Synthesis and Characterization of Selective Fluorescent Ligands for the Neurokinin NK_2 Receptor

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Several fluorescent probes for the NK_2 receptor were designed, synthesized, and pharmacologically characterized. These fluorescent ligands are analogues of the selective NK_2 heptapeptide antagonist *N*-α-benzoyl-Ala-Ala-D-Trp-Phe-D-Pro-Pro-Nle-NH_2 (1, GR94800). They were obtained by substitution of 2,*n*-diaminoalkyl amino acid (*n* = 3–6) for Ala[1] and the subsequent coupling of the fluorophore NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl) or fluoresceinthiocarbamyl to the N-ω amino group. The fluorescent derivatives retained high binding affinities for the NK_2 receptor in transfected CHO cells. In contrast, fluorescent derivatives made by replacing the *N*-α-benzoyl group of 1 by NBD or fluorescein were considerably less active. The effect on ligand potency of varying the length of the spacer arm between the peptide moiety and the fluorescent group was also studied. The most potent fluorescent antagonists were *N*-α-benzoyl-Dab(γ-NBD)-Ala-D-Trp-Phe-D-Pro-Pro-Nle-NH_2 (**5B**), $pK_i = 8.87$ for NK_2; *N*-α-benzoyl-Orn(δ-NBD)-Ala-D-Trp-Phe-D-Pro-Pro-Nle-NH_2 (**4B**), $pK_i = 8.84$; and *N*-α-benzoyl-Lys(ε-NBD)-Ala-D-Trp-Phe-D-Pro-Pro-Nle-NH_2 (**3B**), $pK_i = 8.83$. These three compounds were highly selective for NK_2 over NK_3 and NK_1 receptors. We show that these fluorescent ligands are useful tools for the detection of NK_2 receptor expression by flow cytometry. Additionally, these fluorescent probes should prove valuable for fluorescence microscopy and study of ligand–receptor interaction by spectrofluorimetry.

Introduction

The tachykinins (neurokinins) are a family of closely-related mammalian neuropeptides that display a variety of biological activities including pain transmission, smooth-muscle contraction, bronchoconstriction, vasodilation, activation of the immune system, and neurogenic inflammation.[1] Tachykinin actions are mediated by at least three distinct cell-surface receptors, NK_1, NK_2, and NK_3, that show preferential binding for the endogenous agonists substance P (SP), neurokinin A (NKA), and neurokinin B (NKB), respectively.[2] These receptors have been cloned and belong to the superfamily of membrane-bound receptors predicted to have seven transmembrane regions.[2] Neurokinin receptors mediate cellular responses through interaction with heterotrimeric G proteins, presumably of the Gq family, linked to phospholipase C and production of diacylglycerol and inositoltriphosphate second messengers.[3]

Various agonists and antagonists, including radiolabeled analogues, have been developed for the study of NK_2 receptor pharmacology and function,[4] but very few fluorescent ligands have been reported.[5,6] Fluorescently labeled ligands may have several advantages for the study of the nature and function of receptors at high resolution in individual cells.[7] Recently, we described fluorescein-labeled NKA (F-NKA), as a biologically active probe for NK_2 receptors.[6] However, the selectivity of F-NKA for NK_2 receptors over the NK_1 and NK_3 subtypes was low. Studies of receptor localization in tissues and assessment of the importance of the NK_2 receptor in physiological processes would greatly benefit from the availability of high-affinity and selective fluorescent antagonists. In addition, such ligands would be valuable tools for the localization and the study of the ligand binding domain on the receptor using fluorescence spectroscopy.[8]

Here we report the design, synthesis and pharmacological characterization of a series of fluorescent peptide ligands with high affinity and selectivity for the NK_2 receptor. We show that these fluorescent probes can be used for the analysis of NK_2 receptors in cultured cells by flow cytometry.

