Articles

Address and Message Sequences for the Nociceptin Receptor: A Structure−Activity Study of Nociceptin-(1−13)-peptide amide

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Nociceptin (NC) and some of its fragments as well as nociceptin-(1−13)-peptide amide [NC-(1−13)-NH_2] and a series of its analogues were prepared and tested in the mouse vas deferens in an attempt to identify the sequences involved in the activation (message) and in the binding (address) of nociceptin to its receptor. The NC receptor that inhibits the electrically evoked twitches of the mouse vas deferens was demonstrated to be distinct from the δ opioid receptor, since naloxone and Dmt-Tic-OH (a selective δ opioid receptor antagonist) block the δ opioid receptor but have no effect on the nociceptin receptor. Results from structure−activity experiments suggest that (a) the entire sequence of NC may not be required for full biological activities, since NC(1−13)-NH_2 is as active as NC; (b) fragments of NC have however to be amidated as in NC(1−13)-NH_2 in order to be protected from degradation by proteases; (c) cationic residues (as $Arg^{8,12}$, $Lys^{9,13}$) appear to play a functional role, since their replacement with Ala in the sequence of NC(1−13)-NH_2 leads to inactivity; (d) the N-terminal tetrapeptide Phe-Gly-Gly-Phe is essential for activity: its full length and flexibility appear to be required for NC receptor activation and/or occupation; (e) Phe^4 and not Phe^1 appears to be the residue involved in receptor activation, since the replacement of Phe^1 with Leu has no effect, while that of Phe^4 leads to inactivity. Results summarized in this paper indicate that the structural requirements of NC for occupation and activation of its receptor are different from that of opioids, particularly δ agonists.

Introduction

The naturally occurring ligand of the ORL_1/LC_{132} receptor has been recently identified in the rat[1] and porcine brain,[2] and a peptide precursor has been cloned from mouse,[3] rat,[4] and human[5] brains. This novel heptadecapeptide, whose sequence is Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln, has been termed nociceptin (henceforth called NC), or orphanin FQ, and shows structural similarities with the mammalian opioid peptide dynorphin A, except for Phe^1 and a different distribution of the basic residues. In binding[2] as well as in biological assays[1] it was been shown that nociceptin does not interact with the opiate receptors and acts through the activation of a selective and specific functional site (receptor) which has been shown to inhibit the electrically evoked contractions in several isolated organs suspended in vitro.[6−9] When applied in vivo, nociceptin is algogenic[1,2] and recently it has also been found to reverse opioid-mediated analgesia in the mouse.[10,11] It therefore appears that, despite the structural similarities of nociceptin with dynorphin A^{12} and the identity of some biological effects (e.g. inhibition of sympathetic, parasympathetic and peptidergic nervous activities,[6−9] as well as inhibition of the forskolin-induced accumulation of cAMP[1,2]), the functional sites of opioids and that of nociceptin are pharmacologically different.[1,2]

In the present study, we tried to explore the functional roles of the nociceptin molecule and identify the message and address sequences that enter in the receptor interaction. For this purpose, a series of analogs of nocieptin-1(1−13)-peptide amide [NC(1−13)-NH_2], which has been shown to posses full biological activity,[7] were prepared and tested on the mouse vas deferens, a recently described bioassay.[7]

Results and Discussion

Control experiments were performed to establish that the mouse vas deferens is a two receptor system that responds to both deltorphin and nociceptin with a concentration-dependent reduction of the electrically induced twitches. This is shown in Figure 1 (top panel), where the effect of deltorphin I, a δ opioid receptor selective agonist,[13] is found to be inhibited by the nonselective opioid receptor antagonist naloxone[14] (pA_2: 7.5) as well as by the δ opioid receptor selective antagonist Dmt-Tic-OH[15] (pA_2: 8.2). These two antagonists are completely inactive (when applied in concentrations of 1 μM) against the effect of NC [NC(1−17)-OH], (compound **1**) (Figure 1, bottom panel), an agent

