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A synthetic glycine-extended bombesin analogue interacts with the GRP/bombesin receptor

Catherine Oiry, Julie Pannequin, Nicole Bernad, Anne-Marie Artis, Jean-Claude Galleyrand, Chantal Devin, Michèle Cristau, Jean-Alain Fehrentz, Jean Martinez *

Laboratoire des Amino Acides, Peptides et Protéines (L.A.P.P.), UMR CNRS 5810, Universités Montpellier I et II, Faculté de Pharmacie, 15 Av. C. Flahault, 34060 Montpellier Cedex, France

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Abstract

α-amidation of a peptide (which takes place from a glycine-extended precursor) is required to produce biologically active amidated hormones, such as gastrin-releasing peptide (GRP)/Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 (bombesin). It was shown that glycine-extended gastrin mediates mitogenic effects on various cell lines by interacting with a specific receptor, different from the classical CCK_1 or CCK_2 receptors. On the basis of this observation, we have extended the concept of obtaining active glycine-extended forms of others amidated peptides to produce new active analogues. In this study, we have tested the biological behaviour of a synthetic analogue of the glycine-extended bombesin (para-hydroxy-phenyl-propionyl-Gln-Trp-Ala-Val-Gly-His-Leu-Met-Gly-OH or JMV-1458) on various in vitro models. We showed that compound JMV-1458 was able to inhibit specific (3-[^{125}I]iodotyrosyl^{15}) GRP ([^{125}I]GRP) binding in rat pancreatic acini and in Swiss 3T3 cells with K_i values of approximately 10^{-8} M. In isolated rat pancreatic acini, we found that JMV-1458 induced inositol phosphates production and amylase secretion in a dose-dependent manner. In Swiss 3T3 cells, the glycine-extended bombesin analogue dose-dependently produced [^{3}H]thymidine incorporation. By using potent GRP/bombesin receptor antagonists, we showed that this synthetic glycine-extended bombesin analogue induces its biological activities via the classical GRP/bombesin receptor.

Keywords: Gastrin-releasing peptide; Bombesin, synthetic glycine-extended form; (In vitro); Biological effect; Gastrin-releasing peptide/bombesin receptor antagonist

1. Introduction

Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 (bombesin) is a tetradecapeptide isolated from skin frogs (Anastasi et al., 1971), whose mammalian homolog is gastrin-releasing peptide (GRP), originally isolated from porcine non-antral gastric tissue (McDonald et al., 1979). The C-terminal 14 amino acid residues of mammalian GRP are very similar to amphibian bombesin, especially the C-terminal heptapeptide (McDonald et al., 1979). Three separate receptors capable of binding bombesin have been isolated from various human cells: the GRP-preferring receptor found in the central nervous system and the gastro-intestinal tract; the neuromedin B-preferring receptor, which is also found in the central nervous system but with a more limited distribution in the gut; the bombesin receptor subtype-3 (bombesin BB_3 receptor), present in testis and lung cancer (Corjay et al., 1991; Fathi et al., 1993). Recently, a fourth member of the bombesin receptor family (bombesin BB_4 receptor) was isolated from a *Bombina orientalis* brain cDNA library (Nagalla et al., 1995). At present, the mammalian equivalent of the bombesin BB_4 receptor has not been identified (Katsuno et al., 1999). The GRP receptor is a 384-amino-acid protein

* Corresponding author. Tel.: +33-4-67-04-01-83; fax: +33-4-67-41-20-17.

E-mail address: martinez@colombes.pharma.univ-montp1.fr (J. Martinez).

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that is glycosylated to become a 75–85 kDa cell surface receptor (Corjay et al., 1991). Activation of this receptor results in stimulation of phospholipase C with subsequent increases of inositol phosphates and intracellular Ca^{2+} (Corjay et al., 1991; Kroog et al., 1995). Mammalian bombesin-like peptides elicit a broad spectrum of biological responses, including secretion of gastrointestinal, adrenal and pituitary hormones, gastric acid and mucous secretion, regulation of smooth muscle contraction, and modulation of neuronal firing rate. In the central nervous system, these peptides are thought to influence regulation of homeostasis, thermoregulation, metabolism and behaviour (Tache et al., 1988; Lebacq-Verheyden et al., 1990). Bombesin family peptides can also act as mitogens. This has been shown in Swiss 3T3 cells (Rozengurt and Sinnett-Smith, 1983) and in human small cell lung carcinoma where the peptides have been implicated in a subset of tumors to function in autocrine growth loops (Cuttitta et al., 1985).

Prepro GRP is converted to bioactive amidated GRP by sequential enzymatic steps: trypsin-like/subtilisin-like cleavage, carboxypeptidase-like processing and formation of the glycine-extended intermediate, which serves as a substrate for peptidyl glycine α-amidating monooxygenase (Cuttitta, 1993).

