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Structure−Activity Relationships of a Series of Analogues of the RFamide-Related Peptide 26RFa

Olivier Le Marec,[†] Cindy Neveu,[†] Benjamin Lefranc,[†] Christophe Dubessy,[†] Jean A. Boutin,[‡] Jean-Claude Do-Régo,[§] Jean Costentin,[§] Marie-Christine Tonon,[†] Manuel Tena-Sempere,[∥] Hubert Vaudry,*[,†] and Jérôme Leprince[†]

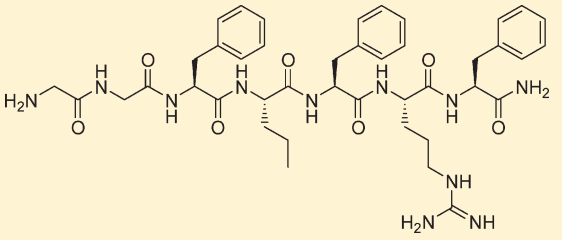
[†]INSERM U982, Laboratory of Neuronal and Neuroendocrine Differentiation and Communication, European Institute for Peptide Research (IFRMP 23), Cell Imaging Platform (PRIMACEN), University of Rouen, 76821 Mont-Saint-Aignan, France

[‡]IdRS, 125 Chemin de la Ronde, 78290 Croissy-sur-Seine, France

[§]Laboratory of Experimental Neuropsychopharmacology, IFRMP 23, University of Rouen, 76183 Rouen, France

[∥]Department of Cell Biology, Physiology, and Immunology, University of Cordoba and CIBER Fisiopatología de la Obesidad y Nutrición, 14004 Cordoba, Spain

ABSTRACT: 26RFa is a new member of the RFamide peptide family that has been identified as the endogenous ligand of the orphan GPCR GPR103. As the C-terminal heptapeptide (26RFa$_{(20-26)}$) mimics the action of the native peptide on food intake and gonadotropin secretion in rodents, we have synthesized a series of analogues of 26RFa$_{(20-26)}$ and measured their potency to induce $[Ca^{2+}]_i$ mobilization in Gα_{16}-*h*GPR103-transfected CHO cells. Systematic replacement of each residue by an alanine (Ala scan) and its D-enantiomer (D scan) showed that the last three C-terminal residues were very sensitive to the substitutions while position 23 tolerated rather well both modifications. Most importantly, replacement of Ser^{23} by a norvaline led to an analogue, [Nva^{23}]26RFa$_{(20-26)}$, that was 3-fold more potent than the native heptapeptide. These new pharmacological data, by providing the first information regarding the structure−activity relationships of 26RFa analogues, should prove useful for the rational design of potent GPR103 receptor ligands with potential therapeutic application.



INTRODUCTION

RFamide-related peptides (RFRPs) constitute a family of regulatory peptides that share the motif Arg-Phe-NH$_2$ at their C-terminus.[1] We have recently isolated a novel 26-amino acid RFRP from the frog brain that we have called 26RFa.[2] Independently, two groups have identified a longer form of 26RFa, termed QRFP (or 43RFa), and shown that both 26RFa and 43RFa act as natural ligands of the orphan receptor GPR103.[3,4] The cDNAs encoding the 26RFa/43RFa precursor have been cloned in chicken,[5] rat,[2,3] mouse,[3,4] ox,[2] and human.[2−4] The 43RFa polypeptide has been purified and characterized from a rat brain extract,[6] and both 26RFa and 43RFa have been isolated from the human hypothalamus and spinal cord.[7] The sequence of 26RFa encompasses a triad of basic amino acids (Arg-Arg-Lys), suggesting that cleavage of the precursor at this site may generate the C-terminal heptapeptide 26RFa$_{(20-26)}$ (Figure 1).

In situ hybridization studies have shown that in the rat and mouse brain, 26RFa and GPR103 mRNAs are expressed in hypothalamic nuclei involved in the control of energy homeostasis including the ventromedial nucleus, the arcuate nucleus, and the lateral hypothalamic area.[6,8,9] Consistent with these neuroanatomical observations, it has been found that intracerebroventricular (icv) administration of 26RFa or 43RFa stimulates food intake in mice.[1,6,8,10−12] The GPR103 gene is also expressed in extra-hypothalamic regions of the central nervous system (CNS) and in various peripheral organs including the kidney, pituitary, testis, adrenal gland, and bone.[3,4,13] As a matter of fact, different studies have shown that 26RFa and/or 43RFa stimulates LH secretion from rat pituitary explants,[14] increases plasma LH, FSH, and aldosterone concentration in rat,[3,14,15] inhibits glucose-induced insulin release from isolated perfused rat pancreas,[16] and evokes a sustained elevation of blood pressure and heart rate.[6] In addition, it has been reported that GPR103 knockout mice suffer from osteopenia and exhibit the characteristic kyphotic hump of osteoporotic patients.[13] These observations suggest that GPR103 could be the target for novel therapeutic agents.

The primary structure of the heptapeptide 26RFa$_{(20-26)}$ has been fully conserved from fish to mammals,[17] suggesting that the C-terminal region of 26RFa and 43RFa is important for their biological activity. Indeed, some of the effects of 26RFa and 43RFa are mimicked by this heptapeptide. In particular, 26RFa$_{(20-26)}$ enhances food consumption in mice[11] and stimulates LH release from rat pituitary gland.[14]