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Table 1. Primary Structures, Abbreviated Names, and Analytical Properties of Nociceptin Fragments and of NC(1−13)-NH_2 Analogues

no.	abbreviated names	primary structures	K' [a]	MH^+ [b]
1	NC(1−17)-OH	H-FGGFTGARKSARKLANQ-OH	3.46	1809
2	NC(1−17)-NH_2	H-FGGFTGARKSARKLANQ-NH_2	2.77	1808
3	NC(1−13)-NH_2	H-FGGFTGARKSARK-NH_2	3.72	1382
4	NC(1−13)-OH	H-FGGFTGARKSARK-OH	2.07	1383
5	NC(1−12)-NH_2	H-FGGFTGARKSAR-NH_2	2.28	1254
6	NC(1−12)-OH	H-FGGFTGARKSA-OH	2.19	1255
7	NC(1−11)-NH_2	H-FGGFTGARKSA-NH_2	2.43	1098
8	NC(1−9)-NH_2	H-FGGFTGARK-NH_2	2.04	940
9	NC(1−5)-NH_2	H-FGGFT-NH_2N	4.27	527
10	NC(1−4)-NH_2	H-FGGF-NH_2	5.34	426
11	NC(13-17)-OH	H-KLANQ-OH	3.52	573
12	Ac-Nc(1−13)-NH_2	Ac-FGGFTGARKSARK-NH_2	4.28	1424
13	*N*,*N*-(diallyl)-NC(1−13)-NH_2	*N*,*N*-(diallyl)-FGGFTGARKSARK-NH_2	2.96	1462
14	[D-Phe[1]]-NC(1−13)-NH_2	H-fGGFTGARKSARK-NH_2	2.36	1382
15	[D-Phe[4]]-NC(1−13)-NH_2	H-FGGFTGARKSARK-NH_2	2.68	1382
16	[Leu[1]]-NC(1−13)-NH_2	H-LGGFTGARKSARK-NH_2	2.11	1348
17	[Leu[4]]-NC(1−13)-NH_2	H-FGGLTGARKSARK-NH_2	2.21	1348
18	[D-Ala[2],desGly[3]]-NC(1−13)-NH_2	H-FaFTGARKSARK-NH_2	2.67	1339
19	[desGly[3]]-NC(1−13)-NH_2	H-FGFTGARKSARK-NH_2	2.27	1325
20	[Ala[2,3]]NC(1−13)-NH_2	H-FAAFTGARKSARK-NH_2	1.95	1410
21	[Ala[8,9]]NC(1−13)-NH_2	H-FGGFTGAAASARK-NH_2	3.6	1240
22	[Ala[12]]NC(1−13)-NH_2	H-FGGFTGARKSAAK-NH_2	2.38	1297

which is able to induce up to 80% of the inhibitory effect observed with opioids in this preparation.[7]

In another series of experiments we investigated the minimum sequence required for full biological activity. The data presented in Table 2 indicate that nociceptin amide, NC(1−17)-NH_2 (compound **2**), has the same pharmacological effect as naturally occurring peptide, confirming the finding reported by Dooly et al.,[16] who found the same affinity for the two peptides in binding assay to rat brain membranes. Deletion of the four C-terminal (not charged) amino acids as in NC(1−13) gives analogs with different activity, depending on the C-terminal function: NC(1−13)-NH_2 (compound **3**) is a full agonist with the same potency as of NC, while the free acid (compound **4**) shows surprising decrease of potency (more than two log units). Sequence deletion of charged residues from NC(1−13)-NH_2 gives reduction of activity in compound **5**, and analogs **7** and **8** are very weakly active or inactive. These results indicate that (a) the whole sequence of NC may not be required for binding to the receptor[16] and for full biological activity in vitro[7] and perhaps in vivo and (b) the C-terminal amidation in truncated analogs may protect the peptide from enzyme degradation. Such a protection appears to be more efficient on NC(1−13) and NC(1−12) than on NC, probably because the removal of the C-terminal residue from NC is expected to be less effective than the removal from NC(1−13) and from NC(1−12). It is also to be argued that the metabolite released by carboxypeptidases from NC maintain high biological activity while that deriving from NC(1−13) and NC(1−12) are inactive (compounds **7** and **8**). An alternative explanation to the surprising biological activity difference between NC(1−13)-NH_2 and the corresponding free acid NC(1−13)-OH can be sought in the topological (and spatial) proximity of the terminal carboxyl group and the last two basic residues, Arg[12] and Lys.[13] The "message addresses working hypothesis" of NC and dynorphin A are characterized by the presence of several basic residues that, in the case of dynorphin A, are known to interact with the receptor.[17] If we hypothesize that also the basic residues of NC are essential for a good interaction with the NC receptor, it is clear that