Like other amidated peptides, gastrin is synthetized as a precursor that undergoes posttranslational processing to an amidated product on the C-terminus. Posttranslational processing intermediates of gastrin, specifically glycine-extended gastrin (gastrin-Gly), serve as substrate for the amidation reaction (Dockray et al., 1996). Whereas for many years, amidation of gastrin was thought to be an essential prerequisite for biological activity, recent results indicate that non-amidated gastrins can also stimulate cells proliferation in vitro (Seva et al., 1994; Singh et al., 1995). Interestingly, it was shown that proliferative effects induced by the glycine-extended progastrin were mediated through a specific receptor, different from the classical CCK_1 or CCK_2 receptors.

On the basis of the data obtained with the glycine-extended progastrin, we hypothesized that the concept of obtaining active glycine-extended forms of amidated peptides could be generalized to other amidated peptide hormones. As an example, we decided to investigate the biological activities of Gly-extended forms of bombesin. In this study, we present the biological behaviour of a synthetic analogue of the glycine-extended bombesin (para-hydroxy-phenyl-propionyl-Gln-Trp-Ala-Val-Gly-His-Leu-Met-Gly-OH or JMV-1458) on various in vitro models. We have compared JMV-1458 and amidated bombesin for their ability to induce inositol phosphates production and amylase release in rat pancreatic acini and to stimulate DNA synthesis in Swiss 3T3 cells. By using specific GRP/bombesin receptor antagonists, we have studied the pharmacological profile of the JMV-1458-activated receptor.

2. Materials and methods

2.1. Chemicals

Collagenase EC 3.4.24.3 was obtained from Serva (Heidelberg, FRG). NaCl, KCl, NaH_2PO_4, $MgCl_2$, $MgSO_4$, $CaCl_2$, K_2CO_3, LiCl, KH_2PO_4, $NaHCO_3$, sodium pyruvate, sodium glutamate, glutamine, ammonium formate, glucose, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), trypsin inhibitor, trichloroacetic acid and Krebs–Henseleit buffer were from Sigma (St. Louis, MO, USA). Bovine serum albumin fraction V was from Euromedex (France). The protein concentration was evaluated using the Bio-Rad protein assay (Bio-Rad, Richmond, CA, USA), based on the Bradford dye-binding procedure. Dowex AG1-X8 anion exchange resin (100–200 mesh, formate form) was also from Bio-Rad. Phadebas amylase test reagent was from Pharmacia (France). (3-[^{125}I]iodotyrosyl^{15}) GRP ([^{125}I]GRP; 2000 Ci/mmol), *myo*-[2-^{3}H]inositol (16.5 Ci/mmol) and [^{3}H]thymidine (24 Ci/mmol) were purchased from Amersham (Buckinghamshire, UK). Glutamine, penicillin/streptomycin, fetal bovine serum, amino acid mixture and essential vitamin mixture were from Gibco Life Technologies (Scotland). Dulbecco's modified Eagles' medium (DMEM) was from Bio Whittaker (Verviers, Belgium).

Bombesin, JMV-1458, D-Phe-Gln-Trp-Ala-Val-Gly-His-*N*-(1-amino-1-isobutyl-2-hydroxy-hexane) (JMV-641), *N*-((para-hydroxy-phenyl)-propionic)-Gln-Trp-Ala-*N*-3 (*S*) amino-2-oxo-1-azepine acetic)-His-Leu-methyl ester (JMV-1799) and *N*-((para-hydroxy-phenyl)-propionic)-Gln-Trp-Ala-*N*-(3 (*S*) amino-2-oxo-1-azepine acetic)-His-(1-amino-1-isobutyl-2-hydroxy-hexane) (JMV-1802; Fig. 1) were synthesized in our laboratory. A stock solution of each bombesin analogue was prepared in pure dimethyl sulfoxide and stored at −20°C. Dilutions were made in experiment incubation medium and the maximal final concentration did not contain more than 1% dimethyl sulfoxide.

2.2. Experiments on dispersed rat pancreatic acini

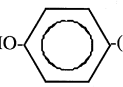
2.2.1. Preparation of dispersed rat pancreatic acini

Male Wistar rats (200–300 g) were obtained from the Pharmacological Breeding Center of Montpellier University (France). Dispersed acini were prepared as previously described (Peikin et al., 1978) with some modifications (Jensen et al., 1982) in buffer 1 containing (in mM) HEPES (pH 7.4) 25.5, NaCl 98, KCl 6, NaH_2PO_4 2.5, sodium pyruvate 5, sodium glutamate 5, glutamine 2, $CaCl_2$ 1.5, glucose 11.5, $MgCl_2$ 1, trypsin inhibitor 0.01% (p v^{-1}), amino acid mixture 1% (v v^{-1}) and essential vitamin mixture 1% (v v^{-1}).