Design and Synthesis of Fluorescent NK_2 Antagonists

Fluorescent ligands for the NK_2 receptor were designed using the highly potent and selective antagonist GR94800 (1).[9] Based on structure–activity relationship studies carried out during the development of GR94800, two modifications were investigated in order to introduce a fluorophore into the structure while the affinity and selectivity for the NK_2 receptor were preserved. First, the effect of the replacement of the N-terminal bulky lipophilic benzoyl group in 1 by the fluorescent group fluorescein or 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) was investigated. Secondly, Ala[1] was replaced by Lys or Orn or Dab or Dap respectively and the newly introduced side-chain amino group was derivatized with fluorescein or NBD (Figure 1). Fluorescein was selected as a fluorophore for its high absorbance and fluorecence properties. NBD, which has spectral properties similar to fluorescein, was selected because its fluorescence is sensitive to the medium and is much higher in a hydrophobic environment.[10] This feature could be exploited to investigate the interactions of ligands with the NK_2 receptor, an integral membrane protein, and to assess the hydrophobicity of the binding pocket. In addition, NBD is sterically more compact than fluorescein and therefore less likely to perturb receptor binding affinity when introduced into the ligand.

The heptapeptides **2A**–**6A** (Figure 1) were synthesized by automatic solid-phase methodology using Fmoc chemistry (see Experimental Section). The synthetic peptides

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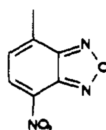
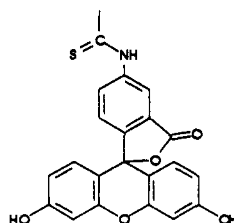
Compound	Structure
1 (GR94800)	PhCO-Ala-Ala-D-Trp-Phe-D-Pro-Pro-Nle-NH2
2	R-Ala-Ala-D-Trp-Phe-D-Pro-Pro-Nle-NH2
3	PhCO- Lys(ε-R)-Ala-D-Trp-Phe-D-Pro-Pro-Nle-NH2
4	PhCO- Orn(δ-R)-Ala-D-Trp-Phe-D-Pro-Pro-Nle-NH2
5	PhCO- Dab(γ-R)-Ala-D-Trp-Phe-D-Pro-Pro-Nle-NH2
6	PhCO-Dap(β-R)-Ala-D-Trp-Phe-D-Pro-Pro-Nle-NH2

A R = H



B R = NBD



C R = Flu

Figure 1. Molecular structure of the heptapeptide ligands.

were purified to >95% purity by HPLC and were analyzed by amino acid analysis and electrospray mass spectrometry.

The peptides **2A** and **3A** were derivatized with fluorescein by reaction with 10 equiv of the amino group selective reagent fluorescein isothiocyanate (FITC) in 50 mM sodium borate, pH 9.5:DMF 1:1 at 4 °C for 1.5 h. The crude mixture was purified by hydrophobic interaction ("reversed-phase") HPLC. The major reaction product was the peptide substituted with one fluoresceinyl group (**2C**, **3C**). The peptides **2A**–**6A** were allowed to react with 3 molar equiv of NBD fluoride as described above for the fluorescein derivatives to yield the NBD heptapeptides **2B**–**6B**. The molecular structure was confirmed by electrospray mass spectrometry (ES-MS), and the presence of fluorescein or NBD, was detected by visible spectrometry at 442 or 475 nm, respectively.

