Articles

Structure−Activity Relationships of a Series of Analogues of the Octadecaneuropeptide ODN on Calcium Mobilization in Rat Astrocytes[§]

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The octadecaneuropeptide ODN (QATVGDVNTDRPGLLDLK), originally characterized as an endogenous ligand for central-type benzodiazepine receptors, increases intracellular calcium concentration ($[Ca^{2+}]_i$) in rat astroglial cells. A series of ODN analogues was synthesized, and each compound was studied for its ability to induce Ca^{2+} mobilization in cultured rat astrocytes. Replacement of each amino acid by an L-alanine residue (AlaScan) showed that the N-terminal region of the molecule was relatively tolerant to alanine substitution (**2−8**, **10**), except for the Ala⁹-substituted analogue (**9**) which was totally devoid of activity. Pyroglutamization (**21**) and acetylation (**22**) of the Gln¹ residue reduced the Ca^{2+} response suggesting that a free N-terminal amine function is required for full activity of ODN. Alanine substitution of the residues in the C-terminal region of the molecule (**11−14**, **16−18**) significantly reduced the biological activity of ODN. In particular, modifications of the Leu¹⁵ residue (**15**, **20**) abolished the Ca^{2+}-mobilizing activity. The analogues [Ala⁹]ODN (**9**), [Ala¹⁵]ODN (**15**), [D-Thr⁹]ODN (**19**), and [D-Leu¹⁵]ODN (**20**) partially antagonized the Ca^{2+} response evoked by ODN. Most importantly, the octapeptide $ODN_{11−18}$ (OP, **24**) produced a dose−response curve that was superimposable to that obtained with ODN, indicating that the C-terminal region of the molecule possesses full biological activity. Finally, the AlaScan of OP revealed that replacement of the Leu⁵ residue by Ala (**29**) or D-Leu (**33**) totally suppressed the calcium response, confirming the crucial contribution of the Leu¹⁵ residue of ODN to the biological activity of the neuropeptide.

Introduction

The search for endogenous ligands of benzodiazepine receptors has led to the discovery of an 86-amino acid polypeptide termed diazepam-binding inhibitor (DBI) which, like β-carbolines, acts as an inverse agonist of central-type benzodiazepine receptors.[1−3] Proteolytic cleavage of DBI generates several biologically active peptides including the triakontatetraneuropeptide TTN ($DBI_{17−50}$)[4] and the octadecaneuropeptide ODN ($DBI_{33−50}$).[2] The generic term "endozepines" is generally used to designate DBI and its processing fragments.[3,5]

Intracerebroventricular administration of endozepines induces proconflict behavior,[1,2,6] provokes anxiogenic responses,[7] and inhibits apomorphine-induced yawning and penile erection.[8] In vitro studies have shown that endozepines exert a large array of biological activities such as modulation of $GABA_A$ receptors in pituitary cells,[9] inhibition of glucose-induced insulin release,[10,11] and stimulation of steroid hormone[12−14] and neurosteroid biosynthesis.[15] Short-chain analogues of endozepines may thus have potential therapeutic applications in the treatment of neurological and/or endocrine disorders. At the molecular level, ODN appears to modulate central-type benzodiazepine receptors associated with the $GABA_A$−receptor complex,[2,16] while TTN is a selective ligand of peripheral-type (mitochondrial) benzodiazepine receptors.[16,17] In addition, it has been recently demonstrated that ODN also activates a membrane receptor positively coupled to phospholipase C through a PTX-sensitive G-protein.[18] Therefore, the design of specific ODN agonists and antagonists may prove useful to investigate the mechanisms of action of endozepines on the different types of receptors.

