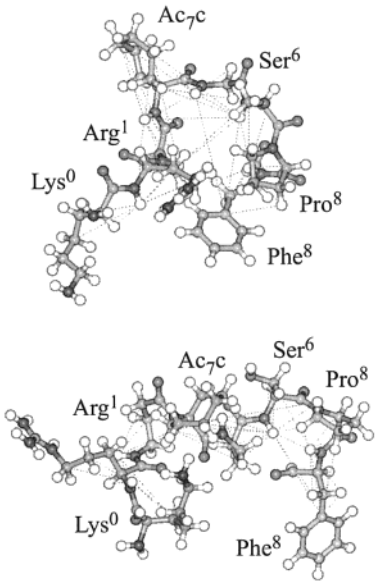

Figure 1. Representative structures of **3** (top) and **5** (bottom) illustrating the NOE-derived distance constraints.

Both **3** and **5** display two sets of ^{1}H NMR signals arising from *cis* and *trans* isomers about the Ser^6-Pro^7 amide bond, with the *cis* isomer accounting for less than 20% of the population. Most of the resonances from the different isomers were well-resolved and therefore standard NMR-based refinement methods could be utilized. This is in contrast to the Ado-containing peptide[3] in which overlapping of the *cis* and *trans* isomers required the use of a novel procedure for overlapping NOEs.[5] A representative NOESY spectrum of **3** is given in the Supporting Information. A total of 51 and 56 NOEs were unambiguously assigned and converted to distances for **3** and **5**, respectively.

The structure refinement, based on metric matrix DG calculations and energy minimization, resulted in a number of low-energy structures consistent with the NOEs (no violations greater than 0.2 Å). The structural variability of the ensemble of structures was ascertained by calculation of the dihedral angle order parameters for ϕ and ψ[6] (data not shown). High values (>0.8), indicating a convergence of the structures, are observed for **3**, with the exception of Arg^1-Gly^2, which is a point of flexibility indicated by order parameter values lower than 0.4. Displaying representative structures in a Ramachandran plot (Supporting Information) indicates that these residues adopt two different families of conformations, with Gly^2 adopting conformations centered around −60°,−60° and 60°, 60°. The C$^\alpha$-protons of Gly^2 are involved in only sequential NOEs, consistent with this structural variability. In Figure 1 the NOEs observed for **3** are depicted on a representative structure, illustrating the relative NOE density dispersed throughout the peptide. We attribute the positive ϕ dihedral angle of Arg^1 to the interaction of the positively charged amino acid with the zwitterionic lipid environment, which affords sufficient energy to overcome the preference for negative ϕ dihedral angles as observed for cytosolic proteins. Previously we have observed *cis*

peptide bonds (not involving Pro), induced by the interaction of the peptide with the lipid environment.[7] The remainder of the residues are found to cluster in small regions of the ϕ,ψ plot.

The resulting ensemble of structures from the refinement of **5** is quite different. There is less convergence of the structural features, indicated by a limited number of dihedral angle order parameters greater than 0.8 (ϕ,ψ of Ac_7c^3 and Pro^7, ϕ of Phe^8, ψ of Arg^1). This difference is illustrated in the Ramachandran map of representative structures taken from the ensemble (Supporting Information). The increased structural variability is expected given the increased length of the linker, γAbu versus Gly. For most of the amino acids there are two or three different families of conformations consistent with the experimental observations. Interestingly, the Ac_7c^3 residue adopts a semiextended conformation with the ϕ dihedral angle at 180°, leading to a more elongated structure of the peptide. This conformation is supported by a large number of NOEs involving the amide proton of Ac_7c^3. The semiextended conformation is not commonly observed for Ac_nc amino acids. However, in **5** the Ac_7c is surrounded by γAbu residues with two additional methylene groups (in comparison to Gly) which may favor more extended conformations. The NOEs used in the structural refinement calculations of **5** are illustrated in Figure 1.

To quantify the consequences of the linker length on the topological arrangement of the termini of the peptide, the distances between them and the side chains of Arg^1 and Phe^8 were calculated for the resulting ensembles of **3** and **5**. We have previously utilized these distances in the characterization of the Ado-containing Lys-BK analogue.[3] For **3**, the average distance between the termini is 8.0 Å, ranging from 4.1−11.8 Å for members of the ensemble. This should be compared with an average of 15.0 Å (ranging from 11.5−19.2 Å) for **5**.