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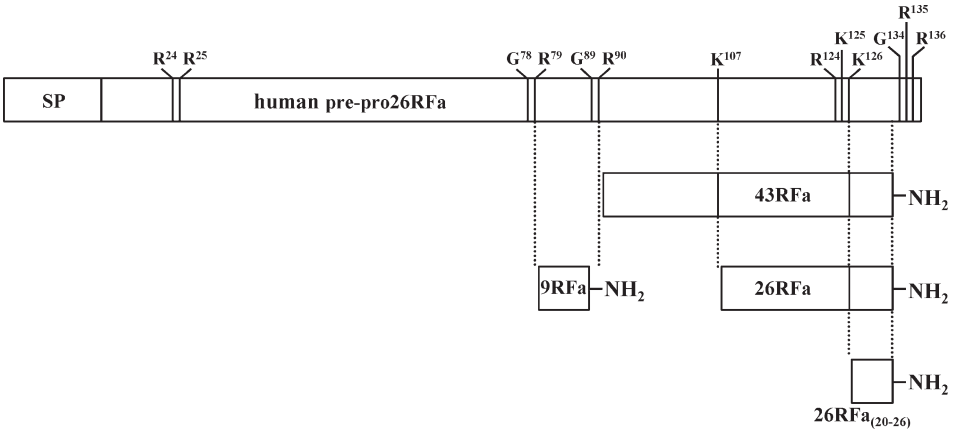
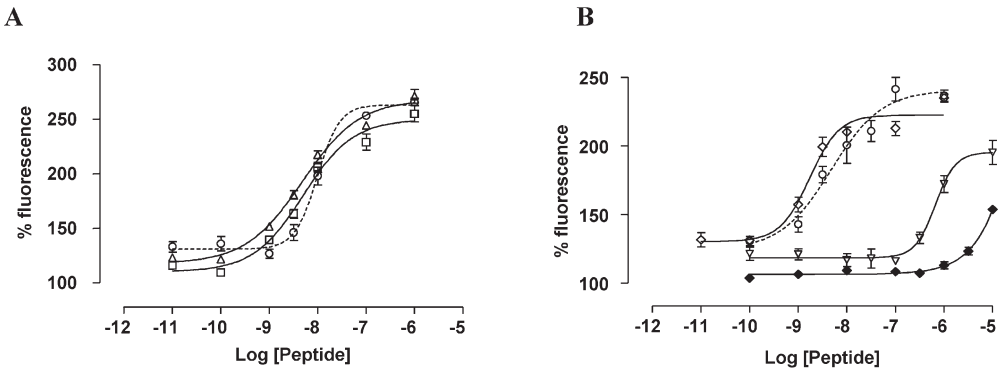


Figure 1. Schematic representation of the human precursor of 26RFa, prepro-26RFa, and the other putative RFamide peptides generated by the cleavage at different basic sites. Basic residues that represent potential cleavages sites and glycine residues that serve for amidation are indicated. SP indicates signal peptide.



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compd	peptide	sequence[a]	EC_{50} (nM)[b]
1	*h*43RFa	QDEGSEATGFLPAAGEKTSGPLGNLAEELNGYSRKKGGFSFRF-NH_2	7.7 ± 1.5
2	*r*43RFa	QDSGSEATGFLPTDSEKASGPLGTLAEELSSYSRRKGGFSFRF-NH_2	3.5 ± 0.4
3	*h*26RFa	TSGPLGNLAEELNGYSRKKGGFSFRF-NH_2	10.4 ± 1.5
4	*r*26RFa	ASGPLGTLAEELSSYSRRKGGFSFRF-NH_2	4.9 ± 2.8
16	$26RFa_{(20-26)}$	GGFSFRF-NH_2	739 ± 149
5	9RFa	EHAGCRFRF-NH_2	2910 ± 653

Figure 2. Effect of human and rodent 26RFa-related peptides potentially generated from the 26RFa precursor on $[Ca^{2+}]_i$ mobilization in *h*GPR103-transfected cells. (A, B) Representative concentration−response curves: □, *h*43RFa, △, *r*43RFa, ○, *h*26RFa, ◇, *r*26RFa, ▽, $26RFa_{(20-26)}$, ◆, 9RFa. (C) Sequence alignments of peptides potentially generated from the human and rat 26RFa precursors and their EC_{50} calculated from dose−response curves similar to those shown in parts A and B. Data are the mean ± SEM of at least three separate experiments.

The aim of the present study was to investigate the structure−activity relationships of 26RFa in order to design low molecular weight and lipophilic ligands of GPR103 that could lead to the development of therapeutic compounds as previously done with ACE inhibitors.[18] We have synthesized a collection of 26RFa analogues in order to determine the minimal sequence retaining Ca^{2+}-mobilizing activity in $G\alpha_{16}$-*h*GPR103-transfected CHO cells and to identify the position-related structural requirements for agonistic and, possibly, antagonistic behavior.

RESULTS AND DISCUSSION

To compare the potencies and efficacies of all the RFRPs that may be generated from the human and rat 26RFa precursors (Figure 1), we have studied the effects of the 26- and 43-amino acid peptides (*h*26RFa, *r*26RFa, *h*43RFa, *r*43RFa) as well as the fully conserved C-terminal heptapeptide $26RFa_{(20-26)}$ and the human-specific peptide 9RFa on $[Ca^{2+}]_i$ in cultured $G\alpha_{16}$-*h*GPR103-transfected CHO cells (Figure 2, Table 1, compounds **1**−**5**, **16**). Concentration−response curves showed that rat 26RFa and 43RFa were slightly more potent than their human

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Table 1. Chemical Data for Compounds 1−62