Bombesin

Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

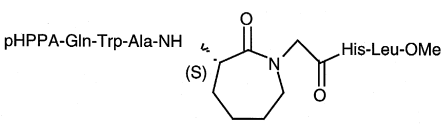
JMV-1458

HO-  -$(CH_2)_2$-CO-NH-Gln-Trp-Ala-Val-Gly-His-Leu-Met-Gly-OH

JMV-641

H-D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-ψ (CHOH)–($CH_2)_3$-CH_3

JMV-1799



JMV-1802

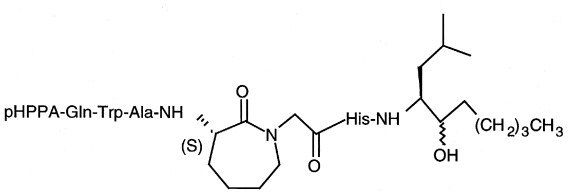


Fig. 1. Structures of the bombesin analogues used in the various experiments.

2.2.2. Binding of [^{125}I]GRP

The incubation medium used for binding experiments (buffer 2) was Krebs–Henseleit buffer (pH 7.4) supplemented with 1% bovine serum albumin (p v^{-1}). For displacement experiments, dispersed rat acini (0.5 mg protein) were incubated with 20 pM of [^{125}I]GRP (1/10 K_d) for 60 min at 37°C in a final volume of 0.5 ml in the presence of various concentrations of bombesin analogues in polypropylene tubes. Non-specific binding was determined in the presence of 10 μM unlabeled bombesin and was always less than 25% of the total binding. Incubation was terminated by adding 3 ml of buffer 2 at 4°C supplemented with 4% bovine serum albumin (p v^{-1}). Aliquots were then centrifuged at 4°C for 10 min at 3000 rpm. The supernatants were discarded and the radioactivity bound to the pellet was measured. Incubations were performed in duplicate and mean values were used for calculations.

2.2.3. Measurement of inositol phosphates production

Intracellular inositol phosphates were determined according to Qian et al. (1993) with some modifications. Rat pancreatic acini (6 mg protein ml^{-1}) were incubated in buffer 1 with 400 μCi *myo*-[2-^{3}H]inositol for 2 h at 37°C. Acini were then washed three times in the same buffer and incubated (15 min, 37°C) in 20 ml of buffer 3 (buffer 3 contained (in mM) HEPES (pH 7.4) 20, NaCl 116, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 1, glucose 11, $MgSO_4$ 1.2, $NaHCO_3$ 5) supplemented with 20 mM LiCl. Aliquots of [^{3}H]inositol-loaded acini (0.4 ml) were incubated (15 min, 37°C) with buffer 3 or with various compounds as described in each individual experiment in a final volume of 0.5 ml. The incubation was terminated by adding 500 μl $HClO_4$ 5% (v v^{-1}). Each tube subsequently received 155 μl of K_2CO_3 2 M and 25 μl of HEPES 0.4 M, and the contents were vortexed and centrifuged (3000 rpm, 10 min). 900 μl of each tube were collected and applied to a column containing 1.6 ml of a 1:4 (p p^{-1}) Dowex AG-1-X8 anion-exchange resin in distilled water. The columns were washed in the following manner: 10 ml distilled water, 4 ml of 40 mM ammonium formate. Inositol phosphates were eluted with 5 ml of 1 M ammonium formate. The eluates were then assayed for their radioactivity after the addition of 10 ml Complete Phase Combining System for liquid scintillation counting solution to each vial.

2.2.4. Amylase release test

Dispersed acini (0.5 mg) were suspended in 0.5 ml of buffer 1 and incubated for 30 min at 37°C in the same buffer. Amylase release was measured as previously described (Jensen et al., 1978; Sekar et al., 1991) using the Phadebas reagent (Ceska et al., 1969). Incubations were performed in duplicate and mean values were used for calculations.

2.3. Experiments on Swiss 3T3 cells

2.3.1. Cells culture

Swiss 3T3 cells were a gift from Dr. Abello (Inserm U45, Lyon, France). Cells were maintained at 37°C in a humidified atmosphere containing 10% CO_2 by serial passages in DMEM supplemented with 10% fetal bovine serum, 1 nM glutamine and 1% (v v^{-1}) penicillin/streptomycin.

2.3.2. Binding of [^{125}I]GRP

The day before the binding experiment, cells were seeded into 24-well plates (10^5 cells per well) and incubated for 24 h at 37°C in maintenance medium. After 24 h incubation time, cells were incubated with 20 pM of [^{125}I]GRP (1/10 K_d) for 60 min at 22°C in 1 ml of maintenance medium without fetal bovine serum supplemented with 0.2% bovine serum albumin (p v^{-1}), in the presence of various concentrations of bombesin analogues. Non-specific binding was determined in the presence of 10 μM unlabeled bombesin and was always less than 15% of the total binding. After 1 h incubation, cells were

washed twice with cold phosphate buffer saline supplemented with 0.2% bovine serum albumin (p v^{-1}) and solubilized in 1 ml of 1 M NaOH. Samples were removed from the plates, placed in tubes and associated radioactivity was determined. Incubations were performed in duplicate and mean values were used for calculations.