Pharmacology

The fluorescent peptides were assayed for NK_2 binding affinity by competitive displacement binding analysis with the NK_2-selective antagonist [^{3}H]GR100679,[11] using CHO cells stably transfected with human ileal NK_2 receptors (CHO/T cells, ca. 45 000 receptors/cell).[12] NK_2 receptors in CHO cells have been shown to display pharmacological properties similar to those of the NK_2 receptors found in rabbit pulmonary artery (RPA) with respect to antagonist potencies.[13] The results are summarised in Table 1. Compounds **2B** and **2C** showed an important decrease, 560- and 6580-fold, respectively, in affinity for the NK_2 receptor compared to 1, indicating that the bulk and the nature of the lipophilic N-terminal group in the parent compound is an important determinant for NK_2 receptor recognition. In contrast, the fluorescent peptides **3B**, **4B**, and **5B**, obtained by replacement of Ala[1] in 1, maintained a relatively high affinity for the NK_2 receptor with only an 8–15-fold decrease in affinity compared to 1. There were significant variations in binding affinity as a function of the length of the side chain at the amino acid in position 1. Although shortening of the chain from lysyl to diaminobutyl resulted in little change in affinity, the removal of another methylene group to give the diaminopropyl analogue **6B** caused a further 5-fold decrease in affinity.

Table 1. Potencies of NK_2 Antagonist Peptides to Compete for [^{3}H]GR100679 Binding in CHO/T Cells[a]

compd	pK_i	n
1	9.81 ± 0.07	8
2B	7.06 ± 0.02	3
2C	5.99 ± 0.10	3
3B	8.83 ± 0.06	3
3C	7.61 ± 0.19	2
4B	8.84 ± 0.07	4
5B	8.87 ± 0.11	5
6B	8.18 ± 0.09	3

[a] pK_i values were calculated, using the Cheng–Prusoff equation,[20] from IC_{50} values obtained in assays of competitive inhibition of [^{3}H]GR100679 binding to NK_2 receptors on CHO cells by receptor ligands. Hill slopes were not significantly different from unity. Data are mean ± SE; n represents the number of separate assays performed in triplicate.

Comparison of **2B** and **2C**, or **3B** and **3C**, indicated that peptides substituted with fluorescein were less active than their NBD counterparts, presumably reflecting the molecular volume of the substituents. The most potent fluorescent analogues were **5B**, **4B**, and **3B** with pK_i of 8.87, 8.84, and 8.83, respectively, compared to 9.81 for the heptapeptide 1 (GR94800).

Functional activation of neurokinin receptors can be assayed by measuring agonist-stimulated release of Ca^{2+} from intracellular stores in transfected CHO cells using the fluorescent Ca^{2+}-chelating agent fura-2.[14] To determine whether the fluorescent peptide **5B** was still an antagonist at the NK_2 receptor, we assayed for its ability to mobilize Ca^{2+} in CHO cells (Figure 2). As predicted, peptide **5B** showed no activity at 1 μM concentration. However, at the same concentration **5B** inhibited NKA-stimulated calcium release. These results demonstrate that the fluorescent peptide **5B** is an antagonist at the NK_2 receptor. Furthermore, **5B** was not able to inhibit Ca^{2+} release elicited by SP or NKB in CHO cells transfected with NK_1 or NK_3 receptors, respectively (Figure 2). This observation confirmed that compound **5B** was highly selective for the NK_2 receptor in a functional assay.

Fluorescent Peptide 5B as a Probe for the NK_2 Receptor in Cell Lines

The ability of the fluorescent peptide **5B** to recognize and label the NK_2 receptor was determined by flow cytometry using stably transfected CHO cells expressing human NK_2 receptors. A typical fluorescence histogram is shown in Figure 3 (panel A). The degree of fluorescence was measured as mean fluorescence intensity (MFI) of whole cells at different concentrations of **5B**, in the absence or presence of 10 μM of the antagonist GR100679. At 5 nM **5B**, a shift in fluorescence intensity was routinely detected. At the same concentration, the more potent fluoresceinyl-NKA[6] gave a MFI slightly and consistently higher. However, peptide **5B** was much more selective than fluoresceinyl-NKA for NK_2 over NK_1 and NK_3