		HPLC		MS	
compd	peptide	t_R (min)[a]	purity (%)	calcd[b]	obsd[c]
1	*h*43RFa	22.2	99.9	4501.19	4501.76
2	*r*43RFa	23.2	99.9	4551.19	4552.10
3	*h*26RFa	27.3	99.9	2830.45	2831.26
4	*r*26RFa	23.3	99.9	2818.45	2819.48
5	9RFa	16.4	99.1	1120.53	1121.50
6	*h*26RFa$_{(4-26)}$	21.7	99.9	2585.35	2586.55
7	*h*26RFa$_{(7-26)}$	19.3	99.9	2318.19	2319.27
8	*h*26RFa$_{(10-26)}$	17.7	99.9	2020.03	2020.99
9	*h*26RFa$_{(13-26)}$	18.5	98.9	1648.86	1649.85
10	*h*26RFa$_{(14-26)}$	18.5	99.9	1534.82	1535.81
11	*h*26RFa$_{(15-26)}$	18.3	98.8	1477.79	1478.79
12	*h*26RFa$_{(16-26)}$	18.1	99.9	1314.73	1315.73
13	*h*26RFa$_{(17-26)}$	18.1	99.8	1227.70	1228.70
14	*h*26RFa$_{(18-26)}$	18.2	99.9	1071.60	1072.60
15	26RFa$_{(19-26)}$	18.2	99.0	943.50	944.40
16	26RFa$_{(20-26)}$	19.3	99.9	815.41	816.41
17	26RFa$_{(21-26)}$	18.6	99.9	758.39	759.47
18	26RFa$_{(22-26)}$	18.4	99.9	701.36	702.44
19	*h*26RFa$_{(8-16)}$	14.0	99.2	994.46	995.49
20	*h*26RFa$_{(4-17)}$	18.3	99.9	1532.80	1533.80
21	*h*26RFa$_{(1-16)}$	20.0	99.9	1620.76	1621.70
22	*h*26RFa$_{(1-18)}$	19.8	99.9	1904.96	1906.18
23	[Ala^{20}]26RFa$_{(20-26)}$	19.1	99.9	829.42	830.38
24	[Ala^{21}]26RFa$_{(20-26)}$	19.1	99.9	829.42	830.35
25	[Ala^{22}]26RFa$_{(20-26)}$	15.9	99.9	739.38	740.31
26	[Ala^{23}]26RFa$_{(20-26)}$	19.2	99.9	799.41	800.39
27	[Ala^{24}]26RFa$_{(20-26)}$	15.3	99.9	739.38	740.22
28	[Ala^{25}]26RFa$_{(20-26)}$	20.1	99.9	730.34	731.21
29	[Ala^{26}]26RFa$_{(20-26)}$	15.6	99.9	739.38	740.44
30	[D-Phe^{22}]26RFa$_{(20-26)}$	18.5	99.9	815.41	816.44
31	[D-Ser^{23}]26RFa$_{(20-26)}$	18.4	99.9	815.41	816.51
32	[D-Phe^{24}]26RFa$_{(20-26)}$	17.8	99.9	815.41	816.41
33	[D-Arg^{25}]26RFa$_{(20-26)}$	17.2	99.9	815.41	816.47
34	[D-Phe^{26}]26RFa$_{(20-26)}$	18.1	99.9	815.41	816.44
35	[Leu^{23}]26RFa$_{(20-26)}$	21.0	99.9	841.46	842.50
36	[Nle^{23}]26RFa$_{(20-26)}$	21.4	99.9	841.46	842.58
37	[Ile^{23}]26RFa$_{(20-26)}$	20.6	99.9	841.46	842.54
38	[Tle^{23}]26RFa$_{(20-26)}$	20.3	99.8	841.46	842.36
39	[Val^{23}]26RFa$_{(20-26)}$	19.7	99.9	827.44	828.44
40	[Nva^{23}]26RFa$_{(20-26)}$	20.3	99.9	827.44	828.40
41	[Abu^{23}]26RFa$_{(20-26)}$	48.6	99.9	813.43	814.43
42	[Cha^{23}]26RFa$_{(20-26)}$	23.4	99.9	881.49	882.59
43	[hSer^{23}]26RFa$_{(20-26)}$	18.5	99.9	829.42	830.51
44	desamide-*h*26RFa	22.2	99.9	2831.43	2833.37
45	desamide-26RFa$_{(20-26)}$	20.1	99.9	816.39	817.65
46	*N*,*N*-dibenzyl-*h*26RFa	26.5	99.0	3010.54	3011.39
47	*N*, *N*-piperidinyl-*h*26RFa	23.7	99.3	2898.51	2899.39
48	*N*,*N*-dibenzyl-26RFa$_{(20-26)}$	27.7	99.9	995.50	996.63
49	*N*, *N*-piperidinyl-26RFa$_{(20-26)}$	22.9	98.9	883.47	884.59
50	*N*-ethyl, *N*-benzyl-26RFa$_{(20-26)}$	25.0	99.9	933.49	934.63
51	*N*-benzyl-*h*26RFa	23.7	99.9	2920.50	2921.63
52	*N*-benzyl-26RFa$_{(20-26)}$	23.1	98.6	905.45	906.65
53	*N*-benzyl-*h*26RFa$_{(1-25)}$	22.4	99.9	2773.43	2774.33
54	*N*-benzyl-26RFa$_{(20-25)}$	20.1	99.9	758.39	759.39
55	Ac-26RFa$_{(20-26)}$	20.2	98.9	857.42	858.42
56	Piv-26RFa$_{(20-26)}$	23.3	99.9	899.47	900.47
57	Bz-26RFa$_{(20-26)}$	23.5	99.9	919.43	920.44
58	Z-26RFa$_{(20-26)}$	24.9	99.9	949.44	950.44
59	Adam-26RFa$_{(20-26)}$	28.1	99.9	977.51	978.52
60	Fur-26RFa$_{(20-26)}$	23.7	99.9	909.41	910.42
61	Tmg-26RFa$_{(20-26)}$	20.7	98.8	913.49	914.49
62	*p*FPha-26RFa$_{(20-26)}$	26.2	99.9	951.44	952.45

[a] Retention time determined by RP-HPLC. [b] Theoretical monoisotopic molecular weight. [c] MH^+ value assessed by MALDI-TOF-MS.

counterparts (Figure 2A,B) while the N-terminally elongated forms (*h*43RFa and *r*43RFa) were in the same range of potency as the corresponding 26RFa forms (Figure 2C). We have previously shown that in methanol solution the Pro^4-Arg^{17} region of 26RFa adopts a well-defined α-helical structure.[19] The slightly higher potency of *r*26RFa, compared to *h*26RFa, can likely be accounted for by the propencity of the Thr^7, Ser^{13}, and Ser^{14} residues in the rat sequence (compared to the Asn^7, Asn^{13}, and Gly^{14} residues in the human sequence) to stabilize this α-helix in lipid bilayer.[20] The C-terminal heptapeptide, 26RFa$_{(20-26)}$, whose sequence is fully conserved from fish to human,[17] was substantially less potent (75-fold) but as efficacious as *h*26RFa to increase $[Ca^{2+}]_i$ (Figures 2B,C). Finally, in agreement with a previous report,[4] we found that 9RFa possesses a very weak agonistic activity, indicating that this peptide cannot be considered as an endogenous ligand of GPR103. Conversely, it has been reported that the affinity of 9RFa for another RFamide receptor, NPFF2, is similar to that of *h*26RFa,[21] suggesting that 9RFa could activate receptor(s) other than GPR103.