2.3.3. Swiss 3T3 cells proliferation studies by [^{3}H]thymidine incorporation

Swiss 3T3 cells (10^5 cells) were plated in 1 ml of maintenance medium DMEM supplemented with 10% fetal bovine serum (v v^{-1}) and allowed to attach overnight. Then, cells were cultured for 24 h in serum free medium supplemented with 0.2% bovine serum albumin (p v^{-1}). Cells were then treated for 24 h with various concentrations of test compounds. DNA synthesis was estimated by measurement of [^{3}H]thymidine incorporation into trichloroacetic acid precipitable material. The [^{3}H]thymidine (0.5 mCi per well) was added during the last hour of the 24 h treatment period for 4 h duration. Then, the cells were washed twice with phosphate buffer saline supplemented with 0.2% bovine serum albumin (p v^{-1}) to remove unincorporated [^{3}H]thymidine. DNA was precipitated with 5% trichloracetic acid (p v^{-1}) at 4°C for 30 min. Precipitates were washed twice with 95% ethanol, dissolved in 1 ml of 1 M NaOH and analyzed in a liquid scintillation counter after neutralization with 1 ml of 1 M HCl. Incubations were performed in duplicate and mean values were used for calculations.

3. Results

3.1. [^{125}I]GRP binding experiments

The effects of some bombesin analogues on [^{125}I]GRP binding were investigated. Bombesin, JMV-1458, JMV-641 (Azay et al., 1996; Llinares et al., 1999), JMV-1799, and JMV-1802 were tested for their potency to inhibit specific binding of [^{125}I]GRP to rat pancreatic acini and Swiss 3T3 cells. In rat pancreatic acini, the amidated bombesin exhibited high affinity for bombesin receptor ($K_i = 1.8 \pm 0.8$ nM); the synthetic glycine-extended bombesin analogue JMV-1458 had a 10-fold lower affinity ($K_i = 15 \pm 3$ nM) (mean ± S.D. from three independent experiments performed in duplicate; Fig. 2A). However, the glycine-extended bombesin was only moderately active ($K_i = 106 \pm 10$ nM). In Swiss 3T3 cells, bombesin and JMV-1458 inhibited [^{125}I]GRP binding in a dose-dependent manner with K_i values, respectively, of 1.6 ± 0.7 and 21 ± 12 nM (mean ± S.D. from three independent experiments performed in duplicate; Fig. 2B). Again, the glycine-extended bombesin was not very potent in inhibiting [^{125}I]GRP binding to Swiss 3T3 cells ($K_i = 500 \pm 21$ nM). As shown in Table 1, the three potent bombesin receptor antagonists JMV-641, JMV-1799 and JMV-1802 were able to inhibit [^{125}I]GRP binding in a dose-dependent manner both on rat pancreatic acini and Swiss 3T3 cells with high affinities (K_i values of JMV-641, JMV-1799 and JMV-1802 were, respectively, 1.0 ± 0.1, 3.1 ± 2.1 and 3.8 ± 2.9 nM in rat pancreatic acini and 0.8 ± 0.1, 1.2 ± 0.6 and 0.8 ± 0.4 nM in Swiss 3T3 cells; mean ± S.D. from three independent experiments performed in duplicate).

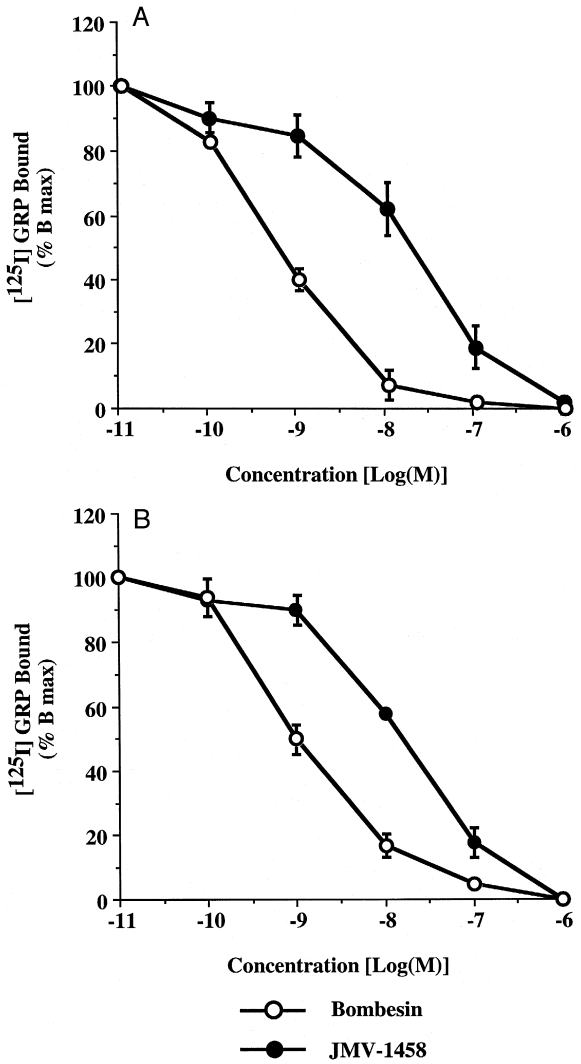


Fig. 2. Binding experiments of [^{125}I]GRP on rat pancreatic acini (A) and Swiss 3T3 cells (B). The specific binding of [^{125}I]GRP was measured in the presence of various concentrations of bombesin (○) and JMV-1458 (●). Results are the means ± S.D. of three experiments, each performed in duplicate.