Discussion

The goal of the research carried out here is the characterization of the structural features required for agonist activity at the B1 BK receptor. This receptor is an important pharmacological target given its role in asthma, inflammatory joint diseases, pancreatitis, hypertension, myocardial ischemia, and most recently multiple sclerosis.[8−11] Beginning with the natural peptide ligands, $desArg^9$-BK and Lys^0-$desArg^9$-BK, strides have been made via a series of substitutions and deletions to define the biologically important residues.[1,12−14] Replacement of the central portion of BK, Pro^2-Pro^3-Gly^4-Phe^5, with alkyl spacers has been used as a first step to reduce the peptide character of the compounds and therefore increase the enzymatic stability.[1,2] These compounds also lend themselves to structural studies, with the aim of defining the topological arrangement of the terminal (both N- and C-termini) residues, which is optimal for selectivity/activity at the B1 BK receptor.[3]

The structural consequences of the incorporation of an Ac_nc residue have been extensively examined.[15−20] In general, this residue favors a helical conformation (both the α-helix and 3_{10} helix) as well as stabilizes β-turns. The achiral nature of the Ac_nc residue results in equal populations of the left- and right-handed conformations (both positive and negative ϕ,ψ values). Recently, an Ac_nc scan has been used to map the binding site of phosphopeptides to the Grb2-SH2 domain.[21]

The Ac_nc residue was utilized here to probe the importance of a centrally located hydrophobic residue for activity at the B1 BK receptor. Simultaneously, this cyclic amino acid adds conformational constraint, which limits the topological arrangement of the residues at the N- and C-termini. This structural feature was found to be an important factor for biological function at the B1 BK receptor.[1−3]

Both of the peptides examined here contain a pseudo-β-turn at the C-terminus, a feature we observed previously for **1**.[3] For all of these peptides the observed backbone conformation of Ser^6-Pro^7-Phe^8 can be superimposed almost perfectly onto three-quarters of a βI-turn (positions i, $i+1$, and $i+2$), with the Pro residue at the $i+1$ position. A number of $i,i+2$ NOEs between Ser^6 and Phe^8 are observed for both peptides. This conformation is favored despite the absence of the standard 10-membered hydrogen bond of β-turns (there is no residue at the $i+3$ position to donate the amide proton). Certainly the central Pro residue is determinant for the adoption of this structure. From numerous structural investigations a C-terminal β-turn has been defined as an important feature for activity at the B2 BK receptor and used in the rational design of BK analogues.[22−28] The observation of a similar structural motif for the B1 receptor-selective analogues, in the absence of Arg^9, is important for understanding the basis for receptor selectivity. The structural findings would suggest that the selectivity for the B1 receptor of the $desArg^9$ compounds is simply from the loss of the Arg^9 side chain and the associated positive charge and not the result of large conformational changes. It is important to note that a single point in the B1 receptor has been identified as determinant for the observed ligand specificity.[29] The B1 receptor contains a Lys at this position, while in the B2 receptor a Ser is found at position 111. The positively charged Lys residue would lead to unfavorable Coulombic interactions with Arg^9-containing analogues, resulting in a high selectivity for $desArg^9$ analogues.[29]

In our previous analysis of **1** distances between the residues of the termini were used as a simple measure of the relative topology of the N- and C-termini.[3] Here, we observe a good correlation between the distance between the side chains of Arg^1 and Phe^8 and the biological activity. For **3** an average distance of 8.0 ± 2.3 Å is calculated for the ensemble of structures. This is to be compared with a value of just over 10 Å for the average distance observed for **1**. In contrast, an average distance of 15 Å was calculated for **5**, an analogue that is 1 log unit less active in the rat ileum assay (Table 1). The optimal distance between the C-terminal β-turn (or pseudo-β-turn) and the charged residues of the N-terminus is obtained with the Gly-flanking residues. Increasing the length of the central linker (i.e. βAla or γAbu as flanking residues) reduces the biological response. The lack of activity of **2**, without flanking residues, suggests that a minimum distance is required. Likewise, the inactivity of **10** clearly indicates that the central linker should contain a conformationally constrained, hydrophobic residue, possibly responsible for an interaction with the membrane environment of the

G-protein coupled receptor. The results from **1** clearly indicate a strong interaction of Ado with the membrane, a step that may be required for recognition and interaction with the receptor. A membrane-associated pathway for the interaction of peptide hormones with their G-protein coupled receptors has been suggested by a number of authors.[30−34] This theory dictates that the ligand first interacts with the membrane surface, which induces the peptide to adopt the optimal orientation and conformation for interaction with the receptor. Our previous results with **1** and those presented here clearly indicate the requirement for a hydrophobic residue and its tendency to associate with the membrane environment. The central region in the natural sequences of BK and Lys-BK consisting of Pro-Pro-Gly-Phe could certainly play such a role.

Experimental Section

Abbreviations: γAbu, 4-aminobutyric acid; βAla, 3-aminopropionic acid; Ac$_n$c, 1-aminocycloalkane-1-carboxylic acids; Ado, 12-aminododecanoic acid; BK, bradykinin; doxyl, 4,4-dimethyl-3-oxazolidinyloxy; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DG, distance geometry; DMF, dimethylformamide; DPC, dodecylphosphocholine; ESI-MS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; NMM, *N*-methylmorpholine; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancements; NOESY, nuclear Overhauser enhancement spectroscopy; PEG-PS, poly(ethylene glycol) polystyrene; RP-HPLC, reverse-phase high-performance liquid chromatography; ROESY, rotational Overhauser enhancement spectroscopy; TFA, trifluoroacetic acid.

