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Structure-activity relationships of non-opioid [des-Arg⁷]-dynorphin A analogues for bradykinin receptors

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Abstract

In our earlier studies, bradykinin receptors (BRs) were identified as a potential target for the neuroexcitatory effects of dynorphin A (Dyn A) in the central nervous system (CNS), and [des-Arg⁷]-Dyn A-(4-11) (**6**) was discovered as a lead ligand to modulate Dyn A-(2-13) induced neuroexcitatory effects in the CNS as an antagonist. In an effort to gain insights into key structural features of the Dyn A for the BRs, we pursued further structure-activity relationships (SAR) study on the [des-Arg⁷]-Dyn A analogs and confirmed that all of the [des-Arg⁷]-Dyn A analogues showed good binding affinities at the BRs.

Keywords

Non-opioid dynorphin A; Structure-Activity relationship; Bradykinin receptors; Chronic pain; pH sensitivity

Chronic pain is difficult to treat properly by current methods using opioids due to serious side effects such as tolerance, addiction, and constipation.^{1,2} Current treatment modalities for the chronic pain can cause different even more pain with prolonged use.³ This is because of changes in gene expression that are related to treatment attempts: nervous system adaptation/change.⁴ Therefore, to treat chronic pain efficiently without the serious side effects, there is a need to develop drugs through novel approaches considering the possible changes in pain pathways. From this perspective, dynorphin A (Dyn A, H-Tyr¹-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln¹⁷-OH), which is an endogenous opioid ligand for three subtypes (μ , δ , and κ) of opioid receptors, can be a potential target to pursue with regard to possible system changes, because the ligand shows very distinctive biological roles in the pain pathway: neuroinhibitory (opioid) effects or neuroexcitatory (non-opioid) effects.^{5,6,7} While Dyn A's neuroinhibitory effects are well

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understood along with its opioid receptor interactions, the mechanism of its neuroexcitatory effects are not established yet despite its frequent observation in animal models.

In our earlier study, we identified the bradykinin receptor (BR) as a potential neuroexcitatory target of Dyn A in the central nervous system (CNS).⁷ Under chronic pain or nerve injury conditions, Dyn A is up-regulated in the spinal cord and the excess of Dyn A interacts with central BRs to cause hyperalgesia.⁷⁻⁹ On the basis of this fact, we hypothesized that Dyn A structure-based BR antagonists could be discovered to modulate the hyperalgesia in the CNS. Therefore, we have been developing BRs antagonists by utilizing the Dyn A structure as a template. In our earlier studies, we identified a good pharmacophore for the BRs in rat brain: an amphipathic non-opioid fragment, [des-Arg⁷]-Dyn A-(4-11) (**6**).¹⁰ Ligand **6** inhibited the actions of Dyn A-(2-13)'s hyperalgesic effects in the CNS.^{10, 11} We also demonstrated that the deletion of Arg⁷ residue in Dyn A-(4-11) does not affect binding affinity in rat brain BRs. This SAR result is remarkable because two Arg residues in positions 6 and 7 are known to be important for the biological activity of Dyn A and in general, deletion of one amino acid residue in the middle of a bioactive sequence typically causes significant topographical changes and a different biological profile.^{12,13} In contrast, our SAR results exhibited that both analogues have the same range of binding affinity (Dyn A-(4-11): IC₅₀ = 140 nM; **6**: IC₅₀ = 190 nM) at the BRs. NMR studies of Dyn A-(4-11) and **6** showed that the two analogues have the same distorted type 1 – turn structure at the C-terminus, which is considered a key region for binding to the BRs.¹⁰ Therefore, we pursued further structure-activity relationship studies on the [des-Arg⁷]-Dyn A analogues and confirmed that all of the [des-Arg⁷]-Dyn A analogs show the same range of binding affinities to the BRs as respective Dyn A analogs (Table 1).

It was shown that the BRs recognition predominantly depended upon the basicity of the C-terminal amino acid, and the modification of the C-terminal carboxylate group to an amide reduced the binding affinity dramatically.¹⁰ Therefore our SAR study on [des-Arg⁷]-Dyn A analogues was limited to analogues with a basic amino acid residue at the C-terminus. All of the [des-Arg⁷]-Dyn A analogues were synthesized by standard solid phase peptide synthesis using the *N*^α-Fmoc strategy on amino acid pre-loaded Wang resin in high yields (overall yields > 40%). Each coupling reaction used 3 eq. 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HBTU)/3 eq *N*-hydroxybenzotriazole (HOBt)/6eq. diisopropylethylamine (DIPEA) for 50 min at rt and each Fmoc-deprotection used 20% piperidine/*N,N*-dimethylformamide (DMF) for 20 min at rt. After chain elongations, resins were cleaved by the use of a 92% trifluoroacetic acid (TFA) cocktail solution containing 4% triisopropylsilane (TIS), 2% water, and 2% anisole. The resulting crude peptides (purity: 70-90%) were purified by preparative reverse phase high performance liquid chromatography (RP-HPLC) in a short time (<15 min) thanks to their hydrophilic characters (aLogPs < 0) (Table 2). The purified analogues were validated by analytical HPLC and Mass spectroscopy. The purified analogues were tested for their binding affinities by competition assay using [³H]DALKD or [³H]BK in rat brain membranes where non-specific binding is defined by 10 μM kallidin (Tables 1 and 3).