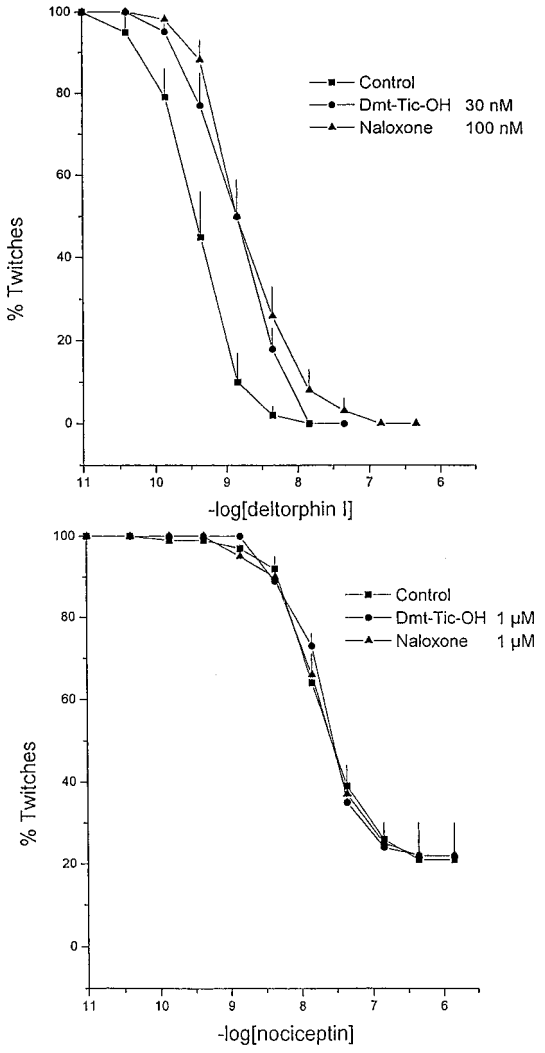

Figure 1. Concentration-response curves to deltorphin I (top panel) and nociceptin (bottom panel) in the absence or in the presence of naloxone or Dmt-Tic-OH in the electrically stimulated mouse vas deferens.

the introduction of an acidic group beside Lys[13] amounts to a "neutralization" of this residue. This view is substantiated by the almost identical decrease of activ-

Table 2. Apparent Affinities (pEC_{50}) and Maximum Effects of Nociceptin and Nociceptin Related Peptides on the Electrically Stimulated Mouse Vas Deferens[g]

		agonist		
no.	abbreviated names	pEC_{50} ($CL_{95\%}$)[b]	E_{max},[c] %	antagonist[a]
1	NC(1–17)-OH	7.84 (0.07)	−83 ± 3	ND[d]
2	NC(1–17)-NH_2	7.74 (0.11)	−79 ± 4	ND
3	NC(1–13)-NH_2	7.75 (0.14)	−84 ± 4	ND
4	NC(1–13)-OH	5.65 (0.35)	−73 ± 7	ND
5	NC(1–12)-NH_2	6.06 (0.51)	−79 ± 7	ND
6	NC(1–12)-OH	I[e]		I
7	NC(1–11)-NH_2	crc incom[f]		I
8	NC(1–9)-NH_2	I		I
9	NC(1–5)-NH_2	I		I
10	NC(1–4)-NH_2	I		I
11	NC(13–17)-OH	I		I

[a] The antagonistic properties of these compounds were tested using NC(1–13)-NH_2 as agonist. [b] pEC_{50}: the negative logarithm to base ten of the molar concentration of an agonist that produces 50% of the maximal effect. $CL_{95\%}$: 95% confidence limits. [c] E_{max}: the maximal effect induced by an agonist expressed as percent inhibition of electrically induced twitches. [d] ND: not determined because these compounds are full agonists. [e] I: inactive at 10 μM. [f] crc incom: crc incomplete indicates that only a slight effect (<50% inhibition) was detected at the highest concentration tested (10 μM). [g] All the effects of these compounds were not affected by 1 μM naloxone.

Table 3. Apparent Affinities (pEC_{50}) and Maximum Effects of NC(1–13)-NH_2 Analogues on the Electrically Stimulated Mouse Vas Deferens[g]

		agonist		
no.	abbreviated names	pEC_{50} ($CL_{95\%}$)[b]	E_{max},[c] %	antagonist[a]
12	Ac-NC(1–13)-NH_2	5.82 (0.21)	−79 ± 6	ND[d]
13	*N*,*N*-(diallyl)-NC(1–13)-NH_2	I[e]		I
14	[D-Phe[1]]NC(1–13)-NH_2	crc incom[f]		I
15	[D-Phe[4]]NC(1–13)-NH_2	crc incom		I
16	[Leu[1]]NC(1–13)-NH_2	7.55 (0.26)	−80 ± 12	ND
17	[Leu[4]]NC(1–13)-NH_2	I		I
18	[D-Ala[2],desGly[3]]NC(1–13)-NH_2	crc incom		I
19	[desGly[3]]NC(1–13)-NH_2	I		I
20	[Ala[2,3]]NC(1–13)-NH_2	I		I
21	[Ala[8,9]]NC(1–13)-NH_2	crc incom		I
22	[Ala[12]]NC(1–13)-NH_2	crc incom		I

