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Novel nociceptin analogues★✪

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A series of new nociceptin analogues containing cysteine was tested for their nociceptive effects in tail-flick test on mice after *icv* injection. The cysteines were introduced in order to get irreversibly binding analogues based on the assumption that the cysteines in the ligand can interact with the cysteines from the receptor to form an S–S bridge. *In vivo* tests revealed that Cys^1-nociceptin (1-13)-NH_2 (Cys1-NC) is an antagonist, whereas Cys7-NC is an agonist. Gly^1,[Phe(p-NO_2)]^4-NC was less active indicating that the antagonist properties of Cys1-NC are associated with the presence of the sulfhydryl group of cysteine. The analogues D-Cys2 and Cys3 were also almost inactive.

In the early 1990s the experiments on cloning of opioid receptors revealed the existence of a new receptor highly homologous (60–70%) to opioid receptor. Surprisingly, the classical opioids such as enkephalins did not bind well to the newly discovered receptor, hence it was named orphan receptor. In 1995 a native ligand was discovered and according to its unusual pharmacological properties was named nociceptin or orphanin FQ. The name nociceptin was based on the fact that the new neuropeptide shortened the latency time in response to pain stimulus, an effect opposite to that of morphine.

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Abbreviations: ANTG, antagonist; *icv*, intracerebroventricular; NC, nociceptin.

Nociceptin (FGGFTGARKSARKLANQ) binds poorly to opioid receptors and exerts its effect *via* its own receptor [1–4].

RESULTS AND DISCUSSION

We have synthesized and tested nociceptin analogues containing cysteine in positions 1, 2, 3 and 7. The positions were chosen on the assumption that amino-acid residues at those positions are not essential for biological activity. Previously published papers revealed that the replacement of amino acids located at aforementioned positions are tolerated. The so-called L-Ala scan revealed that the side chains of amino acids at positions 3 and 7 are not necessary for binding [5]. D-Ala scan suggested that at position 2 only a small D-amino acid is tolerated [5]. On the other hand, the replacement Phe1→Leu1 resulted in a diminished binding affinity (3 times only) therefore we decided to test the Cys1 analogue [6–7].

The peptides tested in this study are schematically depicted in Table 1. The cysteine analogues were designed in order to bind irreversibly to the nociceptin receptor. The receptor contains Cys residues located in all transmembrane domains (with the exception of TM5). In addition the extracellular loops E2 and E3 are connected with an S–S bridge (Cys123–Cys200 in human receptor) and one Cys residue is in E4.

We assume that those cysteines may act as an anchor for cysteine-containing analogues by formation of a new S–S bridge with the sulfhydryl group of the analogue.

The peptides were tested biologically with tail-flick test after *icv* injection of 10 nmol of the compound in order to assess their properties. Table 1 and Figs. 1 and 2 show the results. Control injections of solvent (phosphate-buffered saline) did not change mean latency time significantly as compared to untreated mice (Table 1). Nociceptin was tested at three doses of 0.1, 1 and 10 nmol, but *icv* in-

Table 1. Structure of the investigated compounds and the mean latency times

Substance	Structure	Mean latency times in tail-flick test				
		0 min	*15 min*	*30 min*	*45 min*	*60 min*
Control	-	8.1 ±0.6	7.1 ±0.9	7.4 ±0.8	6.9 ±0.6	7.7 ±0.7
Solvent	phosphate buffered saline	7.9 ±0.7	9.0 ±0.4	7.3 ±0.2	6.5 ±0.7	7.0 ±0.5
BN-16	GGGF(p-NO2)TGARKSARKLANQ-NH_2	8.1 ±0.8	11.1 ±1.3 *	8.7 ±0.3	7.7 ±0.6	8.1 ±0.7
Cys7-NC	FGGFTGCRKSARK-NH_2	8.1 ±0.5	4.5 ±0.4 *	5.3 ±0.4 *	6.0 ±0.5	8.4 ±1.4
Cys3-NC	FGCFTGARKSARK-NH_2	8.3 ±1.0	8.7 ±0.9	7.7 ±0.6	7.0 ±1.1	7.8 ±1.1
D-Cys2-NC	FcGFTGARKSARK-NH_2	7.3 ±0.7	6.6 ±1.3	7.4 ±0.8	8.1 ±0.9	7.1 ±0.8
Cys1-NC	CGGFTGARKSARK-NH_2	8.0 ±0.6	10.2 ±0.3 *	9.5 ±0.8	8.4 ±0.7	8.6 ±1.0
ANTG	N-Ph- GGFTGARKSARKLANQ-NH_2	7.7 ±0.8	10.3 ±0.6 *	10.7 ±0.8 *	8.6 ±0.7	8.1 ±0.4
NC	FGGFTGARKSARKLANQ-NH_2	8.5 ±1.2	4.1 ±0.3 *	5.3 ±0.7 *	6.0 ±0.6 *	9.7 ±2.1