Endozepines are widely distributed in the central nervous system[19−21] and in peripheral tissues.[19,22−24] In the brain, it has been shown that the DBI gene is exclusively expressed in glial cells.[25,26] In fact, several observations suggest that endozepines can act as autocrine factors regulating the activity of astroglial cells: (i) rat astrocytes can release substantial amounts of endozepines[27] and (ii) exposure of rat astrocytes to nanomolar concentrations of ODN causes a dose-dependent increase in cytosolic calcium concentration ($[Ca^{2+}]_i$).[27,28]

Table 1. Chemical Data for Compounds **1–20**

compd	peptide	t_R (min)[a]	<Glu (%)[b]	$[MH]^+$ (amu)[c]
1	ODN	18.71	1.9	1912.2
2	[Ala[1]]ODN	18.84		1855.8
3	[Ala[3]]ODN	18.65	1.9	1883.4
4	[Ala[4]]ODN	18.43	5.8	1885.5
5	[Ala[5]]ODN	18.87	6.9	1927.6
6	[Ala[6]]ODN	18.81	4.7	1868.4
7	[Ala[7]]ODN	18.41	7.4	1884.9
8	[Ala[8]]ODN	18.95	7.3	1870.0
9	[Ala[9]]ODN	18.75	2.7	1882.6
10	[Ala[10]]ODN	19.25	1.6	1868.2
11	[Ala[11]]ODN	18.77	1.4	1827.5
12	[Ala[12]]ODN	18.95	1.7	1886.3
13	[Ala[13]]ODN	19.11	1.4	1926.1
14	[Ala[14]]ODN	16.79	1.0	1869.7
15	[Ala[15]]ODN	16.85	1.6	1871.0
16	[Ala[16]]ODN	18.65	1.1	1869.0
17	[Ala[17]]ODN	16.68	1.4	1871.0
18	[Ala[18]]ODN	20.20	5.1	1855.9
19	[D-Thr[9]]ODN	18.84	2.1	1912.3
20	[D-Leu[15]]ODN	19.96	2.4	1912.5

[a] Retention time determined by RP-HPLC. [b] Percentage of pyro-glutamic acid residue contained in each analogue as assessed by RP-HPLC. [c] m/z values measured by FAB-MS.

The aim of the present study was to design potent and selective ligands of the endozepine receptors based on the primary structure of ODN. We have synthesized a collection of analogues of ODN in order to determine the minimal sequence for activity and to identify the position-related structural requirements for agonistic and antagonistic behavior. For the assessment of biological activity, we have taken advantage of the highly sensitive calcium-mobilizing response of rat astroglial cells to investigate the structure–activity relationships of the ODN-related peptides.

Results and Discussion

Replacement of each amino acid residue by an alanine moiety is a classical approach to investigate the structure–activity relationships of regulatory peptides.[29–32] To determine the amino acid side chains of ODN involved in ligand–receptor interaction, a series of L-alanine-substituted analogues was first synthesized (Table 1, compounds **2–18**) and the effectiveness of these peptides to increase $[Ca^{2+}]_i$ in cultured rat astrocytes was assessed by microfluorimetry (Figure 1A). Alanine replacement in the N-terminal region (compound **2** and compounds **4–8**) or at position 10 (compound **10**) did not significantly affect the activity of the peptide (Figure 1B). In particular, replacement of the Gly[5] residue by Ala, which is expected to restrict the conformational freedom of the peptide backbone at this point, did not impair the $[Ca^{2+}]_i$-mobilizing activity of the peptide. Similarly, the fact that compound **3** exhibited 70% of the efficacy of ODN indicated that Thr[3] was relatively tolerant to Ala substitution. The high activity that was retained upon substitution of each residue in the N-terminal domain (Gln[1] to Asn[8]) with alanine implies that the side chains in this region are not intimately involved in receptor contact.