3.2. Measurement of inositol phosphates production in dispersed rat pancreatic acini

It is well known that following bombesin binding, a series of early events occurs, including inositol phosphates

Table 1
K_i values of various bombesin analogues to inhibit [^{125}I]GRP binding in rat pancreatic acini and Swiss 3T3 cells. Results are the means ± S.D. of three independent experiments, each performed in duplicate

Compounds	K_i (nM)	
	Rat pancreatic acini	Swiss 3T3 cells
JMV-641	1.0 ± 0.1	0.8 ± 0.1
JMV-1799	3.1 ± 2.1	1.2 ± 0.6
JMV-1802	3.8 ± 2.9	0.8 ± 0.4

Table 2
K_i values of various bombesin receptor antagonists on inositol phosphates production induced by 10^{-7} M bombesin and 10^{-6} M JMV-1458 in rat pancreatic acini. Results are the mean ± S.D. of three independent experiments, each performed in duplicate

Compounds	K_i (nM)	
	Bombesin-induced inositol phosphates production	JMV-1458-induced inositol phosphates production
JMV-641	0.19 ± 0.16	0.08 ± 0.03
JMV-1799	2.40 ± 2.10	2.20 ± 2.10
JMV-1802	0.30 ± 0.17	0.38 ± 0.23

generation (Corjay et al., 1991; Kroog et al., 1995). We have tested the capacity of bombesin and of the synthetic glycine-extended bombesin analogue JMV-1458 to stimulate inositol phosphates production. We showed that these two compounds were able to stimulate inositol phosphates production in a dose-dependent manner (Fig. 3). The maximal stimulation was obtained with 10^{-7} M bombesin and with 10^{-6} M JMV-1458, both compounds having the same efficacy inducing the same maximal response. As compared to the basal value, 10^{-7} M bombesin and 10^{-6} M JMV-1458 produced, respectively, a 67.5 ± 2.5% and a 64.0 ± 2.9% increase of inositol phosphates production (mean ± S.D. from four independent experiments performed in duplicate). The effective concentrations producing 50% of the maximal response (EC_{50}) were, respectively, 2.3 ± 0.6 and 46 ± 38 nM for bombesin and JMV-1458 (mean ± S.D. from three independent experiments performed in duplicate).

We have tested the potency of the bombesin analogues JMV-641, JMV-1799 and JMV-1802 for their ability to stimulate the inositol phosphates production and to inhibit the inositol phosphates production induced by 10^{-7} M bombesin and by 10^{-6} M glycine-extended bombesin analogue JMV-1458. Our results indicated that none of the tested compounds affected the basal inositol phosphates production even at doses as high as 10^{-5} M. On the other hand, JMV-641, JMV-1799 and JMV-1802 were able to inhibit inositol phosphates production induced by bombesin and by the glycine-extended bombesin analogue in a dose-dependent manner, displaying the same inhibition profiles. The K_i values of these antagonists are reported in Table 2.

3.3. Amylase release test in dispersed rat pancreatic acini

As already described (Llinares et al., 1999), our results showed that bombesin was dose-dependently able to stimulate enzyme secretion from isolated rat pancreatic acini. In this study, we showed that the synthetic glycine-extended bombesin analogue JMV-1458 stimulated amylase release in a dose-dependent manner (Fig. 4). The maximal stimu-

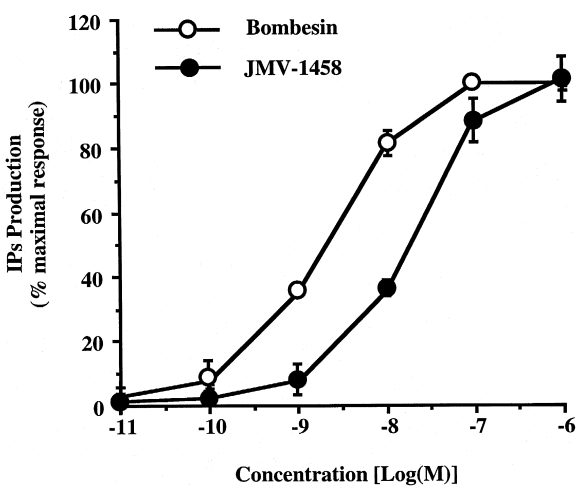


Fig. 3. Dose–response curves for bombesin (○) and JMV-1458 (●) on inositol phosphates production in dispersed rat pancreatic acini. After subtraction of the basal inositol phosphates production, data were expressed as percentage of the response obtained with 10^{-7} M bombesin or 10^{-6} M JMV-1458. For each agonist, the mean control value was 1400 dis/min with a magnitude of stimulation of 3. Results are the means ± S.D. of three experiments, each performed in duplicate.