To further investigate the contribution of the N-terminal, central, and C-terminal regions of the peptide in the $[Ca^{2+}]_i$ mobilizing activity, we have generated downsized analogues of *h*26RFa (Table 1, compounds **6**−**22**). Deletion of three (Table 2, compound **6**), six (Table 2, compound **7**), and nine amino acids (Table 2, compound **8**) from the N-terminus did not markedly affect the biological activity of the peptide, suggesting that the amino tail and the N-terminal extremity of the α-helix do not play a crucial role in the biological activity of *h*26RFa. Additional deletion of three (Table 2, compound **9**), four (Table 2, compound **10**), and five residues (Table 2, compound **11**) led to analogues that were 10- to 20-fold less potent than *h*26RFa (Table 2). Shorter C-terminal peptides, i.e., 26RFa$_{(16-26)}$, 26RFa$_{(17-26)}$, and 26RFa$_{(18-26)}$ (Table 2, compounds **12**−**14**), were approximately 30 times less potent than *h*26RFa. Finally, 26RFa$_{(19-26)}$ and 26RFa$_{(20-26)}$ were about 75-fold less potent than *h*26RFa (Table 2, compounds **15** and **16**) while 26RFa$_{(21-26)}$ and 26RFa$_{(22-26)}$ were totally devoid of activity (Table 2, compounds **17** and **18**), indicating that the biological activity of the peptide is harbored in its C-terminal

Table 2. Effects of N-Terminal, Central, and C-Terminal Truncated *h*26RFa Analogues on Basal $[Ca^{2+}]_i$ and *h*26RFa-Induced $[Ca^{2+}]_i$ Increase in CHO-Gα_{16}-*h*GPR103-Transfected Cells

		sequence																												
compd	peptide				5						10					15						20						25			EC_{50} (nM)[a]	IC_{50} (nM)[a]
3	*h*26RFa	T	S	G	P	L	G	N	L	A	E	E	L	N	G	Y	S	R	K	K	G	G	F	S	F	R	F		10.4 ± 1.50	
6	*h*26RFa$_{(4-26)}$				P	L	G	N	L	A	E	E	L	N	G	Y	S	R	K	K	G	G	F	S	F	R	F		43.2 ± 40.3	
7	*h*26RFa$_{(7-26)}$							N	L	A	E	E	L	N	G	Y	S	R	K	K	G	G	F	S	F	R	F		30.2 ± 11.6	
8	*h*26RFa$_{(10-26)}$										E	E	L	N	G	Y	S	R	K	K	G	G	F	S	F	R	F		37.5 ± 15.2	
9	*h*26RFa$_{(13-26)}$													N	G	Y	S	R	K	K	G	G	F	S	F	R	F		95.3 ± 40.7***	
10	*h*26RFa$_{(14-26)}$														G	Y	S	R	K	K	G	G	F	S	F	R	F		185 ± 52**	
11	*h*26RFa$_{(15-26)}$															Y	S	R	K	K	G	G	F	S	F	R	F		138 ± 37**	
12	*h*26RFa$_{(16-26)}$																S	R	K	K	G	G	F	S	F	R	F		237 ± 57***	
13	*h*26RFa$_{(17-26)}$																	R	K	K	G	G	F	S	F	R	F		282 ± 69***	
14	*h*26RFa$_{(18-26)}$																		K	K	G	G	F	S	F	R	F		233 ± 51***	
15	*h*26RFa$_{(19-26)}$																			K	G	G	F	S	F	R	F		1710 ± 521***	
16	*h*26RFa$_{(20-26)}$																				G	G	F	S	F	R	F		739 ± 149***	
17	*h*26RFa$_{(21-26)}$																					G	F	S	F	R	F		NC	
18	*h*26RFa$_{(22-26)}$																						F	S	F	R	F		ND	
19	*h*26RFa$_{(8-16)}$								L	A	E	E	L	N	G	Y	S												ND	ND
20	*h*26RFa$_{(4-17)}$				P	L	G	N	L	A	E	E	L	N	G	Y	S	R												ND	ND
21	*h*26RFa$_{(1-16)}$	T	S	G	P	L	G	N	L	A	E	E	L	N	G	Y	S												ND	ND
22	*h*26RFa$_{(1-18)}$	T	S	G	P	L	G	N	L	A	E	E	L	N	G	Y	S	R	K										ND	ND

[a] Data are the mean ± SEM of at least three distinct experiments. NC, not calculable. ND, not detectable. (**) $p < 0.01$ and (***) $p < 0.001$ vs control (*h*26RFa) as assessed by Mann−Witney test.

Table 3. Effects of L-Alanine and D-Residue Substituted 26RFa$_{(20-26)}$ Analogues on Basal $[Ca^{2+}]_i$ and *h*26RFa-Induced $[Ca^{2+}]_i$ Increase in CHO-Gα_{16}-*h*GPR103-Transfected Cells

		sequence								
compd	peptide	20	21	22	23	24	25	26	EC_{50} (nM)[a]	IC_{50} (nM)[a]
16	26RFa$_{(20-26)}$	G	G	F	S	F	R	F	739 ± 149	
23	[Ala^{20}]26RFa$_{(20-26)}$	A							3350 ± 668**	
24	[Ala^{21}]26RFa$_{(20-26)}$		A						2985 ± 862**	
25	[Ala^{22}]26RFa$_{(20-26)}$			A					1333 ± 321	
26	[Ala^{23}]26RFa$_{(20-26)}$				A				1308 ± 724	
27	[Ala^{24}]26RFa$_{(20-26)}$					A			ND	ND
28	[Ala^{25}]26RFa$_{(20-26)}$						A		ND	ND
29	[Ala^{26}]26RFa$_{(20-26)}$							A	ND	ND
30	[D-Phe^{22}]26RFa$_{(20-26)}$			f					ND	ND
31	[D-Ser^{23}]26RFa$_{(20-26)}$				s				4975 ± 2305*	
32	[D-Phe^{24}]26RFa$_{(20-26)}$					f			ND	ND
33	[D-Arg^{25}]26RFa$_{(20-26)}$						r		ND	ND
34	[D-Phe^{26}]26RFa$_{(20-26)}$							f	ND	ND

[a] Data are the mean ± SEM of at least three distinct experiments. ND, not detectable. (*) $p < 0.05$ and (**) $p < 0.01$ vs control (26RFa$_{(20-26)}$) as assessed by the Mann−Witney test.

part. Indeed, neither central segments such as *h*26RFa$_{(8-16)}$ and *h*26RFa$_{(4-17)}$ nor N-terminal fragments (Table 2, compounds **19**−**22**) exhibited agonistic or antagonistic activities in GPR103-transfected cells.

The gradual decline in the biological activity observed with the N-side truncated analogues has to be correlated to the decrease in the affinity of these fragments for GPR103.[3] All these data clearly indicate that the biologically active domain of 26RFa is located in the C-terminal region and that 26RFa$_{(20-26)}$, which may be processed in vivo by prohormone convertases and whose sequence is fully conserved from fish to mammals, is the shortest analogue that retains full efficiency to increase $[Ca^{2+}]_i$ in GPR103-transfected cells. 26RFa$_{(20-26)}$ is also relatively hydrophobic and thus more suitable for the development of molecules with therapeutic value. For all these reasons, 26RFa$_{(20-26)}$ naturally stood out as the ideal molecular scaffold for the design of GPR103 peptide ligands with low molecular weight.

