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Synthesis and biological activity of branched enkephalin analogues containing two amino acids in a side chain

Irina Bobrova*, Natalia Mishlakova, Anette Selander, Elena Mekshun, Guntis Rozental

Latvian Institute of Organic Synthesis, 21 Aizkraukles, LV-1006 Riga, Latvia, and Experimental Alcohol and Drug Addiction Research, Karolinska Institute, S-17176 Stockholm, Sweden

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1. Enkephalins

Endogenous opioid pentapeptides have attracted considerable attention since their discovery in 1975 [1], most notably, with respect to the design of a potential analgesic drugs and also as possible useful tools for investigating opioid receptors (ORs) [2, 3]. Recently we have synthesized and described the opioid activity of branched enkephalin pentapeptides containing one amino acid in side chain [4, 5]. The new modification gave rise to an increase in opioid activity of analogues. The branched compounds were more active than the respective linear or cyclic analogues, given intracisternally to mice ('tail pinch' method). Unlike linear compounds the new analogues were active after intravenous (i.v.) administration.

The present investigation was undertaken to explore the effect of the extension of the branching side chain of enkephalin peptides by a second amino acid on opioid activity. The structure of peptides is based on the common enkephalin sequence: Tyr-Gly-Gly-Phe with replacement of 2-glycine by D-ornithine (D-lysine) derivatized by the attachment of two amino acid residues (Asn-Leu-, Asn-Arg-, Arg-Asn-) to the δ-amino group of D-ornithine (or the ε-amino group of D-lysine).

The peptides were synthesized by classical methods of peptide chemistry by a stepwise elongation and/or fragment condensation. Pentafluorophenyl activated esters of Boc-amino acids and diphenylphosphoryl-azide coupling methods were employed. The analytical data of peptides are shown in *table I*.

2. Biological activity and discussion

The incorporation of two amino acid residues in the side chain of the new branched analogues increased their opioid activity (*table II*). Compared to the analogues, containing one amino acid residue in the side chain, the resulting compounds showed 10–150 fold increase in analgesic potency, determined by the 'tail pinch' method [6] after intracisternal administration to mice. The extension of side chain in analogue **5** with leucine attached to the δ-amino group of D-ornithine by a polar asparagine residue enhanced analgesic activity of compound **1** by 10 times. The extension of the side chain in analogue **6**, containing the polar amino acid residue asparagine attached to the δ-amino group of D-ornithine, by a basic arginine residue induced a 12-fold increase in potency of the analogue **3**. The C-terminal amidation of peptide **3** resulted in 150 times improvement in potency of compound **4** as compared to compound **6**, containing one amino acid residue in the side chain or in 75 times compared to the corresponding amide **6a**. The peptide with an Arg-Asn-side chain in position 2 in conjunction with amidation exhibited a remarkable increase in potency. The branched enkephalin analogue **4** possessed pronounced activity and turned out to be the most active among the branched peptides. It was 43500 times more potent than [Leu^5]-enkephalin (LE) and 300 times more potent than morphine under these conditions (*table II*).

*Correspondence and reprints

Table I. Analytical data of enkephalin analogues.

No	Compound	[a]D^{22}(°) 0.2M AcOH (c)	TLC[a] R_f(A)	TLC[a] R_f(B) R_f(C)	Amino acid analysis Tyr	Gly	Phe $Phe(NO_2)$	Orn Lys	Leu Arg	Asn	HPLC[b] Mobile phase composition	HPLC[b] (k')[c]
1	Tyr-D-Orn-Gly-Phe Asn-Leu	+34.5 (0.20)	0.65	0.28 0.18	0.87	1.00	1.01	0.93	0.89	0.89	18:82	1.2
2	Tyr-D-Lys-Gly-Phe(NO_2)-NH_2 Asn-Arg	+33 (0.13)	0.53	0.36 0.19	1.02	1.00	1.10	0.86	0.92	1.05	25:75	1.5
3	Tyr-D-Orn-Gly-Phe Arg-Asn	+33 (1.00)	0.32	0.16	0.86	1.00	0.92	0.93	0.79	0.98	5:95	1.1
4	Tyr-D-Orn-Gly-Phe-NH_2 Arg-Asn	+45 (1.00)	0.42	0.21	0.93	1.00	1.07	0.97	1.00	1.03	5:95	1.3

[a]Thin layer chromatography (TLC) was carried out on Merck precoated 0.25 mm analytical silica-gel plates 60 F_{254} using the solvent systems: A, *n*Butanol–pyridine–AcOH–H_2O (15:10:3:6); B, $CHCl_3$–MeOH–AcOH–H_2O (30:20:4:6); C, *n*Butanol–AcOH-H_2O (4:1:1). [b]RP-HPLC was performed on a Dupont model 8800 HPLC system using a column (4.6 x 110 mm) packed with reverse phase sorvent Silasorb C_{18} (Lachema); mobile phase was: CH_3CN: 0.1 M acetate buffer. [c]Capacity factor.

Table II. Analgesic activity of enkephalin analogues ('tail pinch' method in mice).