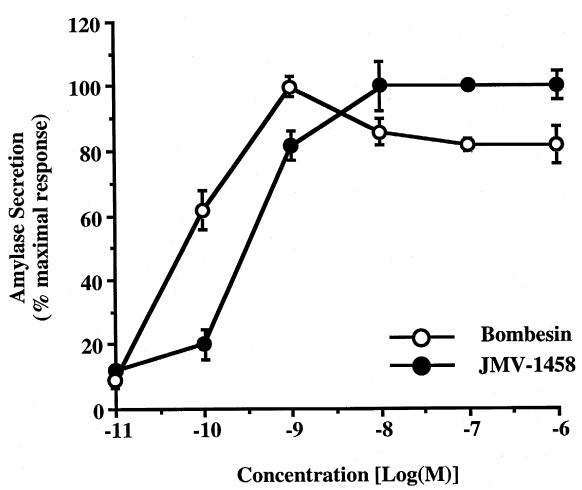


Fig. 4. Dose–response curves for bombesin (○) and JMV-1458 (●) on amylase secretion in dispersed rat pancreatic acini. After subtraction of the basal amylase secretion, data were expressed as percentage of the response obtained with 10^{-9} M bombesin and 10^{-8} M JMV-1458. For each agonist, the magnitude of stimulation was 20. Results are the means ± S.D. of three experiments, each performed in duplicate.

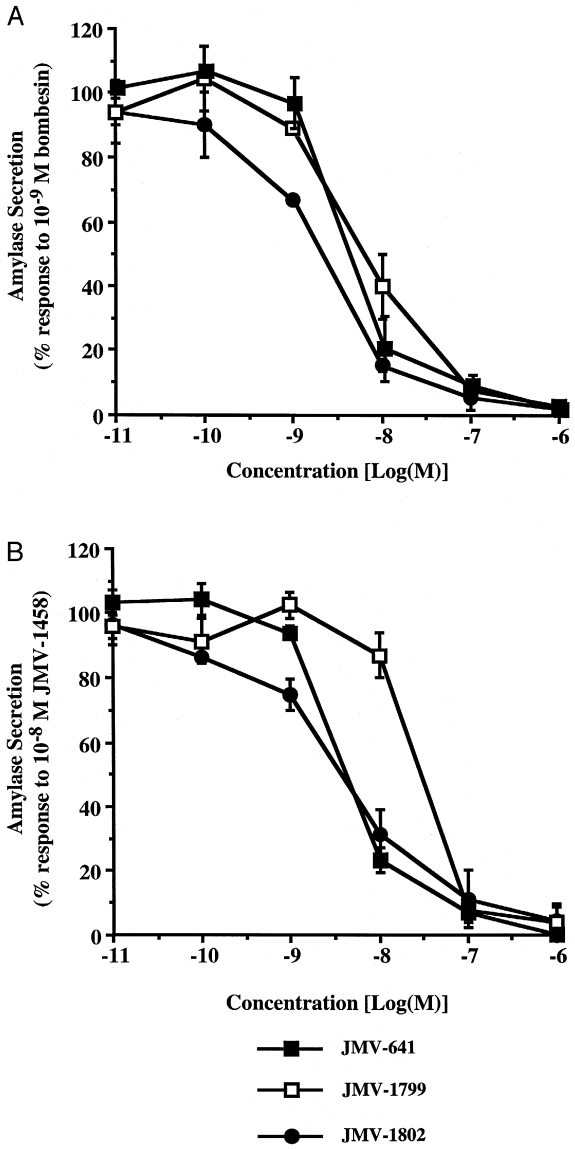


Fig. 5. Effect of JMV-641 (■), JMV-1799 (□) and JMV-1802 (●) on amylase secretion induced by 10^{-9} M bombesin (A) and 10^{-8} M JMV-1458 (B) in dispersed rat pancreatic acini. After subtraction of the basal amylase secretion, data were expressed as percentage of the response obtained with 10^{-9} M bombesin (A) and 10^{-8} M JMV-1458 (B). For each agonist, the magnitude of stimulation was 20. Results are the means ± S.D. of three experiments, each performed in duplicate.

lation was obtained with 10^{-9} M bombesin and with 10^{-8} M JMV-1458. Considering the amylase release obtained with 10^{-9} M bombesin as the reference, the JMV-1458 compound is a full agonist inducing the same maximal response. The apparent effective concentration producing 50% of the maximal amylase secretion (EC_{50}) was 0.07 ± 0.04 and 0.5 ± 0.7 nM for bombesin and JMV-1458, respectively (mean ± S.D. from three independent experiments performed in duplicate).

Compounds JMV-641, JMV-1799 and JMV-1802 were tested for their capacity to stimulate amylase release and to inhibit the amylase release induced by 10^{-9} M bombesin and 10^{-8} M JMV-1458. Our results showed that none of these compounds affected the basal amylase release even when tested at doses as high as 10^{-5} M. On the other hand, JMV-641, JMV-1799 and JMV-1802 inhibited, in a dose-dependent manner, amylase release induced by bombesin and by the glycine-extended bombesin analogue. The K_i values of JMV-641, JMV-1799 and JMV-1802 in inhibiting amylase secretion induced by bombesin were 2.1 ± 1.1, 21 ± 3 and 3.3 ± 1.1 nM, respectively (mean ± S.D. from three independent experiments performed in duplicate; Fig. 5A). The K_i values of JMV-641, JMV-1799 and JMV-1802 in inhibiting amylase secretion induced by JMV-1458 were 3.1 ± 2.2, 12 ± 5 and 2.7 ± 0.3 nM, respectively (mean ± S.D. from three independent experiments performed in duplicate; Fig. 5B).