In contrast, L-alanine substitution in the C-terminal region of the molecule (compounds **11–18**) as well as on the 9th position (compound **9**) significantly reduced the activity of ODN on $[Ca^{2+}]_i$. In particular, compounds **9** and **15**, in which the Thr[9] and Leu[15] residues had been substituted, were totally devoid of activity for
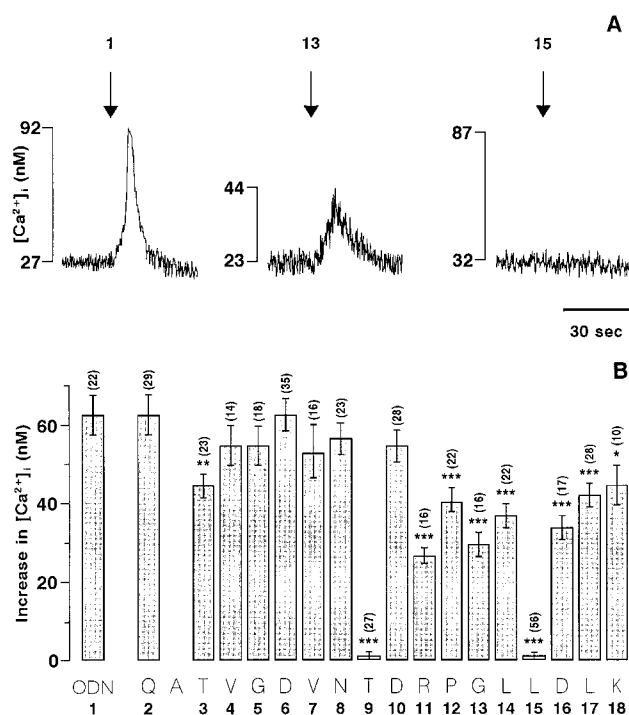

Figure 1. Effect of L-alanine-substituted ODN analogues (**2–18**) on $[Ca^{2+}]_i$ in cultured rat astrocytes. Panel A shows three typical profiles recorded from different cells. A 2-s pulse (arrows) of ODN (**1**), [Ala[13]]ODN (**13**), or [Ala[15]]ODN (**15**) (10^{-8} M each) was administered in the vicinity of cells. Panel B shows the effects of ODN (**1**; 10^{-8} M) and L-alanine-substituted ODN analogues (**2–18**; 10^{-8} M each) on the amplitude of the calcium response. Each value represents the mean amplitude (±SEM) of the calcium response calculated from at least four different dishes from two independent cultures. The number of cells studied is indicated in parentheses. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs ODN (Student's t-test).

Table 2. Effects of [Ala[9]]ODN (**9**) and [Ala[15]]ODN (**15**) on Basal $[Ca^{2+}]_i$ and ODN-Induced $[Ca^{2+}]_i$ Increase in Cultured Rat Astrocytes

compd	peptide(s)	concn (M)	$[Ca^{2+}]_i$ variation (nM)[a]	
9	[Ala[9]]ODN	10^{-10}	ND[b]	(42)[c]
		10^{-8}	ND	(27)
		10^{-6}	ND	(14)
15	[Ala[15]]ODN	10^{-10}	ND	(40)
		10^{-8}	ND	(97)
		10^{-6}	ND	(40)
1	ODN	10^{-8}	62 ± 5	(22)
1 + 9[d]	ODN + [Ala[9]]ODN	$10^{-8} + 10^{-6}$	39 ± 3***	(15)
1 + 15[d]	ODN + [Ala[15]]ODN	$10^{-8} + 10^{-6}$	36 ± 5***	(11)

[a] Values are the mean ± SEM obtained from at least four different dishes from two independent cultures. [b] ND, not detectable. [c] Number of cells studied. [d] The cells were incubated for 15 min with [Ala[9]]ODN (**9**) or [Ala[15]]ODN (**15**) (10^{-6} M each) before a 2-s pulse of ODN (**1**; 10^{-8} M) was administered. $***p < 0.001$ compared to ODN alone (Student's t-test).