The contribution of the amino acid side chains to the biological activity of 26RFa$_{(20-26)}$ was evaluated by systematic alanine replacement of each residue of the peptide sequence (Table 1, compounds **23**−**29**).[22−24] Alanine substitution in the N-terminal region (Table 3, compounds **23**−**26**) did not substantially affect the activity of the peptide (Table 3). In particular, replacement of the two glycine residues by Ala, which is expected to restrict the conformational freedom of the peptide backbone at these points, did not markedly impair the $[Ca^{2+}]_i$-mobilizing activity of the peptide (Table 3, compounds **23** and **24**). In contrast, L-alanine substitution in the C-terminal part of the molecule led to analogues totally devoid of stimulating activity for concentrations ranging from 10^{-12} to 10^{-5} M (Table 3, compounds **27**−**29**). Similarly, [Ala^{24}]-, [Ala^{25}]-, and [Ala^{26}]26RFa$_{(20-26)}$ did not reduce the *h*26RFa-evoked increase in $[Ca^{2+}]_i$, suggesting that the Phe-Arg-Phe triad is directly involved in GPR103 activation as already reported for NPFF and PrRP, which are two other peptides from the RFamide superfamily.[25,26] In order to explore the importance of the orientation of the amino acid side chains in the biological activity of the peptide, a series of D-isomer-substituted peptides was synthesized (Table 1, compounds **30**−**34**) and the ability of the analogues to elicit $[Ca^{2+}]_i$ increase was measured (Table 3, compounds

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Table 4. Effect of Ser^{23} Residue-Substituted $26RFa_{(20-26)}$ Analogues on Basal $[Ca^{2+}]_i$ in CHO-Gα_{16}-*h*GPR103-Transfected Cells

compd	Peptide	side-chain formula	side-chain name	EC_{50} (nM)[a]
16	$26RFa_{(20-26)}$		hydroxymethyl	739 ± 149
35	$[Leu^{23}]26RFa_{(20-26)}$		secbutyl	1471 ± 795
36	$[Nle^{23}]26RFa_{(20-26)}$		butyl	741 ± 160
37	$[Ile^{23}]26RFa_{(20-26)}$		isobutyl	817 ± 244
38	$[Tle^{23}]26RFa_{(20-26)}$		tertbutyl	1820 ± 724*
39	$[Val^{23}]26RFa_{(20-26)}$		isopropyl	1631 ± 1027
40	$[Nva^{23}]26RFa_{(20-26)}$		propyl	233 ± 72*
41	$[Abu^{23}]26RFa_{(20-26)}$		ethyl	355 ± 151
42	$[Cha^{23}]26RFa_{(20-26)}$		methylcyclohexyl	1103 ± 108
43	$[hSer^{23}]26RFa_{(20-26)}$		hydroxyethyl	374 ± 110

[a] Data are the mean ± SEM of at least three distinct experiments. (*) $p < 0.05$ vs control ($26RFa_{(20-26)}$) as assessed by the Mann−Witney test.

Table 5. Effects of C-Terminally Modified *h*26RFa and $26RFa_{(20-26)}$ Analogues on Basal $[Ca^{2+}]_i$ and *h*26RFa-Induced $[Ca^{2+}]_i$ Increase in CHO-Gα_{16}-*h*GPR103-Transfected Cells

compd	peptide	EC_{50} (nM)[a]	IC_{50} (nM)[a]
3	*h*26RFa	10.4 ± 1.5	
16	$26RFa_{(20-26)}$	739 ± 149	
44	desamide-*h*26RFa	531 ± 319***	
45	desamide-$26RFa_{(20-26)}$	ND	ND
46	*N*,*N*-dibenzyl-*h*26RFa	166 ± 61**	
47	*N*,*N*-piperidinyl-*h*26RFa	282 ± 134***	
48	*N*,*N*-dibenzyl-$26RFa_{(20-26)}$	ND	NC
49	*N*,*N*-piperidinyl-$26RFa_{(20-26)}$	1198 ± 286	
50	*N*-ethyl,*N*-benzyl-$26RFa_{(20-26)}$	1088 ± 199	
51	*N*-benzyl-*h*26RFa	39 ± 5.5	
52	*N*-benzyl-$26RFa_{(20-26)}$	ND	ND
53	*N*-benzyl-$h26RFa_{(1-25)0}$	67 ± 26**	
54	*N*-benzyl-$26RFa_{(20-25)}$	ND	ND

[a] Data are the mean ± SEM of at least three distinct experiments. NC, not calculable. ND, not detectable. (**) $p < 0.01$ and (***) $p < 0.001$ vs control (*h*26RFa) as assessed by the Mann−Witney test.

30−**34**). D-Amino acid replacement at positions 22, 24, 25, and 26 (Table 3, compounds **30**, **32**−**34**) resulted in a complete loss of the $[Ca^{2+}]_i$ response, while this substitution was rather well tolerated at position 23 (Table 3, compound **31**). Altogether, these data demonstrate that not only the chemical structure but also the correct orientation of the side chain of each residue plays a critical role in the activity of the peptide.

Since the Ser^{23} residue tolerated rather well the ala- and D-isomer substitutions, we assumed that this could be a suitable position to generate superior analogues of $26RFa_{(20-26)}$. We thus designed seven peptides in which the Ser side chain was replaced by differently branched aliphatic moieties (Table 1, compounds **35**−**43**). Substitution of the hydroxymethyl side chain of Ser by butyl and isobutyl moieties did not modify the activity of the compounds compared to the native heptapeptide (Table 4, compounds **36** and **37**). In contrast, replacement of the hydroxymethyl group by a short and hindered *sec*-butyl ($[Leu^{23}]26RFa_{(20-26)}$), *tert*-butyl ($[Tle^{23}]26RFa_{(20-26)}$), or isopropyl moiety ($[Val^{23}]26RFa_{(20-26)}$) led to compounds with a 2- to 2.5-fold reduced potency (Table 4, compounds **35**, **38**, and **39**). A 2-fold decrease of the potency was also observed with the $[Cha^{23}]26RFa_{(20-26)}$ analogue bearing a bulky and nonpolar methylcyclohexyl moiety (Table 4, compound **42**). In contrast, the presence of a short side chain such as propyl and ethyl enhanced the activity of the corresponding analogues on $[Ca^{2+}]_i$ (Table 4, compounds **40** and **41**). In particular, $[Nva^{23}]26RFa_{(20-26)}$, which exhibited a 3-fold lower EC_{50} than $26RFa_{(20-26)}$, was the most potent downsized peptide of this series (Table 4, compound **40**). These data suggest that the residue at position 23 may interact with a narrow hydrophobic binding pocket within the receptor. Finally, the $[hSer^{23}]26RFa_{(20-26)}$, in which the Ser hydroxymethyl side chain is elongated by a single methylene group, was 2 fold more potent than $26RFa_{(20-26)}$ (Table 4, compound **43**). This could be accounted for by the formation of hydrogen bonds, allowing additional forces to anchor the peptide in the receptor. Alternatively, it is well established that uncharged amino acids exhibit distinct proclivity for folding peptides and proteins.[27] For instance, Ile and to a lesser extent Nle have a greater propensity to form a β-structure than the helix inducers Leu and Ala,[28] while Val has been shown to promote a γ-turn formation in peptide models.[29] These conformational preferences are usually governed by a membrane-mimicking environment.[20] In water and methanol, the C-terminal region of 26RFa, corresponding to $26RFa_{(20-26)}$, does not exhibit a particular structure.[19] However, we assume that in a lipid bilayer this region adopts a typical conformation that could be strongly stabilized by uncharged amino acids with short and linear side chains such as norvaline and 2-aminobutyric acid.