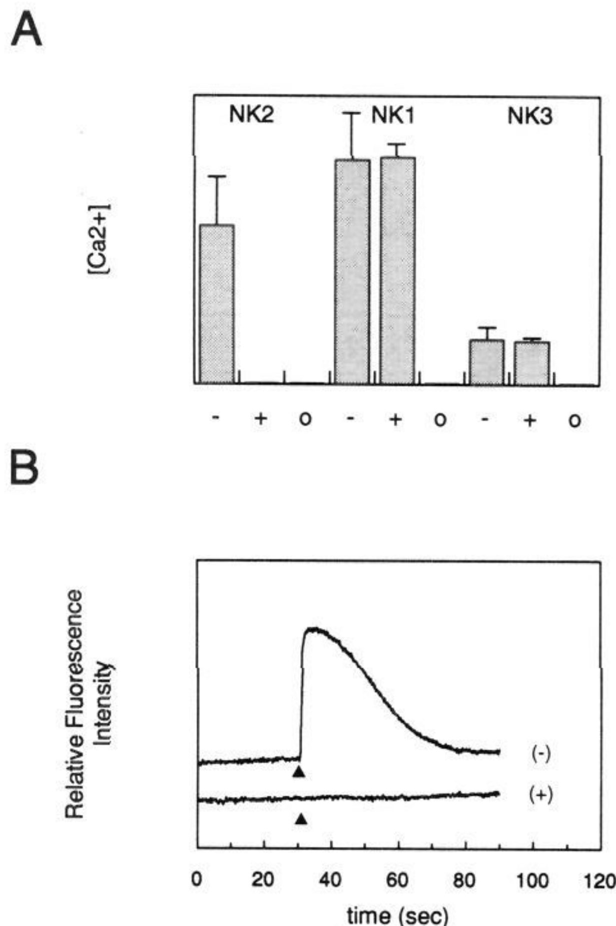


Figure 2. Neurokinin receptor-mediated Ca^{2+} responses in transfected CHO cells. (Panel A) Intracellular Ca^{2+} release from NK_2-, NK_1-, or NK_3-expressing CHO cells loaded with fura-2 and stimulated with 10 nM NKA, SP, or NKB, respectively, in the presence (+) or absence (–) of 1 μM **5B**, or with 1 μM **5B** alone (O). Data are mean ±SE. One typical experiment for NK_2 is shown in panel B. Arrowheads indicate addition of the peptides. Data were recorded in four individual experiments.

receptors. This was shown by flow cytometric analysis of stable CHO cell clones selectively expressing each tachykinin receptor at $>10^5$ receptors/cell (Figure 3). At various concentrations of **5B** (20–100 nM), only CHO cells expressing NK_2 receptors were labeled, whereas the same concentrations of fluoresceinyl-NKA gave positive results for all three tachykinin cell lines.

Conclusion

We have developed highly fluorescent NK_2 heptapeptide antagonists (**3B**, **4B**, and **5B**) from **1** (GR94800). In these studies we found that replacement of the Ala[1] methyl side chain by longer chains bearing the terminal bicyclic fluorophore NBD (**3B**, **4B**, **5B**) caused only a small decrease in potency, whereas direct attachment of NBD to the Ala[1] methyl group (**6B**) markedly reduced the affinity for NK_2. This may presumably reflect unfavorable steric or electronic interactions between the NBD and residues in the receptor binding pocket. Alternatively, intramolecular interactions with NBD may prevent the peptide from adopting the optimal receptor binding conformation. Previous work had shown the importance of the lipophilic N-terminal benzoyl substituent in **1**.[9] The present study further indicates that increasing the size of this substituent (**2B**,**C**) greatly reduced the potency.

We have demonstrated in a functional Ca^{2+} mobilization assay and flow cytometry binding assay that the fluorescent heptapeptide **5B** is a highly potent NK_2 antagonist. Furthermore, **5B** is much more selective for the NK_2 receptor than fluoresceinyl-NKA previously synthesized in our laboratory.[6] These new fluorescent probes should be useful tools for the localization and characterization of NK_2 receptors in tissues and for the study of ligand–receptor interactions by spectrofluorimetry.