[a] The antagonistic properties of these compounds were tested using NC(1–13)-NH_2 as agonist. [b] pEC_{50}: the negative logarithm to base ten of the molar concentration of an agonist that produces 50% of the maximal effect. $CL_{95\%}$: 95% confidence limits. [c] E_{max}: the maximal effect induced by an agonist expressed as percent inhibition of electrically induced twitches. [d] ND: not determined because these compounds are full agonists. [e] I: inactive at 10 μM. [f] crc incom: crc incomplete indicates that only a slight effect (<50% inhibition) was detected at the highest concentration tested (10 μM). [g] All the effects of these compounds were not affected by 1 μM naloxone.

ity in going from NC(1–13)-NH_2, to NC(1–12)-NH_2 in which Lys[13] is altogether suppressed.

The results obtained with the N-terminal fragments (compounds **8**–**10**) raise another point: in contrast to μ and δ opioid receptors, which can be activated by a minimum tetrapeptide sequence,[18–20] nociceptin fragments (compound **8**–**10**) are completely inactive, suggesting that the nociceptin receptor can be occupied and activated only by a rather large sequence that includes an N-terminal message site and a C-terminal cationic segment, which probably contributes to address.

This hypothesis was tested with the series of compounds presented in Table 3, which are analogs of NC-(1–13)-NH_2. Firstly, the N-terminal amino function was modified by acetylation (compound **12**) or by dialylation (compound **13**): both modifications led to very significant decrease or total elimination of activity. Moreover analog **13** was not found to act as an antagonist, contrary to opioids, where this modification leads to antagonism.[21,22] Other changes were made in positions 1 and 4 to explore the functional role of the aromatic residues. Configurational requirements are evidenced by the replacement of Phe[1] or Phe[4] with the corresponding D-residues (compound **14** and **15**): this replacement leads to drastic decrease of activity, indicating that the message domain of nociceptin, similarly to opioid peptides,[23,24] requires a defined steric arrangement of the side chains of the two aromatic residues. These results are only partially in accord with those reported for similar analogs by Civelli et al.[25] using receptor binding and forskolin-stimulated cAMP accumulation techniques. Replacement of Phe[1] or Phe[4] with an aliphatic residue (Leu), to eliminate aromaticity, was well-tolerated in position 1 (compound **16**) but not in position 4 (compound **17**), in contrast to the opioid peptides where Tyr[1] is essential for receptor activation.[23,24] It should be also pointed out that studies about NC based on the conventional "alanine scan" indicated that Phe[1] or Phe[4] replacement leads to complete loss of affinity and activity on the NC receptor.[16,25] The use of a residue with a large hydrophobic side chain has allowed, in the present study, to determine the difference in the functional role of Phe[1] and Phe;[4] aromaticity is essential only in position 4.

To explore further the message sequence we shortened the chain by one residue (compound **19**) and replaced Gly with D-Ala (compound **18**). Both analogs were found to be completely inactive, again contrasting with what has been reported for opioid peptides where Tyr-D-Xaa-Phe acts as a message sequence selective for μ and δ opioid receptors.[26] These results indicate that not only the active site of NC is different from that of opioids but also the steric requirements of the message sequence of the two group of peptides (NC and opioids) are different. This is also shown in compound **20**, where the two Gly residues were replaced with Ala to stabilize the conformation of the message sequence; the analog was completely inactive, indicating that the side chain has a negative influence on the bioactive conformation of the NC message sequence. Finally two other compounds were prepared to evaluate the role of the cationic residues at the C-terminal site of NC(1–13)-NH_2; as shown by the results obtained with compound **21** and **22**, the replacement of Arg[8] and Lys[9] with Ala, as well as that of Arg,[12] leads to complete loss of activity, suggesting that positive charges at the C-terminal end play a substantial role for activity, probably by influencing the peptide affinity (binding) to the receptor. Indeed, both compounds were found to be inactive as antagonists.