It is well established that C-terminal amidation of peptides prevents extracellular inactivation by peptidases and often contributes to the biological activity.[30−33] Consistent with these notions, desamide-*h*26RFa was at least 50-fold less potent than *h*26RFa, and desamide-$26RFa_{(20-26)}$ was totally devoid of agonistic and antagonistic activity (Table 1, compounds **44** and **45**). These findings are in agreement with previous data showing that desamide-*h*26RFa has a 400-fold lower affinity for GPR103 than 26RFa.[3] Therefore, we next investigated the contribution of the terminal carboxamide to the biological activity by using a series of mono- and disubstituted *h*26RFa, $h26RFa_{(1-25)}$, and $26RFa_{(20-26)}$ analogues (Tables 1 and 5, compounds **46**−**54**). The disubstituted analogues, i.e., *N*,*N*-dibenzyl-*h*26RFa, *N*,*N*-piperidinyl-*h*26RFa, and *N*-ethyl,*N*-benzyl-$26RFa_{(20-26)}$ (Table 5, compounds **46**, **47**, and **50**), showed a strong decrease in Ca^{2+}-mobilizing activity compared to the native peptide, suggesting that the hydrogen atoms of the primary amide participate in some interactions either with GPR103 or with a carboxy function of the peptide. Since the C-terminal extremity of 26RFa presents a random-coil structure in water and methanol, we assume the presence of intermolecular interactions.[19] In support of this notion, the monosubstituted carboxamide derivative of *h*26RFa, *N*-benzyl-*h*26RFa, was almost as potent as *h*26RFa (Table 5, compound **51**). However, *N*-benzyl-$26RFa_{(20-26)}$ (Table 5, compound **52**) was totally devoid of agonistic or antagonistic activities, indicating that this analogue was unable to bind to GPR103. This is an unexpected result if the same mode of action is accepted for both 26RFa and its C-terminal heptapeptide $26RFa_{(20-26)}$. It has been demonstrated that in all mammalian RFRPs the Arg-Phe-NH_2 motif is essential for bioactivity.[25] Interestingly, we observed that the phenylalanine residue can be deleted without loss of activity if the benzyl side chain is reported to the carboxamide function as in

Table 6. Effect of N-Terminally Modified 26RFa$_{(20-26)}$ Analogues on Basal $[Ca^{2+}]_i$ in CHO-Gα_{16}-*h*GPR103-Transfected Cells

compd	peptide[a]	EC_{50} (nM)[b]
16	26RFa$_{(20-26)}$	739 ± 149
55	Ac-26RFa$_{(20-26)}$	1443 ± 741
56	Piv-26RFa$_{(20-26)}$	2545 ± 694**
57	Bz-26RFa$_{(20-26)}$	722 ± 139
58	Z-26RFa$_{(20-26)}$	826 ± 166
59	Adam-26RFa$_{(20-26)}$	588 ± 141
60	Fur-26RFa$_{(20-26)}$	695 ± 270
61	Tmg-26RFa$_{(20-26)}$	455 ± 76
62	*p*FPha-26RFa$_{(20-26)}$	830 ± 292

[a] Ac, acetyl; Adam, adamantanoyl; Bz, benzoyl; Fur, furoyl; *p*FPha, *p*-fluorophenylacetyl; Piv, pivaloyl; Tmg, tetramethylguanidinyl; Z, benzyloxycarbonyl. [b] Data are the mean ± SEM of at least three distinct experiments. (**) $p < 0.01$ vs control (26RFa$_{(20-26)}$) as assessed by the Mann−Witney test.

N-benzyl-*h*26RFa$_{(1-25)}$ (Table 5, compound **53**). Unfortunately, the truncated *N*-benzyl-26RFa$_{(20-25)}$ analogue did not follow the paradigm (Table 5, compound **54**). We thus propose that either the C-terminal amide favors the formation of at least one hydrogen bond within the binding pocket of GPR103 or the cavity is too narrow for harboring sterical hindered moieties. To the best of our knowledge, only one structure−activity relationship study reports the chemical modulation of a carboxamide function of a regulatory peptide, i.e., substance P, and demonstrates the involvement of the primary amide in the peptide−receptor interaction through hydrogen bound in very much the same way as for 26RFa.[34]

Acylation of the N-terminal extremity of regulatory peptides affords protection from exopeptidase degradation notably by dipeptidylaminopeptidases.[35,36] Acylation can also modulate the intrinsic biological activity of peptides. Thus, acetylation is required for the biological activity of α-MSH whereas it strongly attenuates the morphinomimetic activity of β-endorphin.[37] Therefore, we next examined the effect of N-terminal acylation of 26RFa$_{(20-26)}$ on intracellular calcium concentration (Tables 1 and 6, compounds **55**−**62**). Whatever the physicochemical feature of the acyl group, i.e., aliphatic or aromatic and small or bulky, the potency of all N-acylated heptapeptides tested was in the same range as that of the native peptide, providing further evidence that the N-terminal region does not participate in the activation of the GPR103.