concentrations ranging from 10^{-10} to 10^{-6} M (Figure 1, Table 2). In fact, compounds **9** and **15** at micromolar concentrations reduced by 38% and 42%, respectively, the ODN-evoked increase in Ca^{2+} (Table 2), suggesting that the Thr[9] and Leu[15] residues are involved in receptor activation rather than receptor binding. D-Amino acid replacement at positions 9 and 15 (compounds **19** and **20**, respectively) resulted in complete loss of the $[Ca^{2+}]_i$ response (Table 3), confirming the importance of the side chains of the Thr[9] and Leu[15] residues in the biological

Table 3. Effects of [D-Thr^9]ODN (**19**) and [D-Leu^{15}]ODN (**20**) on Basal [Ca^{2+}]$_i$ and ODN-Induced [Ca^{2+}]$_i$ Increase in Cultured Rat Astrocytes

compd	peptide(s)	concn (M)	[Ca^{2+}]$_i$ variation (nM)^a	
19	[D-Thr^9]ODN	10^{-10}	ND^b	(56)^c
		10^{-8}	ND	(80)
		10^{-6}	ND	(45)
20	[D-Leu^{15}]ODN	10^{-10}	ND	(65)
		10^{-8}	ND	(91)
		10^{-6}	ND	(70)
1	ODN	10^{-8}	62 ± 5	(22)
1 + 19^d	ODN + [D-Thr^9]ODN	$10^{-8} + 10^{-6}$	31 ± 3***	(19)
1 + 20^d	ODN + [D-Leu^{15}]ODN	$10^{-8} + 10^{-6}$	25 ± 3***	(17)

^{a} Values are the mean ± SEM obtained from at least four different dishes from two independent cultures. ^{b} ND, not detectable. ^{c} Number of cells studied. ^{d} The cells were incubated for 15 min with [D-Thr^9]ODN (**19**) or [D-Leu^{15}]ODN (**20**) (10^{-6} M each) before a 2-s pulse of ODN (**1**; 10^{-8} M) was administered. ***$p <$ 0.001 compared to ODN alone (Student's t-test).

**Table 4.** Chemical Data for Compounds **21**−**33**

compd	peptide	t_R (min)^a	purity (%)^b	[MH]$^+$ (amu)^c
21	[<Glu^1]ODN	19.47	98.5	1895.1
22	Ac-ODN	19.09	97.8	1953.7
23	Ac-[Ala^1]ODN	19.85	99.9	1898.0
24	OP	18.43	99.9	911.3
25	[Ala^1]OP	19.25	99.9	826.0
26	[Ala^2]OP	18.51	99.5	886.0
27	[Ala^3]OP	18.40	99.8	925.0
28	[Ala^4]OP	15.55	99.9	969.0
29	[Ala^5]OP	15.78	99.9	869.0
30	[Ala^6]OP	18.18	99.7	867.0
31	[Ala^7]OP	15.40	99.7	869.0
32	[Ala^8]OP	19.91	99.9	854.6
33	[D-Leu^5]OP	19.77	99.9	911.3

^{a} Retention time determined by RP-HPLC. ^{b} Assessed by RP-HPLC. ^{c} m/z values measured by FAB-MS.

activity of ODN. Interestingly, [D-Thr^9]ODN and [D-Leu^{15}]ODN (compounds **19** and **20**) exhibited a weak antagonistic activity (Table 3) in very much the same way as the Ala^9- and Ala^{15}-substituted analogues.

Reversed-phase HPLC analysis of ODN and its L-Ala analogues (with the exception of compound **2**) revealed that the pyroglutamic acid derivatives may be spontaneously formed (Table 1). The <Glu residue of several regulatory peptides plays an important role in determining the biological activity of the peptide[33] and/or in conferring metabolic stability toward aminopeptidases.[34] To determine whether <Glu formation had any effect on the intrinsic activity of ODN, [<Glu^1]ODN (compound **21**) was synthesized (Table 4). As shown in Figure 2, [<Glu^1]ODN only exhibited 61% of the efficacy of the native molecule. Similarly, N$^\alpha$-acetylation of ODN (compound **22**) and of [Ala^1]ODN (compound **23**) reduced by 38% and 39%, respectively, the efficacy of the analogue, suggesting that a free N-terminus is required for full bioactivity (Figure 2).