As shown in Table 1, [des-Arg⁷]-Dyn A analogues **1-10** exhibited a similar pattern of binding affinities as Dyn A analogues for rat brain BRs at both pH 6.8 and pH 7.4. Since pH

7.4 is considered the physiological condition in medicine and biology and also is the condition Dyn A analogues have been tested for their binding affinities at opioid receptors, Dyn A analogues were first tested at pH 7.4. Considering the important role of C-terminal basic amino acid in binding, the receptor recognition was considered to be through an electrostatic interaction between positive charges of Dyn A analogues and negative charges of the BRs. In an effort to investigate the potential electrostatic interactions, the pH condition was varied from 7.4 to 6.8. As the result, it was observed that binding affinities of Dyn A analogues are pH sensitive and are enhanced at the lower pH (6.8). This observation may be an indication of the electrostatic interactions, which generally depend on pH conditions. This study confirmed that the optimal binding conditions for the BRs to Dyn A analogues are at pH 6.8, which is consistent with the binding conditions previously established for BRs.¹⁴ Therefore, the pH for the binding assays was changed from 7.4 to 6.8 and most of the [des-Arg⁷]-Dyn A analogues were re-tested at pH 6.8 for their binding affinities at rat brain BRs (Table 1).

It is clear that deletion of the Arg residue at position 7 did not affect binding affinities at all (column 4 and 5 in Table 1). Even some [des-Arg⁷]-Dyn A analogues (**2** – **4**) increased their binding affinities 4 - 8 folds (**2**: IC₅₀ = 41 nM; Dyn A-(3-13): IC₅₀ = 320 nM). The increase seemed to be caused by relatively low binding affinities of their parent ligands Dyn A-(3-13), Dyn A-(4-13), and Dyn A-(5-13). While the lower binding affinities of Dyn A-(3-13), Dyn A-(4-13), and Dyn A-(5-13) were slightly recovered in Dyn A-(3-11), Dyn A-(4-11), and Dyn A-(5-11) by the truncation of Lys and Leu at the C-terminus, all of [des-Arg⁷]-Dyn A analogues decreased their binding affinities gradually with the truncation of C-terminal and/or N-terminal amino acid residues. To investigate the role of Arg at positions 6 and 7, the Arg residue was replaced by an Ala in ligands **11**-**14**. Binding affinity results showed different SAR in fragments Dyn A-(5-13) and Dyn A-(4-11). While deletion or substitution of an Arg residue in the fragment Dyn A-(5-13) (IC₅₀ = 470 nM) increased its binding affinity in **4** (IC₅₀ = 30 nM), **11** (IC₅₀ = 100 nM), and **12** (IC₅₀ = 120 nM), the Ala substitution in the fragment Dyn A-(4-11) (IC₅₀ = 110 nM) reduced the binding affinity in **13** (IC₅₀ = 260 nM) and **14** (IC₅₀ = 740 nM). Although the changes in binding affinities are small, the SAR results suggest that localization, replacement, and deletion of the Arg play a role in the change of topographical structure cooperatively. The SAR results by the Ala substitutions implicated that two Arg residues are necessary in Dyn A-(4-11) by showing lower binding affinities of **13** and **14**. But contrarily [des-Arg⁷]-Dyn A-(4-11) **6** retained good binding affinity (IC₅₀ = 69 nM) even by the deletion of an Arg residue. This SAR result is an example showing that an Ala scan can be misleading. The Pro residue at position 10 in Dyn A, which contributes to the turn structure affect the binding affinity more than other residues. The length between the Pro and the two Arg residues are the key factor for determining the structure, and fulfil a requirement for amphipathic character of Dyn A. There is no additive effect on the binding observed in ligand **15**, which has two arginines at position 9. Therefore it is clear that although electrostatic interactions are considered to be a main force for the BRs recognition, increasing the net charge for the interaction by adding more basic amino acid residue may not be an option, since the localization of positive charges appears to be more important for receptor recognition. From the SAR results, we again suggest a simple amphipathic pharmacophore for the BRs .