26RFa has been shown to exert multiple effects in the CNS. Thus, icv injection of 26RFa or 43RFa stimulates food intake,[1,6,8,10−12] activates the hypothalamo−pituitary−gonadal axis,[14,15] modulates nociceptive stimuli transmission,[38] and increases arterial blood pressure and heart rate in rodents.[6] In addition, the wide distribution of GPR103 mRNAs in peripheral organs suggests that the 26RFa/GPR103 system may play other physiological functions.[3,4,13] In agreement with this notion, in rat, 43RFa stimulates aldosterone secretion in vivo[3] and 26RFa enhances LH and FSH release in vitro.[14] Phenotyping analysis of GPR103$^{-/-}$ mice revealed massive reduction in bone density, notably in females that exhibit the characteristic kyphotic hump of osteoporetic patients.[13] Altogether, these observations validate the development of GPR103 agonists and antagonists that could selectively cross or not the blood−brain barrier for the treatment of neuroendocrine and metabolic disorders.

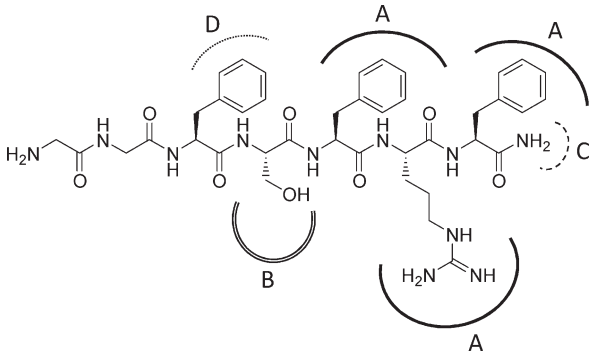

Figure 3. Hypothetical model of interaction between 26RFa$_{(20-26)}$ and GPR103. The curved lines illustrate the different motifs of the ligand that potentially interact with GPR103. (A) The bold lines aim at the three motifs that appear to play a role in receptor activation. (B) The doubled line designates the serine residue that likely fits into the narrow hydrophobic pocket. (C) The dashed line illustrates the identified H-bond interaction. (D) The dotted line points to an unknown interaction with the GPR103 receptor.

In summary, this first structure−activity relationship study of 26RFa revealed that (i) 26RFa is as potent as 43RFa while the conserved C-terminal heptapeptide 26RFa$_{(20-26)}$ is 75-fold less potent, (ii) the Phe-Arg-Phe triad is involved in GPR103 activation, (iii) position 23 of 26RFa$_{(20-26)}$ can be optimized using diverse residues for improving GPR103 activation, and (iv) the C-terminal primary amide is involved in a hydrogen bound probably with specific residue of GPR103 in contrast to the N-terminal function of 26RFa$_{(20-26)}$ (Figure 3). Our data also demonstrate that a suitable modification of 26RFa$_{(20-26)}$ such as Nva[23] substitution could open up new opportunities for the design of potent GPR103 receptor ligands with potential therapeutic applications.

EXPERIMENTAL SECTION

Materials. All Fmoc amino acid residues, Boc-Thr(Bzl)-OH, Boc-Gly-OH, and Z-Gly-OH were purchased from Senn Chemicals (Dielsdorf, Switzerland) or Bachem (Weil am Rhein, Germany). The preloaded 4-hydroxymethylphenoxymethyl-copolystyrene−1%-divinylbenzene (HMP) resins, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), and dichloromethane (DCM) were from Applera-France (Courtaboeuf, France). Acetonitrile and *N*-methylpyrrolidinone (NMP) were from Carlo Erba (Val-de-Reuil, France). The Rink amide 4-methylbenzhydrylamine (MBHA) resin was from VWR International (Fontenay-sous-Bois, France). The preloaded 2-methoxy-4-alkoxybenzyl alcohol resins were from Bachem. Diisopropylethylamine (DIEA), piperidine, trifluoroacetic acid (TFA), the nutrient mixture F-12 HAM, L-glutamine, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), penicillin−streptomycin solution, probenecid, M2 antibodies, and other reagents were from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Phenol, Geneticin (G-418 sulfate), hygromycin B, Hank's balanced salt solution (HBSS), and pluronic F-127 were from Gibco Life Technologies (Cergy-Pontoise, France). Fetal bovine serum was from Lonza (Viviers, Belgium).

Peptide Synthesis. All peptides were synthesized on a 433A Applied Biosystems peptide synthesizer (Applera-France) using the standard manufacturer's procedures. C-Terminal amidated analogues, C-terminal N- and N,N-modified amidated analogues, and C-terminal free analogues of 26RFa were synthesized (0.1 mmol scale) on a Rink amide MBHA resin, on preloaded 2-methoxy-4-alkoxybenzyl alcohol resin (Fmoc-Phe-SASRIN or

Fmoc-Arg(Pbf)-SASRIN), and on preloaded HMP resin (Fmoc-Ser(^{t}Bu)-HMP, Fmoc-Lys(Boc)-HMP, Fmoc-Arg(Pbf)-HMP), or Fmoc-Phe-HMP), respectively. All Fmoc-amino acids (1 mmol, 10 equiv) were coupled by in situ activation with HBTU/HOBt (1.0 mmol/1.0 mmol, 10 equiv) and DIEA (1 mmol, 10 equiv) in NMP. Reactive side chains were protected as follows, unless otherwise stated: Thr, Ser, and Tyr, *tert*-butyl (^{t}Bu) ether; Lys, *tert*-butyloxycarbonyl (Boc) carbamate; Glu, *O*-*tert*-butyl (O^{t}Bu) ester; Asn, trityl (Trt) amide; and Arg, pentamethyldihydrobenzofuran (Pbf) sulfonylamide.

After completion of the chain assembly, N^{α}-acylation of 26RFa$_{(20-26)}$ was performed on the resin by addition of a mixture of acetic anhydride or acyl chloride/DIEA (0.4 mmol, 4 equiv; 0.5 mmol, 5 equiv) in NMP at 0 °C for 5 min and at room temperature for 30 min. Reactions were monitored by Kaiser's test. Z-26RFa$_{(20-26)}$ was automatically prepared one step before completion by addition of Z-Gly-OH.

The N- and N,N-modified amidated analogues of 26RFa were prepared by treating Boc-peptidyl-SASRIN with 1% TFA/DCM for 5 min under gentle agitation. After filtration of the resin, the filtrate was neutralized by addition of pyridine. The treatment was repeated with further portions of 1% TFA/DCM until the peptide was split off the resin (TLC; MeOH/H_2O/AcOH 1.3/0.15/0.05 v/v/v). The filtrate was concentrated to 10 mL on a rotary evaporator. The Boc-peptidyl-OH was in situ activated with HBTU/HOBt (1:1, 1 equiv) and DIEA (1 equiv). Primary or secondary amine was added dropwise to the stirred solution, and the mixture was left to stand at 0 °C for 30 min and at room temperature overnight. The reaction was checked for completion by TLC (MeOH/H_2O/AcOH 1.3:0.15:0.05 v/v/v). The mixture was evaporated to dryness, dissolved in AcOEt/Et_2O (9:1), washed successively with 5% $NaHCO_3$ (3 × 10 mL), 5% citric acid (3 × 10 mL), 5% NaCl (3 × 10 mL), and evaporated to dryness.