Experimental Section

Abbreviations. FACS, fluorescence-activated cell sorter; ES-MS, electrospray mass spectrometry; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; Fmoc, 9-fluorenylmethoxycarbonyl; TFA, trifluoroacetic acid; NKA, neurokinin A (substance K); CHO, chinese hamster ovary; HPLC, high-performance liquid chromatography; FITC, fluorescein isothiocyanate; Orn, L-ornithine; Dab, L-2,4-diaminobutyric acid; Dap, L-2,3-diaminopropionic acid; Flu, fluoresceinthiocarbamyl; RP-HPLC, reversed-phase high-performance liquid chromatography; GR100679, *N*-α-cyclohexanoyl-Gly–Ala-D-Trp-Phe-NMe_2.[11]

Materials. All N-Fmoc-amino acid derivatives (side chain amino groups of Lys, Orn, and Dab and the indole nitrogen of DTrp were protected with Boc) were purchased from Bachem Feinchemikalien AG (Budendorf, Switzerland) except for *N*-α-Fmoc *N*-β-Boc-L-diaminopropionic acid, which was purchased from Neosystem Laboratoire SA (Strasbourg, France). FITC was purchased from Fluka AG. NBD fluoride and amino acids used as standards in amino acid analysis were from Sigma Chemical Co. [^{3}H]GR100679 was prepared by Amersham. GR94800 and GR100679 were synthesized by Medicinal Chemistry, Glaxo Group Research, Greenford, U.K.

General Methods for Peptide Synthesis. All peptide analogs were synthesized using an Fmoc strategy and HBTU coupling chemistry (FastMoc).[16] Resin for peptide amides prepared by the Fmoc strategy, Fmoc-4-methoxy-4′-[(γ-carboxypropyl)oxy]benzhydrylamine-alanyl-aminomethyl-polystyrene, with 1% divinylbenzene cross-linking (Bachem Feinchemikalien AG, cat. # D-1600, 0.25 mmol) was used as the solid-phase support, on an Applied Biosystems Inc. Model 431A peptide synthesizer. Each coupling step was performed on the synthesizer using 1 mmol of *N*-Fmoc (side chain Boc- for D-Trp, Lys, Orn, Dab, and Dap) protected amino acid or 2 mmol of benzoic anhydride for the N-terminal acylation, with HBTU coupling cycles. Coupling yields at each cycle were monitored by resin-sampling and colorimetric Kaiser tests.[17] Peptides were deprotected and concurrently cleaved from the resin using reagent K[18] and recovered by precipitation with cold *tert*-butyl methyl ether.

Gradient RP-HPLC was effected on a Waters (Millipore Corp.) system: Model 510 pumps, Model 710B refrigerated sample processor, Model 481 UV/vis spectrophotometer, with Maxima control and acquisition software, using a Nucleosil 300-7 C8 column (Machery-Nagel ET 250/8/4); buffer A, 0.1% (w/v) TFA/water; buffer B, 0.09% TFA/80% acetonitrile/water; Flow rate, 1.0 mL/min; gradient, 0–75% B over 60 min. Peptides were detected by UV absorbance at 214 nm.

L-Leucine was added to each peptide as an internal standard, and samples were hydrolyzed with 6 N HCl containing 1 mg/mL phenol at 112 °C for 16 h, and their amino acid compositions were determined on a Beckman 6300 analyser with Gold data analysis software. L-Ornithine, L-2,4-diamino-*n*-butyric acid, L-2,3-diaminopropionic acid, and L-norleucine were added to the protein hydrolysate standard mixture in appropriate proportions.

Peptides and their fluorescently labeled derivatives were authenticated by ES-MS (VG Trio 2, equipped with a 3000 amu rf generator and operated under Lab-Base software[19]).

General Procedure for the Preparation of Peptides. Crude precipitates were resuspended in 1 M acetic acid/20% acetonitrile/water and desalted on a Sephadex G10 size-exclusion column (Pharmacia). Peak fractions were pooled and lyophilized. Peptides were purified by RP-HPLC (see above) to >95% purity before attachment of the fluorescent probes.