Previous studies have shown that the displacement of [^{3}H]β-carboline and the proconflict activity of ODN are both mimicked by the C-terminal fragment of the peptide.[2] We have thus synthesized the octapeptide RPGLLDLK (OP, compound **24**) corresponding to the sequence ODN$_{11-18}$ (Table 4). The dose−response effects of OP on [Ca^{2+}]$_i$ were compared to those of native ODN (Figure 3). As previously reported,[28] ODN induces a bell-shaped increase in [Ca^{2+}]$_i$, the maximum response being observed at a concentration of $10^{-9}-10^{-8}$ M, while
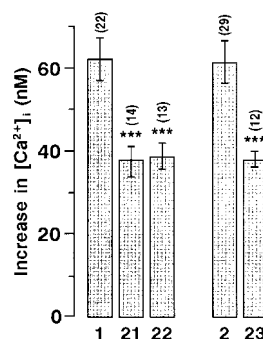
Figure 2. Effect of N-terminal-modified ODN and [Ala^1]ODN analogues (**21**−**23**) on [Ca^{2+}]$_i$ in cultured rat astrocytes. A 2-s pulse of ODN (**1**), [<Glu^1]ODN (**21**), Ac-ODN (**22**), [Ala^1]ODN (**2**), and Ac-[Ala^1]ODN (**23**) (10^{-8} M each) was administered in the vicinity of the cells. Each value represents the mean amplitude (±SEM) of the calcium response calculated from at least four different dishes from two independent cultures. The number of cells studied is indicated in parentheses. ***$p <$ 0.001 vs ODN (**21** and **22**) or [Ala^1]ODN (**23**) (Student's t-test).
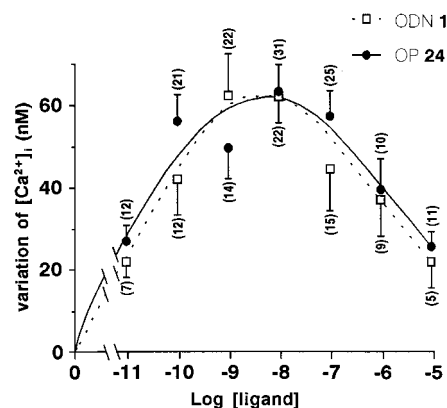
Figure 3. Comparison of the effects of increasing concentrations of ODN (**1**) and OP (**24**) on [Ca^{2+}]$_i$ in cultured rat astrocytes. Each value represents the mean amplitude (±SEM) of the calcium response calculated from at least four different dishes from two independent cultures. The number of cells studied is indicated in parentheses.

at higher concentrations ($10^{-7}-10^{-5}$ M), the amplitude of the [Ca^{2+}]$_i$ response gradually declined. The dose−response obtained with OP was almost superimposable with that of ODN (Figure 3). In particular, at the maximum effective dose of 10^{-8} M, the mean amplitudes of the effects of OP and ODN were strictly identical, indicating that the bioactive domain of ODN is located in the OP region. Consistent with these data, it has been previously reported that OP mimics the proconflict activity of ODN, albeit the activity of the former is 4 times lower than that of the latter.[2]