Experimental Section

General Methods. All peptides reported in this work were prepared by solid phase synthesis performed in a Milligen 9050 synthesizer using a [4-[α-(2′,4′-dimethoxyphenyl)Fmoc-aminomethyl]phenoxy]acetamidonorleucyl-MBHA resin (Rink amide MBHA resin) (0.55 mmol/g; 0.2 g in all syntheses) for peptide amides or [4-(hydroxymethyl)phenoxy]methylcopoly (styrene 1% divinylbenzene) resin (Wang resin) [Fmoc-Lys(Boc)-Wang resin, 0.47 mmol/g, 0.2 g; Fmoc-Gln(Trt)-Wang resin, 0.58 mmol/g, 0.2 g; Fmoc-Arg(Pmc)-Wang resin, 0.52 mmol/g, 0.2 g] for peptide acids, both obtained from Novabiochem AG. All

the resins were mixed with glass beads (1:15 w/w) obtained from Sigma. Protected amino acids were from Backem or Novabiochem AG. Peptide were assembled using Fmoc-protected amino acids and 1,3-diisopropylcarbodiimide and 1-hydroxybenzotriazole as coupling agents, 1 h for each coupling. Side chain protecting groups used were Pmc for Arg, Trt for Asn, Boc for Lys, and tBu for Ser and Thr. Peptides **12** and **13** were synthesized using Ac-Phe-OH or N,N-diallyl-Phe-OH instead of Fmoc-Phe-OH in the final acylation step of the peptide synthesis. The peptides were cleaved from the resin by treatment with TFA/H_2O/phenol/ethanedithiol/thioanisole (reagent K) (82.5:5:5:2.5:5; v/v) at room temperature for 1 h.

Naloxone was from Tocris Cookson. Stock solutions (1 mmol) of peptides were made in distilled water and kept at −20 °C until used. All other reagents were from Sigma Chemical Co. or E. Merck. Krebs solution (gassed with 95% O_2 and 5% CO_2, pH 7.4) had the following composition (in mmol): 118.5 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$, 10 glucose.

Solid Phase Peptide Synthesis of NC(1−17)-NH_2 (2). Rink amide MBHA resin (0.55 mmol/g; 0.2 g) was treated with piperine 20% DMF and linked with N^{α}-Fmoc-N^{γ}-trityl-glutamine via its *N*-hydroxybenzotriazole (HOBt) active ester. The following N^{α}-Fmoc amino acids were sequentially coupled to the growing peptide chain: N^{α}-Fmoc-N^{γ}-Trt-Asn, N^{α}-Fmoc-Ala, N^{α}-Fmoc-Leu, N^{α}-Fmoc-N^{ϵ}-Boc-Lys, N^{α}-Fmoc-N^{ω}-Pmc-Arg, N^{α}-Fmoc-Ala, N^{α}-Fmoc-*O*-tBu-Ser, N^{α}-Fmoc-N^{ϵ}-Boc-Lys, N^{α}-Fmoc-N$^{\omega}$-Pmc-Arg, N^{α}-Fmoc-Ala, N^{α}-Fmoc-Gly, N^{α}-Fmoc-*O*-tBu-Thr, N^{α}-Fmoc-Phe, N^{α}-Fmoc-Gly, N^{α}-Fmoc-Gly, N^{α}-Fmoc-Phe. All the N^{α}-Fmoc amino acids (4 equiv) were coupled to the growing peptide chain by using 1,3-diisopropylcarbodiimide (4 equiv) and 1-hydroxybenzotriazole (4 equiv) in DMF, and the coupling reaction time was 1 h. Piperidine/20% DMF was used to remove the Fmoc group. After deprotection of the last N^{α}-Fmoc group, the peptide resin was washed with methanol and dried in vacuo to yield the protected NC(1−17)-NH_2-resin.

The other peptide amides (compounds **3**, **5**, **7**−**10**, and **12**−**22**) were synthesized in a similar manner. Peptide acids (compounds **1**, **4**, **6**, **11**) were prepared starting from the corresponding Wang resin [Fmoc-Gln(Trt)-Wang resin, 0.58 mmol/g, 0.2 g; Fmoc-Lys(Boc)-Wang resin, 0.47 mmol/g, 0.2 g; or Fmoc-Arg(Pmc)-Wang resin, 0.52 mmol/g, 0.2 g] in the same manner as reported for peptide amides.