General Procedure for Attachment of the Fluorophore. To 5 μmol of peptide in 2.0 mL of 50 mM sodium borate, pH

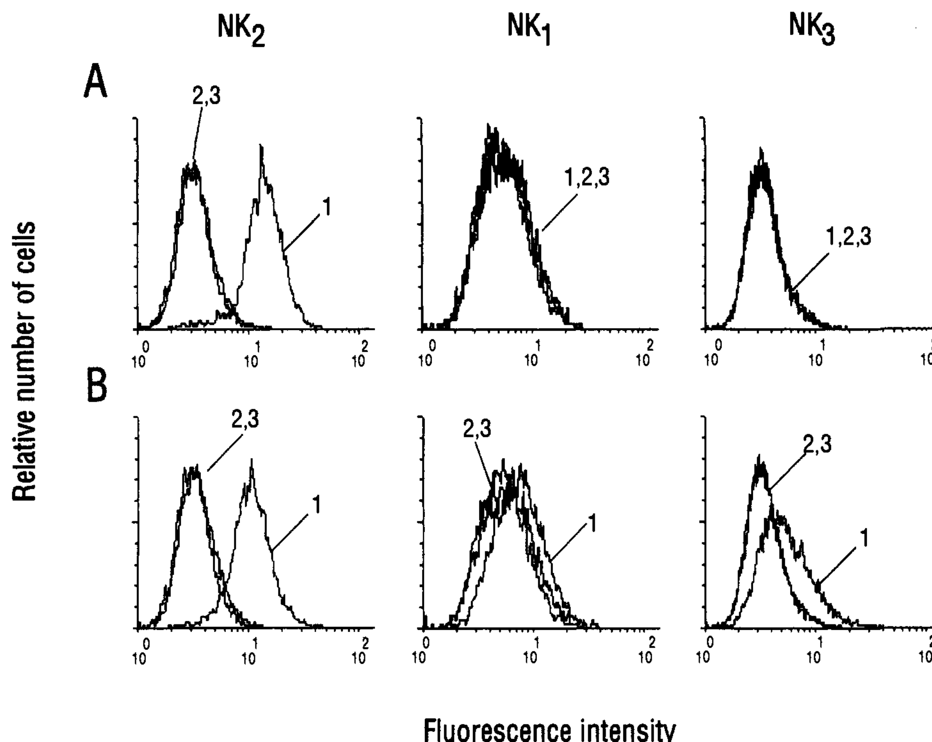


Figure 3. Flow cytometry analysis of tachykinin receptors transfected in CHO cells using the fluorescent antagonist **5B** (panel A) or the fluorescent agonist fluoresceinyl-NKA (F-NKA)[6] (panel B): 1 = 20 nM fluorescent ligand, 2 = 20 nM fluorescent ligand and 20 μM GR100679, and 3 = no ligand (autofluorescence of the cells).

Table 2. Synthesis and Analytical Data

	yield (%)	amino acid analysis Lys	Orn	Dab	Dap	Ala	Phe	Pro	Nle	HPLC[a] t_R (min)	MS (m/z)
2A	64					1.94	1.00	1.98	1.04	44.6	800.8
2B	94					1.73	1.02	2.15	1.07	51.2	964.1
2C	66					1.78	1.04	2.06	1.07	50.6	1188.3
3A	41	0.98				1.02	1.00	2.06	1.07	47.2	961.6
3B	73	0.83				1.04	0.97	1.96	0.99	55.3	1124.9
3C	58	0.93				1.04	1.01	2.08	1.03	54.4	1351.1
4A	58		1.07			1.05	0.97	2.05	0.99	46.9	948.0
4B	66		0.95			1.06	0.96	2.03	0.98	54.4	1110.7
5A	64			0.91		1.05	0.97	2.04	0.98	47.2	933.6
5B	57			0.88		1.03	0.97	1.88	1.00	54.4	1096.6
6A	65				1.02	1.04	0.97	2.00	0.99	47.2	919.0
6B	80				0.51	1.02	0.97	1.88	1.01	54.4	1083.0

[a] See Experimental Section.