Based on the binding affinities of the [des-Arg⁷]-Dyn A analogues at pH 6.8, ligand **6** (IC₅₀ = 69 nM), which had the same high binding affinity as ligand **1** (IC₅₀ = 41 nM), is a good pharmacophore. From the structure of ligand **6**, it is clear that the ligand consists of basic amino acids and hydrophobic amino acids showing amphipathic properties and a Pro residue making a turn structure.¹⁰ The other ligands also show amphipathic properties in their structures. Therefore we further design more analogues to enhance the amphipathic properties in their structure and ligands **16-19** were synthesized. Hydrophobic amino acid residues Leu⁶ and Ile⁸ were replaced by a Nle residue, respectively, in **16** and **17**. These modifications confirmed that both hydrophobic amino acid residues can be replaced by a Nle residue without losing binding affinity. For simplicity, the Lys¹¹ residue was replaced by an Arg, which resulted in a simple amphipathic ligand retaining a good binding affinity (IC₅₀ = 87 nM) at BRs. Based on the binding affinities of both amphipathic ligands **18** (IC₅₀ = 87 nM) and **19** (IC₅₀ = 91 nM), it is clear that ligands will show good binding affinity for BRs as long as amphipathicity is conserved.

In conclusions, we have synthesized a series of [des-Arg⁷]-Dyn A analogues and compared their binding affinities with respective parent Dyn A analogues for rat brain BRs at pH 6.8 and pH 7.4. All of analogues showed the same range of binding affinities as their respective Dyn A analogues with an enhancement at pH 6.8. The enhancement of binding affinities at lower pH along with an important role of basic amino acid residue at the C-terminus suggested that the BRs recognition is mainly through the electrostatic interactions. Further modifications of the amphipathic analogue **6** by replacing Leu⁵ (and/or Ile⁸) and Arg^{6,9} (and/or Lys¹¹) with Nle and Lys (and/or Arg), respectively, retained the same good binding affinities for BRs. Therefore, we conclude that an amphipathic property enabling proper localization of positive charges of basic amino acid residues is the key structure for rat brain BRs recognition.

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Abbreviations

BK	bradykinin
BR	bradykinin receptor
CNS	central nervous system
DALKD	[des-Arg ¹⁰ , Leu ⁹]-kallidin
DIPEA	diisopropylethylamine
DMF	<i>N,N</i> -dimethylformamide
Dyn A	dydnorphin A
Fmoc	9-fluorenylcarboxy
HBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate

HOBt	N- hydroxybenzotriazole
RP-HPLC	reverse phase high performance liquid chromatography
SAR	structure-activity relationship
TFA	trifluoroacetic acid
TIS	triisopropylsilane

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Table 1Binding affinities of [Des-Arg⁷]-Dyn A fragments at BRs in rat brain

no	Dyn A (Tyr ¹ -Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln ¹⁷ -OH) Analogue	BR ^a , [³ H]DALKD				
		pH 6.8		pH 7.4		pH 7.4
		Log[IC ₅₀] ^b	IC ₅₀ (nM)	Log[IC ₅₀] ^b	IC ₅₀ (nM)	IC ₅₀ (nM) ^c
1	[Des-Arg ⁷]-Dyn A-(2-13)	-7.39±0.34	41	n.d.	n.d.	170 (21 ^d)
2	[Des-Arg ⁷]-Dyn A-(3-13)	-7.58±0.06	26	-7.39±0.09	41	320
3	[Des-Arg ⁷]-Dyn A-(4-13)	-7.20±0.08	63	-6.95±0.22	110	390
4	[Des-Arg ⁷]-Dyn A-(5-13) ^e	-7.52±0.09	30	-7.17±0.10	68	470
5	[Des-Arg ⁷]-Dyn A-(3-11)	n.d.	n.d.	-6.88±0.08	130	130
6	[Des-Arg ⁷]-Dyn A-(4-11) ^e	-7.16±0.09	69	-6.71±0.11	190	140
7	[Des-Arg ⁷]-Dyn A-(5-11)	-6.71±0.24	190	-6.74±0.09	180	280
8	[Des-Arg ⁷]-Dyn A-(3-9)	-6.56±0.10	280	-6.21±0.09	620	780
9	[Des-Arg ⁷]-Dyn A-(4-9)	-6.12±0.07	760	-5.67±0.15	2100	1600
10	[Des-Arg ⁷]-Dyn A-(5-9)	-5.66±0.14	2200	-5.03±0.12	9300	3300

^a Competition assays were carried out using rat brain membranes.^b Logarithmic values determined from the nonlinear regression analysis of data collected from at least two independent experiments in duplicate.^c For the comparison, IC₅₀ Values of respective Dyn A fragments in reference 10 are shown in this column.^d Value at pH 6.8.^e Reference 10. n.d.: not determined.