Replacement of each amino acid residue of OP by alanine (Table 4, compounds **25**−**32**) resulted in a significant decrease of the Ca^{2+}-mobilizing activity of all the analogues (Figure 4). A good correlation was generally observed between the relative efficacies of the Ala-substituted OP analogues and the homologous Ala-substituted ODN compounds. In particular, replacement of the Leu^5 residue of OP (compound **29**) resulted in complete loss of the calcium-mobilizing activity of the peptide at a concentration of 10^{-8} M. Similarly, substitution of the L-Leu^5 of OP with a D-Leu residue (compound **33**) totally suppressed the activity (Table 5), confirming the importance of the Leu^{15} residue of ODN
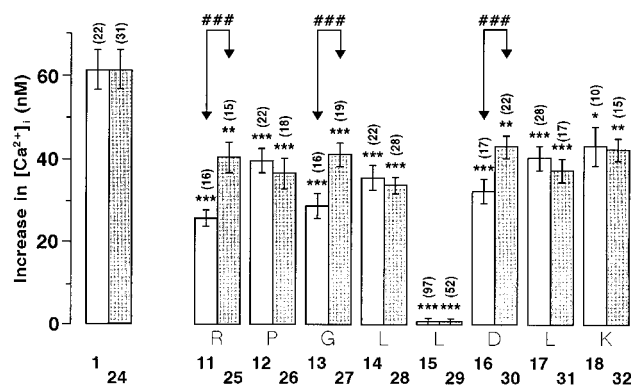
Figure 4. Comparison of the effect of L-alanine-substituted ODN analogues (**11**−**18**) and L-alanine-substituted OP analogues (**25**−**32**) on $[Ca^{2+}]_i$ in cultured rat astrocytes. A 2-s pulse of ODN (**1**) or ODN analogues (**11**−**18**) (open bars) and OP (**24**) or OP analogues (**25**−**32**) (gray bars) was administered at a concentration of 10^{-8} M each in the vicinity of the cells. Each value represents the mean amplitude (±SEM) of the calcium response calculated from at least four different dishes from two independent cultures. The number of cells studied is indicated in parentheses. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs ODN (**11**−**18**) or OP (**25**−**32**), $^{###} p < 0.001$ vs corresponding ODN analogues (Student's *t*-test).

Table 5. Effects of $[Ala^5]OP$ (**29**) and $[D\text{-}Leu^5]OP$ (**33**) on Basal $[Ca^{2+}]_i$ and ODN-Induced $[Ca^{2+}]_i$ Increase in Cultured Rat Astrocytes

compd	peptide(s)	concn (M)	$[Ca^{2+}]_i$ variation (nM)^a	
29	$[Ala^5]OP$	10^{-8}	ND^b	$(52)^c$
		10^{-6}	37 ± 4	(53)
33	$[D\text{-}Leu^5]OP$	10^{-10}	ND	(49)
		10^{-8}	ND	(81)
		10^{-6}	ND	(47)
1	ODN	10^{-8}	62 ± 5	(22)
1 + 33^d	ODN + $[D\text{-}Leu^5]OP$	$10^{-8} + 10^{-6}$	$39 \pm 3***$	(22)

^{a} Values are the mean ± SEM obtained from at least four different dishes from two independent cultures. ^{b} ND, not detectable. ^{c} Number of cells studied. ^{d} The cells were incubated for 15 min with $[D\text{-}Leu^5]OP$ (**33**; 10^{-6} M) before a 2-s pulse of ODN (**1**; 10^{-8} M) was administered. $***p < 0.001$ compared to ODN alone (Student's *t*-test).

for the biological activity of the peptide. In fact, it was found that $[D\text{-}Leu^5]OP$, in very much the same way as $[D\text{-}Leu^{15}]ODN$, significantly reduced the stimulatory effect of ODN on $[Ca^{2+}]_i$ (Table 5). Moreover, the $[Ala^2]$-OP, $[Ala^4]OP$, $[Ala^7]OP$, and $[Ala^8]OP$ analogues (compounds **26**, **28**, **31**, and **32**) exhibited practically the same activity as the corresponding $[Ala^{12}]ODN$, $[Ala^{14}]$-ODN, $[Ala^{17}]ODN$, and $[Ala^{18}]ODN$ analogues (compounds **12**, **14**, **17**, and **18**) (Figure 4). However, OP appeared slightly more tolerant than ODN to Ala substitution. For instance, the $[Ala^1]OP$, $[Ala^3]OP$, and $[Ala^6]OP$ analogues (compounds **25**, **27**, and **30**) retained a stronger biological activity than their Ala-substituted ODN counterparts (Figure 4). In addition, it was noticed that $[Ala^5]OP$ had a weak Ca^{2+}-mobilizing effect at a concentration of 10^{-6} M, whereas $[Ala^{15}]ODN$ was totally devoid of activity at this concentration (Table 5).