9.5:DMF 1:1 was added 0.5 mL of 0.1 M FITC in DMF (50 μmol) or 0.5 mL of 0.03 M NBD fluoride in DMF (15 μmol) and the mixture was kept for 1.5 h in the dark at 4 °C. Fluorescent peptides were purified, directly from the reaction mixture, by small-scale preparative RP-HPLC: column Machery-Nagel SS250/0.5"/10 Nucleosil 7 C18; flow rate, 6 mL/min; gradient, 0–35% B over 35 min, 35–75% B over 80 min, 75–85% B over 5 min. Compounds were detected by UV absorbance at 220 and 260 nm simultaneously (Waters, Model 490). Fractions containing pure material were pooled and lyophilized.

Peptides 2A, 3A, 4A, 5A, and 6A. The peptides were synthesized according to the general procedures described above. Yields are reported in Table 2.

NBD-Labeled Peptides 2B, 3B, 4B, 5B, and 6B. Synthesis started from peptides **2A**, **3A**, **4A**, **5A**, and **6A**, respectively, and NBD fluoride according to the general procedure for attachment of the fluorophore. Yields are reported in Table 2.

Flu-Labeled Peptide 2C and 3C. Synthesis started from peptides **2A** and **3A**, respectively, and FITC according to the general procedure for attachment of the fluorophore. Yields are reported in Table 2.

Cell Culture. All cells were grown as monolayers in a humidified 5% CO_2 atmosphere at 37 °C. CHO/T cells were grown in DMEM/F12 medium supplemented with 10% fetal calf serum, 2% w/v Pen-Strep, 0.5% w/v Nystatin.

Binding Assays. Radioligand binding assays on whole CHO/T cells were performed in 24-well plates with 2.5 × 10^5 cells/well in a total volume of 0.5 mL binding assay medium comprising assay buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 0.02% (w/v) bovine serum albumin), 1.3 nM [^{3}H]-GR100679, and varying concentrations of the test compound (10 pM–10 μM). The plates were incubated at 20 °C for 90 min and then washed three times with ice-cold assay buffer. Optiphase Hi-load (1 mL) was added to each well and the plate was counted on a micro β scintillation counter. Nonspecific binding was determined in the presence of 1 μM GR94800. All measurements were done in triplicate. Data were analyzed using Allfit and IC_{50} values converted to pK_i values using the Cheng–Prusoff equation.

Intracellular [Ca^{2+}] Measurements. Intracellular Ca^{2+} concentrations were determined by using the fluorescent Ca^{2+} chelating agent fura-2. Aliquots of 10^6 cells, at 37 °C, loaded with fura-2 were stimulated with 10 nM neuropeptides in the absence or presence of 1 μM peptide **5B** and fluorescence emission was recorded using a JASCO FP777 spectrofluorimeter (excitation at 340 nm, emission at 505 nm). Other experimental details were as previously reported.[15]

Flow Cytometry Experiments. Cells (about 10^6) were incubated in phosphate-buffered saline (PBS, pH 7.2), containing $MnCl_2$ (3 mM) and bovine serum albumin (0.2 mg/mL), in the presence of the fluorescent antagonist (see text for concentrations)

at 4 °C for 3 h or 20 °C for 1.5 h. The cells were washed twice with ice-cold PBS, pH 7.2, and kept on ice. Nonspecific binding was measured in the presence of GR100679 (20 μM). The cell fluorescence histograms were acquired at room temperature with a Becton-Dickinson FACSCAN instrument using either linear or logarithmic modes with excitation/emission at 488/518 nm.

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