3.4. Swiss 3T3 proliferation studies

We have tested the capacity of bombesin and the synthetic glycine-extended bombesin analogue JMV-1458 to stimulate proliferation of Swiss 3T3 cells by measuring [^{3}H]thymidine incorporation (Fig. 6). Our results showed that bombesin induced [^{3}H]thymidine incorporation in a dose-dependent manner with an EC_{50} value of 0.36 ± 0.06 nM (mean ± S.D. from three independent experiments performed in duplicate). Moreover, the glycine-extended bombesin analogue JMV-1458 was able to induce [^{3}H]thymidine incorporation in a dose-dependent manner with an EC_{50} value of 3.0 ± 1.7 nM (mean ± S.D. from four independent experiments performed in duplicate). JMV-1458 was a full agonist inducing the same maximal response than bombesin.

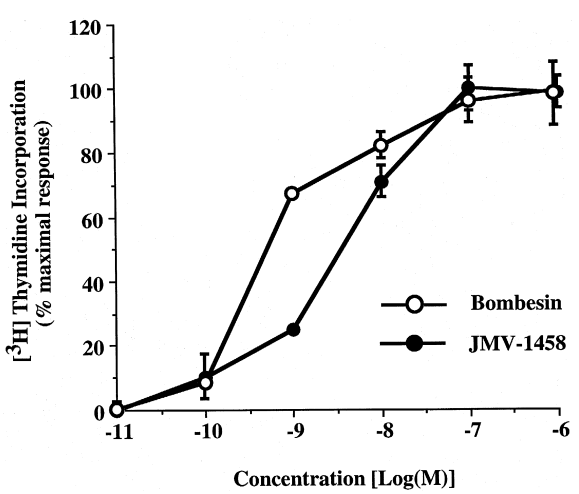


Fig. 6. Dose-response curves for bombesin (○) and JMV-1458 (●) on [^{3}H]thymidine incorporation in Swiss 3T3 cells. After subtraction of the basal [^{3}H]thymidine incorporation, data were expressed as percentage of the response obtained with 10^{-7} M bombesin and 10^{-6} M JMV-1458. For each agonist, the mean control value was 6800 dis/min with a magnitude of stimulation of 8.5. Results are the means ± S.D. of four experiments, each performed in duplicate.

Table 3
K_i values of various bombesin receptor antagonists on [^{3}H]thymidine incorporation induced by 10^{-7} M bombesin and 10^{-6} M JMV-1458 in Swiss 3T3 cells. Results are the means ± S.D. of three independent experiments, each performed in duplicate

Compounds	K_i (nM)	
	Bombesin-induced [^{3}H]thymidine incorporation	JMV-1458-induced [^{3}H]thymidine incorporation
JMV-641	0.10 ± 0.05	0.04 ± 0.01
JMV-1799	7.87 ± 1.57	4.50 ± 2.70
JMV-1802	0.47 ± 0.11	0.10 ± 0.06

We have tested compounds JMV-641, JMV-1799 and JMV-1802 for their capacity to stimulate [^{3}H]thymidine incorporation and to inhibit [^{3}H]thymidine incorporation induced by 10^{-7} M bombesin and 10^{-6} M JMV-1458 (Table 3). None of these compounds were able to stimulate [^{3}H]thymidine incorporation in Swiss 3T3 cells. However, they inhibited the [^{3}H]thymidine incorporation induced by bombesin in a dose-dependent manner with K_i values, respectively, of 0.10 ± 0.05, 7.87 ± 1.57 and 0.47 ± 0.11 nM (mean ± S.D. from three independent experiments performed in duplicate). We also demonstrated that these compounds dose-dependently inhibited JMV-1458-induced [^{3}H]thymidine incorporation with K_i values, respectively, of 0.04 ± 0.01, 4.50 ± 2.70 and 0.10 ± 0.06 nM (mean ± S.D. from three independent experiments performed in duplicate).

4. Discussion

On the basis of the results obtained by some authors on glycine-extended forms of gastrin (Seva et al., 1994; Singh et al., 1995), we have tried to generalize this concept of obtaining active glycine-extended forms of other amidated peptides. In this study, we presented the behaviour of a synthetic glycine-extended bombesin analogue (compound JMV-1458) on various biological effects.