Conclusions

The importance of the various amino acid residues in contributing to the calcium-mobilizing activity of ODN in rat astrocytes can be summarized as follows:

(1) the integrity of the Thr^9 and Leu^{15} residues is crucial for measurable biological activity, (2) analogues substituted by an Ala or a D-amino acid at the 9th and 15th positions exhibit modest antagonistic activity, (3) a free amino group on the Gln^1 residue is required for full agonistic activity, (4) the C-terminal octapeptide (OP) retains full biological activity, and (5) substitution of the Leu^5 residue by Ala or D-Leu in OP abolishes the Ca^{2+}-mobilizing response. Taken together, these data demonstrate the critical importance of the Leu^{15} residue of ODN in determining the biological activity of the molecule. Our results also suggest that Thr^9, which is required for ODN activity but is not present in OP, may play an important role in the correct fit of the N-terminal domain of ODN for receptor activation.

Experimental Section

Materials. All amino acid residues, preloaded 4-hydroxy-methyl-phenoxymethyl-copolystyrene-1%-divinylbenzene resins (Fmoc-Lys(Boc)-HMP, Fmoc-Ala-HMP), *N*-methylpyrrolidin-2-one (NMP), *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), methanol (MeOH), *O*-benzotriazol-1-yl-*N*,*N*,*N*,*N*-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), piperidine, and *N*,*N*-diisopropylethylamine (DIEA) were purchased from Applied Biosystems (St. Quentin en Yvelines, France). Pentachlorophenyl pyroglutamate (<Glu-OPcp) was obtained from Bachem Biochimie (Voisins-le-Bretonneux, France). Trifluoroacetic acid (TFA), phenol, thioanisole, ethanedithiol, and *tert*-butylmethyl ether (TBME) were from Sigma-Aldrich Chimie (St. Quentin Fallavier, France). Dulbecco's modified Eagle's medium (DMEM), F12 culture medium, insulin, and D-(+)-glucose were from Sigma-Aldrich Chimie. Glutamine, the antibiotic−antimycotic solution, and *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid (HEPES) were from Bioproducts (Gagny, France). Fetal bovine serum (FBS) was from Biosys (Compiègne, France). Indo-1-acetoxymethyl ester was from Molecular Probes (Eugene, OR).

Peptide Synthesis. Octadecaneuropeptide (ODN; QATVGDVNTDRPGLLDLK), octaneuropeptide (OP; ODN_{11-18}), $[D\text{-}Thr^9]ODN$, $[D\text{-}Leu^{15}]ODN$, $[D\text{-}Leu^5]OP$, and L-alanine analogues of ODN and OP were synthesized (0.25-mmol scale for ODN, 0.1-mmol scale for all other peptides) on a Fmoc-Lys-(Boc)-HMP resin or a Fmoc-Ala-HMP resin using a 433A Applied Biosystems peptide synthesizer and the standard FastMocΩMonPrevPK procedures. All Fmoc-L-amino acids (1 mmol, 4 or 10 equiv) were coupled by in situ activation with HBTU/HOBt (0.9 mmol, 3.6 or 9 equiv; 1:1, mol/mol) in DMF and DIEA (2 mmol, 8 or 20 equiv) in NMP. Reactive side chains were protected as follows: Gln and Asn, tritylamide (Trt); Thr, *tert*-butyl ether (*t*-Bu); Asp, *tert*-butyl ester (O*t*-Bu); Arg, pentamethylchromansulfonylamide (Pmc); and Lys, *tert*-butyloxycarbonyl (Boc).