Synthesis. The Fmoc derivatives of the Ac$_n$c residues were prepared as previously reported,[2] all other protected amino acid and Fmoc-Phe-Novasyn TGA resin were purchased from Novabiochem (Bubendorf, Switzerland). HATU was from PerSeptive Biosystem (Framingham, MA).

Syntheses were performed on a Milligen 9050 peptide synthesizer (Bedford, MA) using the high-flow protocol on a PEG-PS resin. Coupling cycles of 20 min were carried out with a 3-fold excess of protected amino acids and HATU/NMM as the activating reagent. Fmoc group removal was obtained by treatment with 2% piperidine and 2% DBU in DMF for 5 min. After completion of the synthesis, the resin was washed and dried under vacuum. The peptide was cleaved with reagent K (TFA/phenol/thioanisol/H_2O/ethanedithiol, 82.5:5:5:5:2.5, 120 min at room temperature), precipitated by addition of cold diethyl ether, filtered, dissolved in water, and lyophilized. Crude peptides were purified by semipreparative RP-HPLC on a Beckman System Gold apparatus, using the following conditions: Vydac C_{18} column, 1 × 25 cm; eluant A, 0.1% TFA in H_2O; eluant B, 0.1% TFA in CH_3CN; gradient from 15% to 45% B over 200 min; flow 4 mL/min; UV detection at 210 nm. The final products were characterized by analytical HPLC and ESI-MS (data not shown).

MS analysis was perfomed on a VG Quattro mass spectrometer (Altricham, U.K.) equipped with standard electrospray (ES) ion source. The samples (about 50 μg/mL) were dissolved in a 1:1 mixture of acetonitrile/10 mM ammonium acetate containing 1% acetic acid and introduced by infusion at 5 μL/min using a Harvard Apparatus 11 pump (South Natik, MA). Ionization was obtained using positive ion pneumatically assisted ES, at a nebulizer voltage of 4 kV and a cone voltage of 50−70 V. Nitrogen was used as the nebulization and desolvation gas. The temperature of the ion source was 65 °C. For each sample, either singly or doubly charged quasi-molecular ions were obtained. The experimental and calculated molecular weights in Table 1 are expressed as monoisotopic molecular weights.

Pharmacology. Longitudinal muscles-myenteric plexus preparation of rat ileum were prepared as previously described.[4] Isolated preparations were placed in oxygenated (95% O_2, 5% CO_2) Krebs' solution containing indomethacin, guanethidin (3 μM each), chlorpheniramine, and atropine (1 μM each). Cumulative concentration−response curves to agonists were constructed in the presence of the peptidase inhibitors thiorphan, bestatin, and captopril (1 μM each). Those peptides which did not produce any motor effect (contraction) in the preparation, up to 10 μM concentration, were considered inactive as agonists. Agonist potency of the active peptides was expressed in terms of pD_2 (negative log of the agonist concentration, which produces 50% of the maximal effect). All the values reported are mean ± SEM.

Spectroscopic Investigations. The NMR experiments were carried out on 2.0 mM samples (based on weight) in aqueous solution (90% H_2O−10% D_2O; Cambridge Isotopes) containing approximately 100 mM DPC-d_{38} (98.6%; Cambridge Isotopes). The final pH of the solution was 5.5 (not corrected for isotope effect). Proton spectra were recorded on Varian 500 MHz instrument and processed using VNMR and Felix (Molecular Simulations, Inc.). Chemical shifts were calibrated with respect to internal tetramethylsilane. The resonance assignment, integration of NOEs, and calculation of distance restraints were carried out as described previously for the Ado-containing analogue.[3]

Structure Refinement. A home-written program was utilized to carry out the metric matrix DG calculations following the random metrization algorithm developed by Havel.[35] Informative restraints (those distances more restrictive than the holonomic restraints developed from the molecular constitution) were added to create a distance matrix.[36] The structures of **3** and **5** were first embedded in four dimensions and refined following published procedures.[37,38] The resulting structures were then reduced to three dimensions using random metrization and the optimization procedure repeated. The resulting ensemble of structures was then energy-minimized using the cvff force field within the Discover molecular mechanics program (Molecular Simulations, Inc.). Representative structures were calculated using a home-written clustering program that identifies conformational families based on the backbone dihedral angles. One representative from each structural family was retained and is presented in the illustrations.

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Supporting Information Available: Expanded portions of a NOESY spectrum of **3** and the Ramachandran plots of the resulting ensemble of structures from the DG calculations of **3** and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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