Latency times (in seconds, ± S.E.) tested in 15 minutes intervals after *icv* injection of 10 nmol of compound. The tail flick tests were performed on female mice strain 129 (groups of six animals). Control: untreated mice. Statistical significance was evaluated using ANOVA test (planned comparisons for repeated measures). Results were considered as significant if $P<0.05$. *Statisticaly significant in comparison with mean latency time before injection (0 min).

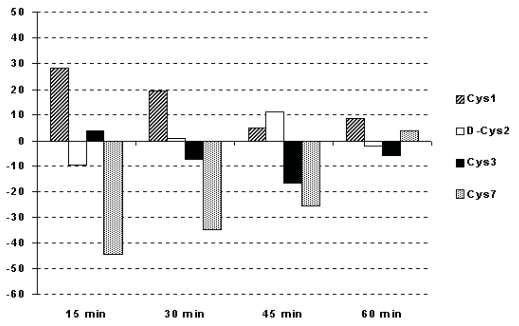

Figure 1. Relative changes (%) of mean latency time in tail-flick test *versus* mean latency time measured before injection.

jection of 0.1 or 1 nmol of the peptide did not result in statistically significant changes in behavior (data not shown). The 10 nmol dose

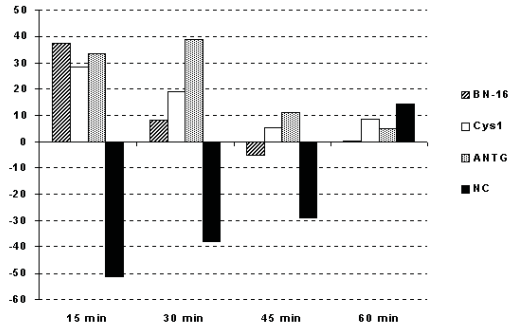

Figure 2. Comparison of Cys1-NC, BN-16, nociceptin (NC), and the antagonist (ANTG) [8] — relative changes (%) of mean latency time in tail-flick test *versus* mean latency time measured before injection.

was sufficient to cause a measurable effect. Figure 1 presents relative changes in the latency time induced by *icv* injection of 10 nmol of cysteine containing analogues. The effects caused by the Cys analogues were compared with the effect of nociceptin (NC) and the known antagonist ([Nphe]^1-nociceptin-NH_2 (ANTG) [8].

Cys1-NC was compared with the non-cysteine analogue Gly^1,Phe(p-NO_2)^4 (BN-16), as well as with nociceptin (NC) and the antagonist (ANTG) (Fig. 2) [8]. The nitro group has been shown to improve agonistic properties of nociceptin*.

We suggest that Cys1-NC(1-13)-NH_2 is an antagonist, whereas Cys7-NC(1-13)-NH_2 is an agonist. The comparison of Cys1-NC with BN-16 revealed that the sulfhydryl group of cysteine in position 1 is necessary for antagonistic properties. Cysteines at positions 2 and 3 have a detrimental effect on biological activity. Weak reactions can be attributed to a weak intrinsic activity. However, mixed agonist-antagonist properties cannot be excluded. The experiments are in progress. It is worth noting that the Cys1-NC analogue (an antagonist) contains Cys in the so-called message domain of nociceptin and Cys7 is located in the address domain.

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