No	Compound	Intracisternal administration ED_{50} nmol/animal	Duration of analgesic effect at $ED_{60\text{-}80}$, min	Relative analgesic activity morphine = 1	Intravenous administration ED_{50} μM/kg	Duration of analgesic effect at $ED_{60\text{-}80}$, min	Relative analgesic activity morphine = 100%
1	Tyr-D-Orn-Gly-Phe Asn-Leu	0.06(0.01-0.29)	45[a]	20	18.2 (3.6-78.3)	60	~100
2	Tyr-D-Lys-Gly-Phe(NO_2)-NH_2 Asn-Arg	0.025(0.05-0.13)	120[a]	48	18.6(4.1-84.9)	90[a]	~100
3	Tyr-D-Orn-Gly-Phe Arg-Asn	0.05(0.03-0.10)	30	24	35 (25-50)	60	50
4	Tyr-D-Orn-Gly-Phe-NH_2 Arg-Asn	0.004(0.003-0.005)	15	300	12 (10-14)	30	150
	Compounds for comparison:						
5	Tyr-D-Orn-Gly-Phe Leu	0.6(0.4-1.0)	30	2	101 (60-173)	30	18
6	Tyr-D-Orn-Gly-Phe Asn	0.6(0.3-1.0)	180	2	32 (21-49)	30	57
6a	Tyr-D-Orn-Gly-Phe-NH_2 Asn	0.29(0.015-5.59)	90[a]	4	21 (9.0-49.2)	45-60[a]	87
7	[Leu^5]-enkephalin	174(102-281)	15	0.007			
8	Morphine	1.2(0.6-2.2)	60	1	18 (15-22)	60	100

[a]ED_{50}.

Table III. Opioid activity of enkephalin analogues.

No	Bioassays *in vitro*					Receptor Binding Assays				
	Guinea Pig Ileum (GPI)		Mouse Vas Deferens (MVD)			[^{3}H]-Naloxone		[^{3}H]-DADLE		
	IC_{50} nM	Relative potency, %	IC_{50} nM	Relative potency, %	GPI_{rel} / MVD_{rel}	IC_{50} nM	Relative potency, %	IC_{50} nM	Relative potency, %	[^{3}H]-Naloxone$_{rel}$ / [^{3}H]-DADLE$_{rel}$
1	178 ± 36	160	580 ± 90	3.4	47	7.00 ± 0.15[a]	146	16.71 ± 2.34	70	2.1
2	26 ± 9	1075	86 ± 17	23.1	46	0.92 ± 0.18	1125			
3	183 ± 40	155	610 ± 120	3.3	47	12.6 ± 1.57	86	80.66 ± 17.93	14	6.1
4	144 ± 32	198	225 ± 32	8.9	22	4.69 ± 0.90	220	80.09 ± 11.27	14	15 7
	Compounds for comparison									
5	364 ± 57	78	680 ± 90	2 9	27	11.47 ± 2.11	90	13.29 ± 2.29	88	1.02
6	380 ± 34	75	2200 ± 540	0 9	83	55.41 ± 12.22	18	327.10 ± 53.87	3.6	5.0
7	285 ± 78	100	20 ± 1	100	1	10.35 ± 1.57	100	11.7 ± 1.7	100	1.00
8	78 ± 19	365	579 ± 224	3 4	107	10.25 ± 1.45	100			

[a] [^{3}H]-DAMGO.

Replacement of Phe^4 by $Phe(NO_2)^4$ gave an increase in the duration of the analgesic effect. The hexapeptide **2** manifested the longest duration: 120 min after intracisternal administration, and 90 min after i.v. administration, 2 and 1.5 times longer than that of morphine under the same conditions (*table II*).

A series of new branched analogues was as active after i.v. administration as morphine (**1**,**2**) or exceeded the activity of morphine (**4**), except for compound **3**. The duration of analgesic action of peptides **1**–**3** was comparable with that of morphine (60 min).

In the GPI assay [5] all branched analogues were more potent than (LE) (*table III*). The extension of the side chain in peptide **5**, containing leucine attached to the δ-amino group of D-ornithine by a polar asparagine residue increased the potency in the GPI of compound **1** 2 times, whereas the potency in the MVD was unchanged. The extension of the side chain in peptide **6**, containing asparagine, by the basic amino acid residue arginine, increased the potency of compound **3** to both μ-and δ-ORs. Amidation **3** → **4** had practically no effect on potency of compound **4** in the GPI and MVD assays. The highest potency (10 times that of (LE)) was displayed by analogue **2**, which also was the most active compound in the MVD bioassay. All peptides demonstrated a preference for the μ-ORs over the δ-ORs.

In the binding assay [5] based on displacement of the μ-selective [H^3]Naloxone from rat brain membranes, the new branched analogues showed the same rank order of potency as in the GPI assay (*table III*). The addition of a basic amino acid residue arginine **6**→ **3** enhanced the affinity to the μ-and δ-ORs in brain. Peptide **3** displayed a 4 fold increased binding to the μ-and δ-ORs with unaltered selectivity. Addition of a polar amino acid residue asparagine **5** → **1** also resulted in an increase of μ-affinity, but had no effect on δ-affinity of **1**. The amidated compound **3**, analogue **4**, was 2 times more potent at the μ-receptor than **3** and showed stronger preference for μ-ORs as compared with δ-ORs than (LE). The peptide **2** was the most active compound, showing a 10 fold increase of μ-receptor affinity relative to that of (LE).

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