Peptide and Side Chain Cleavage and Purification. All peptides were cleaved from the resin and/or deprotected by adding 10 mL of an ice-cold mixture TFA/phenol/H_2O/thioanisole/ethanedithiol (82.5:5:5:5:2.5, v/v/v/v/v) agitated at 0 °C for 5 min and at room temperature for 2 h as previously described.[39,40] Crude peptides were purified by semipreparative reversed-phase HPLC (RP-HPLC) on a Vydac 218TP1022 C_{18} column (2.2 cm × 25 cm; Alltech, Templemars, France) using a linear gradient (10−50% over 50 min) of acetonitrile/TFA (99.9:0.1, v/v) at a flow rate of 10 mL/min. Analytical RP-HPLC analysis, performed on a Vydac 218TP54 C_{18} column (0.46 cm × 25 cm, Alltech), revealed that the purity of all peptides was higher than 98.6% (Table 1). The authenticity of each peptide was verified by MALDI-TOF-MS on a Voyager DE-PRO (Applera-France) in the reflector mode with α-cyano-4-hydroxycinnamic acid as a matrix.

Cloning of *h*GPR103 cDNA. The human GPR103 receptor coding sequence was isolated by RT-PCR from human brain mRNA (Clontech Laboratories, Palo Alto, CA) using the 5′ forward primer (5′-aagcttATGCAGGCGCTTAACATTACC-3′, nt 380−401) and the 3′ reverse primer (5′-CTCCTTTAGACAGTGGGCATTAAggtacc-3′, nt 1653−1675), which were designed according to GenBank data sequence (accession no. NM 198179). Brain mRNA (200 ng) was reverse-transcribed using oligo(dT)12−18 and Superscript II reverse transcriptase (Life Technologies). PCR reactions were performed in a volume of 50 μL containing native Pfu buffer, 0.25 mM dNTP, 2 μL of the single-stranded cDNA preparation, 3× enhancer solution, 0.4 μM primers, and 2 units of the Pfu native polymerase (Life Technologies) with a 35 cycle program of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, and a pre- and postincubation program of 94 °C for 1 min and 72 °C for 5 min, respectively. The amplified cDNA was then subcloned into HindIII and KpnI sites of the pcDNA3.1(+) vector containing an in-frame M2tag (DYKDDDDK carboxy-terminal end) after the KpnI site. The sequence was controlled using dideoxychain termination and automated fluorescent dye ABI3730 DNA sequencer (Applied Biosystems).

Establishment of a Cell Line Stably Expressing *h*GPR103 and the $G\alpha_{16}$ Protein. The preparation of CHO-K1-$G\alpha_{16}$ cells stably expressing the G-protein α_{16} subunit has been previously described.[41] The cells were maintained in Ham-F12 medium supplemented with 10% (v/v) fetal bovine serum (inactivated at 56 °C for 30 min), 2 mM glutamine, 500 IU/mL penicillin, and 100 μg/mL streptomycin. The coding region of the human GPR103 receptor, containing the flag epitope sequence (DYKDDDDK) at its 3′-end, was subcloned into the pcDNA3.1-neo expression vector (Invitrogen, Cergy Pontoise, France) and transfected into CHO-K1-$G\alpha_{16}$ cells using Lipofectamine as described by the manufacturer (Invitrogen). Stably transfected cells were selected with Geneticin (800 μg/mL) and tested by immunofluorescence for their ability to bind the M2 antibody.

Ca^{2+} Mobilization Assays. Changes in intracellular Ca^{2+} concentrations induced by 26RFa analogues in CHO-$G\alpha_{16}$-*h*GPR103-transfected cells were measured on a benchtop scanning fluorometer Flexstation II (Molecular Devices, Sunnyvale, CA). Briefly, 96-well assay black plates with clear bottom (Corning International, Avon, France) were seeded at a density of 40 000 cells/well 24 h prior to assay. For agonist experiments, cells were loaded with 2 μM Fluo-4AM during 1 h, washed 3 times, and incubated 30 min with standard HBSS containing 2.5 mM probenecid and 5 mM HEPES. Compounds to be tested were added at final concentrations ranging from 10^{-12} to 10^{-5} M (HBSS), and the fluorescence intensity was measured during 2 min. Antagonist experiments were conducted using the same protocol except that compounds were manually injected at 10^{-6}, 3.16×10^{-6}, 10^{-5}, and 3.16×10^{-5} M 30 min prior to the injection of the agonist, i.e., *h*26RFa, at a single concentration of 10^{-7} M. A xenon lamp was used as excitation source. The wavelengths of excitation (485 nm) and emission (525 nm) of Fluo-4-AM were selected by two monochromators included in the device equipped with a bottom reading probe.

Statistical Analysis. Calcium experiments were performed in triplicate, and data, expressed as the mean ± SEM, were analyzed with the Prism software (Graphpad Software, San Diego, CA). The EC_{50} values were determined from concentration−response curves using a sigmoidal dose−response fit with variable slope. Differences between 26RFa or 26RFa$_{(20-26)}$ and analogue activities were analyzed by the Mann−Whitney test.

AUTHOR INFORMATION

Corresponding Author

*Phone: (33) 235-14-6624. Fax: (33) 235-14-6946. E-mail: hubert.vaudry@univ-rouen.fr.

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ABBREVIATIONS USED

Abu, 2-aminobutyric acid; Ac, acetyl; Adam, adamantanoyl; Bz, benzoyl; Fur, furoyl; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; *p*FPha, *p*-fluorophenylacetyl; Piv, pivaloyl; RP-HPLC, reversed-phase high performance liquid chromatography; Tle, terleucine; Tmg, tetramethylguanidinyl; Z, benzyloxycarbonyl

■ ADDITIONAL NOTE

Symbols and abbreviations are in accord with recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature: Nomenclature and Symbolism for Amino Acids and Peptides. *Biochem. J.* **1984**, *219*, 345−373 and A Short Guide to Abbreviations and Their Use in Peptide Science. *J. Pept. Sci.* **2003**, *9*, 1−8. In our manuscript, all optically active amino acids are of the L configuration unless otherwise noted.

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