Table 2Analytical Data of [des-Arg⁷]-Dyn A analogues

no	molecular formula	LowMS ^a	HRMS ^b			HPLC ^c	aLogPs ^d
		observed	observed	calcd.	error (ppm)	(t _R , min)	
1	C ₆₀ H ₁₀₅ N ₁₉ O ₁₂	1284.8	642.9168	642.9167	0.1	10.4	-1.45
2	C ₅₈ H ₁₀₂ N ₁₈ O ₁₁	12227.7	614.4057	614.4054	-0.5	10.4	-1.41
3	C ₅₆ H ₉₉ N ₁₇ O ₁₀	1170.7	585.8943	585.8953	1.7	9.7	-1.31
5	C ₄₆ H ₇₉ N ₁₅ O ₉	986.6	493.8169	493.8165	-0.7	9.5	-1.71
7	C ₃₅ H ₆₇ N ₁₃ O ₇	782.5	391.7717	391.7716	-0.2	6.3	-2.05
8	C ₃₅ H ₆₀ N ₁₂ O ₇	761.4	381.2427	381.2427	-0.1	8.2	-2.14
9	C ₃₃ H ₅₇ N ₁₁ O ₆	704.5	352.7316	352.7319	1.0	9.6	-2.00
10	C ₂₄ H ₃₈ N ₁₀ O ₅	557.4	279.1975	279.1977	-0.8	7.0	-2.73
11	C ₅₀ H ₉₅ N ₁₇ O ₁₀	547.9	547.8792	547.8797	0.8	9.2	-1.67
12	C ₅₀ H ₉₅ N ₁₇ O ₁₀	547.9	547.8793	547.8797	0.6	9.2	-1.66
13	C ₄₇ H ₈₁ N ₁₅ O ₉	1000.7	1000.6405	1000.6414	0.9	11.0	-1.52
14	C ₄₇ H ₈₁ N ₁₅ O ₉	1000.7	1000.6435	1000.6414	-2.1	10.3	-1.50
16	C ₄₄ H ₇₆ N ₁₄ O ₈	929.5	929.6030	929.6043	1.4	10.5	-1.60
17	C ₄₄ H ₇₆ N ₁₄ O ₈	929.6	929.6030	929.6043	1.4	11.4	-1.66

^e (M+2H)²⁺.^f (M+3H)³⁺.^a (M + H)⁺, ESI method (Finnigan, Thermoelectron, LCQ classic).^b (M + H)⁺, FAB-MS (JEOL HX110 sector instrument) or MALDI-TOF.^c Performed on a Hewlett Packard 1100 (C-18, Microsorb-MV™, 4.6 mm × 250 mm, 5 μm) using gradient system (10-90% of acetonitrile containing 0.1% TFA within 40 min and up to 100% within additional 5 min, 1 mL/min).^d <http://www.vcclab.org/lab/alogps>.

Table 3Binding affinities of modified Dyn A fragments at BRs in rat brain^a

no	Structure	BR, [³ H]DALKD	
		Log[IC ₅₀]	IC ₅₀ (nM)
11	[Ala ⁶]-Dyn A-(5-13): H- Leu-Ala-Arg-Ile-Arg-Pro-Lys-Leu-Lys-OH	-6.99±0.27	100
12	[Ala ⁷]-Dyn A-(5-13): H- Leu-Arg-Ala-Ile-Arg-Pro-Lys-Leu-Lys-OH	-6.91±0.17	120
13	[Ala ⁶]-Dyn A-(4-11): H-Phe-Leu-Ala-Arg-Ile-Arg-Pro-Lys-OH	-6.58±0.20	260
14	[Ala ⁷]-Dyn A-(4-11): H-Phe-Leu-Arg-Ala-Ile-Arg-Pro-Lys-OH	-6.13±0.22	740
15	[Des-Arg ⁷ , (Arg, Arg) ⁹]-Dyn A-(4-11): H-Phe-Leu-Arg-Ile-Arg-Arg-Pro-Lys-OH ^c	-7.13±0.04	74
16	[Nle ⁵ , Des-Arg ⁷]-Dyn A-(4-11): H-Phe-Nle-Arg-Ile-Arg-Pro-Lys-OH	-7.02±0.06	95
17	[Des-Arg ⁷ , Nle ⁸]-Dyn A-(4-11): H-Phe-Leu-Arg-Nle-Arg-Pro-Lys-OH	-7.04±0.06	91
18	[Nle ⁵ , ⁸ , Des-Arg ⁷ , Arg ¹¹]-Dyn A-(4-11): H-Phe-Nle-Arg-Nle-Arg-Pro-Arg-OH ^c	-7.06±0.10	87
19	[Nle ⁵ , ⁸ , ¹² , Des-Arg ⁷ , Lys ⁶ , ⁹]-Dyn A-(5-13): H-Nle-Lys-Nle-Lys-Pro-Lys-Nle-Lys-OH ^c	-7.04±0.10	91

^b Reference 10^a Details described in Table 1.