Binding experiments indicated that compound JMV-1458 inhibited specific [^{125}I]GRP binding in rat pancreatic acini and in Swiss 3T3 cells with K_i values of approximately 10^{-8} M. These results indicate that JMV-1458 interacts with the classical GRP/bombesin receptor in rat pancreatic acini and in Swiss 3T3 cells. These binding results differs from those obtained by Seva et al. (1994) or Singh et al. (1995) in the sense that they showed that glycine-extended gastrin (2–17) occupies a different binding site than gastrin (2–17). We have already shown that JMV-1458 is specific for the GRP/bombesin receptor subtype. This compound presents a weak affinity (in the micromolar range) for both neuromedin-B and bombesin BB_3 receptor subtypes (data not shown). However, the glycine-extended form of bombesin was only moderately active at the GRP/bombesin receptor.

To study JMV-1458 behaviour in more details, we have tested its ability to induce inositol phosphates production and amylase release in rat pancreatic acini and to stimulate DNA synthesis in Swiss 3T3 cells.

In rat pancreatic acini, we have demonstrated that bombesin and the synthetic glycine-extended bombesin analogue JMV-1458 dose-dependently induced inositol phosphates production and amylase secretion. Compound JMV-1458 was about 10 times less potent that bombesin. Like bombesin, we showed that glycine-extended bombesin analogue JMV-1458 stimulated [^{3}H]thymidine incorporation in a dose-dependent manner in Swiss 3T3 cells. We found an EC_{50} value of approximately 10^{-9} M for the glycine-extended bombesin analogue. In the same cellular model, Mervic et al. (1991) showed that the concentration of bombesin-Gly required to half maximally stimulate [^{3}H]thymidine uptake was 1300 nM. In view of these results, we can hypothesize that the N-terminal modification included in compound JMV-1458 conferred to this glycine-extended bombesin analogue a better affinity for the GRP/bombesin receptor.

Carboxyamidation is a key event in the biosynthetic maturation of peptides. The C-terminal amide function has been shown in a large variety of amidated peptide hormones to be crucial for the expression of biological activity (Hilsted and Rehfeld, 1986). This is particularly true for gastrin (Martinez et al., 1986) and GRP/bombesin (Heimbrook et al., 1989), where suppression of the C-terminal amide resulted in potent antagonist compounds. We have shown in this study that a synthetic glycine-extended bombesin analogue was able to induce, with high potency, the same biological effects than bombesin, its amidated counterpart, on various models. However, whereas glycine-extended forms of gastrin seem to interact with binding sites different from that of gastrin (Seva et al., 1994; Singh et al., 1995), in our case, the glycine-extended analogue of bombesin seems to interact with the same binding sites than the natural amidated peptide. To assess this assumption, we have studied the inhibition profiles of JMV-641, JMV-1799 and JMV-1802 (three potent bombesin receptor antagonists) on inositol phosphates production and amylase release from rat pancreatic acini and on [^{3}H]thymidine incorporation in Swiss 3T3 cells induced by bombesin and JMV-1458. We first tested the capacities of JMV-641 (Azay et al., 1996; Llinares et al., 1999), JMV-1799 and JMV-1802 to inhibit [^{125}I]GRP binding on rat pancreatic acini and on Swiss 3T3 cells. We showed that JMV-641, JMV-1799 and JMV-1802 interacted with the GRP/bombesin receptor with high affinities. These compounds were then tested for their ability to inhibit bombesin-induced inositol phosphates production and amylase release in rat pancreatic acini and for their capacity to inhibit bombesin-induced [^{3}H]thymidine incorporation in Swiss 3T3 cells. On these various models, we

showed that JMV-641, JMV-1799 and JMV-1802 were very potent GRP/bombesin receptor antagonists. We decided to use these compounds to test the implication of the GRP/bombesin receptor on JMV-1458-induced inositol phosphates production and amylase secretion in rat pancreatic acini and on JMV-1458-induced [^{3}H]thymidine incorporation in Swiss 3T3 cells. Our results showed that compounds JMV-641, JMV-1799 and JMV-1802 were very potent in inhibiting the biological effects induced by the glycine-extended analogue of bombesin.

Whatever the agonist tested (bombesin or the glycine-extended analogue of bombesin JMV-1458), we found that the bombesin receptor antagonists JMV-641, JMV-1799 and JMV-1802 inhibited, in a dose-dependent manner, inositol phosphates production and amylase secretion in rat pancreatic acini, as well as [^{3}H]thymidine uptake in Swiss 3T3 cells. In each experiment, these antagonists displayed the same inhibition profile showing similar K_i values. In view of these results, we concluded that the synthetic glycine-extended bombesin analogue JMV-1458 exerted its biological activities by interacting with the classical GRP/bombesin receptor.

In summary, although it was described that carboxyamidation was essential for biological activity of peptide hormones, our results showed that a synthetic analogue of the glycine-extended bombesin was able to induce inositol phosphates production and amylase secretion in rat pancreatic acini and [^{3}H]thymidine accumulation in Swiss 3T3 cells with high potency. We also concluded that an amidated C-terminal residue is not essential for obtaining high affinity and potency in bombesin. Moreover, we showed that biological effects induced by this glycine-extended bombesin analogue were antagonized by very potent GRP/bombesin antagonists, suggesting that compound JMV-1458 interacts with the classical GRP/bombesin receptor.

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