The protected peptide−resin was treated with reagent K (10 mL/0.2 g of resin) for 1 h at room temperature. After filtration of the exhausted resin, the solvent was concentrated in vacuo and the residue triturated with ether. The crude peptide was purified by preparative reverse phase HPLC to yield a white powder after lyophilization.

Peptide Purification and Analytical Determinations. Crude peptides were purified by preparative reversed-phase HPLC using a Water Delta Prep 4000 system with a Waters PrepLC 40 mm Assembly column C_{18} (30 × 4 cm, 300 A, 15 μm spherical particle size column). The peptides were eluted with a gradient of 0−50% B in 25 min at a flow rate of 50 mL/min, and the mobile phases were solvent A (10%, v/v, acetonitrile in 0.1% TFA) and solvent B (60%, v/v, acetonitrile in 0.1% TFA).

Analytical HPLC analyses were performed on a Bruker liquid chromatography LC 21-C instrument fitted with a Vydac C_{18} (4.6 × 250 mm 5 μm particle size) and equipped with a Bruker LC 313 UV variable-wavelength detector. Recording and quantification were accomplished with a chromatographic data processor coupled to an Epson computer system (BX-10). Analytical determination and capacity factor (K') of the peptides were determinated using HPLC conditions in the above solvent systems programmed at flow rates of 1 mL/min for linear gradients from 0% to 100% B in 25 min. All analogues showed less than 1% impurities when monitored at 220 nm.

Amino acid analyses were carried out using Pico-Tag methodology and PITC as amino acid derivatization reagent. Lyophilized samples of peptides (50−1000 pmol) were placed in heat-treated borosilicated tubes (50 × 4 mm), sealed, and hydrolyzed using 200 μL of 6 N HCl containing 1% phenol in the Pico-Tag workstation for 1 h at 150 °C. A Pico-Tag column (15 × 3.9 mm) was employed to separate the PITC-amino acid derivatives. The composition of the peptide was as expected.

The molecular weight of the compounds was determined by a MALDI-TOF analysis using a Hewlett Packard G2025A LD-TOF system mass spectrometer and α-cyano-4-hydroxycinnamic acid as a matrix. The values are expressed as MH^+.

Bioassay. Swiss male mice weighing 25−30 g were used. The prostatic portion of the vas deferens (mVD) was prepared according to the work of Hughes et al.[27] and suspended in 10 mL organ baths containing Mg^{2+} free Krebs solution at 33 °C. The tissues were stimulated through two platinum ring electrodes with supramaximal rectangular pulses of 1 ms duration and 0.1 Hz frequency. The resting tension was maintained at 0.3 g. The electrically evoked contractions were measured isotonically by means of a Basile strain gauge transducer and recorded on a Linseis multichannel chart recorder (model 2005).

After an equilibration period of about 2 h the contractions induced by electrical field stimulation were stable. At this time cumulative concentration−response curves (crc) to nociceptin, nociceptin related peptides, or opioid receptor agonists were performed (0.5 log unit steps). When required, antagonists were added to the Krebs solution 15 min before performing crc to agonists.

Data Analysis and Terminology. The data are expressed as mean ± standard error of the mean of *n* experiments. For pEC_{50} values the confidence limits at 95% are given. Data have been statistically analyzed using the Student two-tailed *t* test or the analysis of variance followed by the Student−Newman−Keuls test for multiple comparison via a software package.[28] *P* values lower than 0.05 were considered to be significant.

The pharmacological terminology adopted in this study is in line with the recent IUPHAR recommendation.[29,30,32] The agonist apparent affinities are given as pEC_{50} = the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect of the agonist. Apparent affinities of antagonists are given in terms of pA_2 = the negative logarithm to base 10 of the molar concentration of an antagonist that makes it necessary to double the concentration of agonist needed to elicit the original submaximal response.[31] Antagonist apparent affinities have been evaluated using the Gaddum Schild equation: $pA_2 = \log (CR - 1/[\text{antagonist}])$ assuming a slope value equal to unity.

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(32) Abbreviations follow IUPAC−IUB Joint Commission on Biochemical Nomenclature for amino acids and peptides: *Eur. J. Biochem.* **1980**, *138*, 9−37. Additional abbreviations used herein are as follows: Ac, acetyl; Boc, *tert*-butyloxycarbonyl; DMF, *N*,*N*-dimethylformamide; Dmt, 2,6-dimethyltyrosine; Fmoc, 9-fluorenylmethyloxycarbonyl; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; NC, nociceptin; PITC, phenyl isothiocyanate; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; tBu, *tert*-Butyl; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Trt, trityl.

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