After completion of the chain assembly, N^α-acetylation of ODN and $[Ala^1]ODN$ was performed on the resin by addition of a mixture of acetic anhydride/DIEA/HOBt (0.4 mmol, 1.6 or 4 equiv; 1:1:1, mol/mol/mol) in NMP for 5 min at 0 °C and 30 min at room temperature. $[<Glu^1]ODN$ was manually prepared one step before completion by addition of <Glu-OPcp (0.3 mmol, 3 equiv) in NMP for 1 h at room temperature. The peptidyl-resins were filtered and washed twice with NMP and DCM. Reactions were monitored by the Kaiser test.[35]

Peptide Cleavage and Purification. Peptides were deprotected and cleaved from the resin by adding 10 mL of the mixture $TFA/phenol/H_2O/thioanisole/ethanedithiol$ (82.5:5:5:5:2.5, v/v/v/v/v; reagent K) for 90 min at room temperature.[36] After filtration, crude peptides were precipitated by addition of TBME, centrifuged (4500 rpm), washed twice with TBME, and lyophilized.

All peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a semiprepara-

tive Vydac C_{18} column (1 × 25 cm; Touzart et Matignon, Courtaboeuf, France) using a linear gradient (10−50% over 40 min) of acetonitrile/TFA (99.9:0.1, v/v) at a flow rate of 5 mL/min. Analytical RP-HPLC (1 mL/min) was performed on a Vydac C_{18} column (0.45 × 25 cm) using a linear gradient (10−40% over 30 min) of acetonitrile/TFA. The purified peptides were characterized by FAB-MS on a conventional EB geometry mass spectrometer JEOL model AX-500 equipped with a DEC data system (JEOL-Europe SA, Croissy-sur-Seine, France) (Tables 1 and 4).

Cell Culture. Primary cultures of rat astrocytes were performed as previously described.[28] Briefly, cerebral hemispheres from newborn Wistar rats were collected in DMEM/F12 culture medium (2:1, v/v) supplemented with 2 mM glutamine, 1% insulin, 5 mM HEPES, 0.4% D-(+)-glucose, and 1% of the antibiotic−antimycotic solution. The tissues were desaggregated mechanically using a Pasteur pipet and filtered through a 82-μm nylon sieve (Tripette et Renaud, Combles, France). Dissociated cells were resuspended in culture medium supplemented with 10% FBS and seeded on coverslips in 35-mm dishes (Dutscher, Brumath, France) at a density of 10^6 cells/dish. The cells were incubated at 37 °C in a moist atmosphere (5% CO_2), and the medium was changed twice a week.

Measurement of Cytosolic Ca^{2+} Concentration. Five- to seven-day old cells were incubated at 37 °C for 45 min in the dark with 5 μM indo-1-acetoxymethyl ester diluted in culture medium. The cells were then washed twice with 2 mL of fresh medium. The $[Ca^{2+}]_i$ was monitored by a dual-emission microfluorimeter system constructed from a Nikon Diaphot inverted microscope, as previously described.[37] The fluorescence emission of indo-1-acetoxymethyl ester, induced by excitation at 355 nm, was recorded at two wavelengths (405 and 480 nm) by separate photometers (Nikon). The 405/480 ratio was determined using an analogic divider (constructed by Dr. B. Dufy, Bordeaux, France) after conversion of single photon currents to voltage signals. All three signals (405 nm, 480 nm, and 405/480 ratio) were continuously recorded with the Jad-Fluo 1.2 software (Notocord Systems, Croissy-sur-Seine, France). The $[Ca^{2+}]_i$ values were calculated as previously described.[38] All secretagogues were ejected for 2 s in the vicinity of individual cells by a pressure ejection system. The doses of peptides indicated correspond to the concentration contained in the ejection pipet.

Statistical Analysis. Data are expressed as mean ± SEM. Differences between ODN and analogue activities were analyzed by the Student's *t*-test.

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