Bombesin, but not amylin, blocks the orexigenic effect of peripheral ghrelin

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Bombesin, but not amylin, blocks the orexigenic effect of peripheral ghrelin. Am J Physiol Regul Integr Comp Physiol 291: R903–R913, 2006. First published April 27, 2006; doi:10.1152/ajpregu.00681.2005.—The interaction between ghrelin and bombesin or amylin administered intraperitoneally on food intake and brain neuronal activity was assessed by Fos-like immunoreactivity (FLI) in nonfasted rats. Ghrelin (13 μg/kg ip) increased food intake compared with the vehicle group when measured at 30 min (g/kg: 3.66 ± 0.80 vs. 1.68 ± 0.42, P < 0.0008). Bombesin (8 μg/kg) injected intraperitoneally with ghrelin (13 μg/kg) blocked the orexigenic effect of ghrelin (1.18 ± 0.41 g/kg, P < 0.0002). Bombesin alone (4 and 8 μg/kg ip) exerted a dose-related nonsignificant reduction of food intake (g/kg: 1.08 ± 0.44, P > 0.45 and 0.55 ± 0.34, P > 0.16, respectively). By contrast, ghrelin-induced stimulation of food intake (g/kg: 3.96 ± 0.56 g/kg vs. vehicle 0.82 ± 0.59, P < 0.004) was not altered by amylin (1 and 5 μg/kg ip; g/kg: 4.37 ± 1.12, P > 0.69, and 3.01 ± 0.78, respectively, P > 0.37). Ghrelin increased the number of FLI-positive neurons/section in the arcuate nucleus (ARC) compared with vehicle (median: 42 vs. 19, P < 0.008). Bombesin alone (4 and 8 μg/kg ip) did not induce FLI neurons in the paraventricular nucleus of the hypothalamus (PVN) and coadministration with ghrelin did not alter ghrelin-induced FLI in the ARC. However, bombesin (8 μg/kg) with ghrelin significantly increased neuronal activity in the PVN approximately threefold compared with vehicle and ~1.5-fold compared with the ghrelin group. Bombesin (8 μg/kg) with ghrelin injected intraperitoneally induced Fos expression in 22.4 ± 0.8% of CRF-immunoreactive neurons in the PVN. These results suggest that peripheral bombesin, unlike amylin, inhibits peripheral ghrelin induced food intake and enhances activation of CRF neurons in the PVN.

c-fos; food intake

IN THE LAST DECADES, VARIOUS peptide hormones from the gastrointestinal (GI) tract have been identified to play a role in the short-term regulation of food intake and satiety by altering the initiation and termination of meals (62). We previously demonstrated in rats that cholecystokinin-8S (CCK) abolished the orexigenic effect of peripherally administered ghrelin and blocked activation of ghrelin sensitive neurons in the hypothalamic arcuate nucleus (ARC) (30). In addition, peripheral interaction of ghrelin with CCK on feeding regulation was no longer observed in rats with disrupted CCKA receptor (18).

Bombesin is a tetradecapeptide that was first isolated in 1971 from the amphibian skin of a European toad Bombina bombina (1). In mammals, several bombesin-like peptides exist with structural homology to bombesin, including the mammalian peptides, gastrin-releasing peptide (GRP), and neuropeptide B (43). Bombesin binds with high affinity to GRP (BB2) and neuropeptide B (BB3) receptors, which are widely spread in the GI tract and also in the central nervous system (43). Bombesin-like peptides are released in the gastric mucosa in response to nutrients (56). It is well established that peripheral or central administration of bombesin or GRP suppresses food intake through interactions with both BB1 and BB2 receptors in a variety of species, including humans (19, 23, 32, 41, 43, 64, 69).

Another satiety peptide released from the GI tract is amylin, a 37-amino acid peptide produced by pancreatic β-cells, which is secreted into the blood stream after food intake (50). Peripheral administration of amylin suppresses food intake in rats and mice (9, 20, 38, 45, 75). Convergent studies established that amylin-induced decreased feeding behavior involves neurons of the area postrema (AP) (39), a circumventricular organ of the brain stem (40), in which amylin receptors are located in high density (58), whereas afferent vagal nerve fibers do not play a role (36).

Ghrelin is a 28-amino acid peptide hormone mainly produced in X/A-like cells in the mucosal layer of the stomach (16, 31, 73). Ghrelin is the first gut-brain peptide being identified to stimulate food intake in rats (46, 66, 71, 72), mice (4, 67), and humans (70). The potent orexigenic effect of peripheral administration of ghrelin (46, 66, 71, 72), the rise in plasma ghrelin levels induced by food deprivation, weight loss or before a meal (14, 66), and the reduction of feeding in response to central administration of growth hormone secre-

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tagogue receptor antagonist (3) in rodents implicate endogenous ghrelin in the initiation of food intake (13). In sharp contrast to ghrelin, bombesin elicits anorexigenic effects and is involved in meal termination (19, 23, 32, 41, 64, 69). Recent studies indicate that vagal afferents-dependent mechanisms may be involved in peripheral injection of ghrelin-induced increase in food intake and Fos-like immunoreactivity (FLI) in the ARC, as demonstrated by the attenuation of the spontaneous feeding behavior induced by ghrelin after surgical deafferentation of the vagal nerve (17). This contrasts with the inhibitory effect of bombesin on food intake that is mainly mediated by spinal afferent nerve fibers (42) and is not solely dependent on afferent vagal nerve fiber integrity (21, 61).

Ghrelin, bombesin, and amylin injected peripherally induce neuronal activation in different brain areas involved in regulation of satiety and hunger, as shown by the induction of the immediate early gene product Fos, a marker indicating neuronal activation in a specific brain area. Ghrelin administered intraperitoneally induces c-fos expression in the ARC in rats and mice (25, 30, 67) and in the paraventricular nucleus of the hypothalamus (PVN) in rats (30, 52). Double labeling showed that peripheral injection of ghrelin activates neurons containing the orexigenic peptides neuropeptide Y (NPY)/Agouti-related protein present in the ARC (67).

Peripheral injection of bombesin induces FLI in the PVN, nucleus tractus solitarius (NTS), and the AP (7, 34). In this context, it is of interest that intracerebroventricular administration of bombesin influences the hypothalamic corticotropin releasing factor (CRF) system as shown by the stimulation of CRF release in the hypothalamus (27, 28, 47). Activation of hypothalamic CRF plays a key role in the endocrine and anxiogenic responses to stress (5) as well as in other behavioral alterations, including the inhibition of food intake (12, 24, 59). A main source of hypothalamic CRF is neurons in the PVN (54).

Peripheral administration of amylin induces c-fos expression in neurons of the AP, NTS, lateral parabrachial nucleus, and in the central nucleus of the amygdala (49) where numerous reciprocal projections exist. Riediger et al. (49) reported that fasting in rats increased Fos-positive neurons in the lateral hypothalamus that are reduced by peripheral administration of amylin (49).

Collectively these findings provide the framework for an antagonistic interaction between ghrelin and bombesin or amylin to regulate food intake. Therefore, in the present study, we investigated whether peripheral bombesin or amylin modulate the orexigenic effects of peripheral ghrelin in freely fed male rats. At first, we examined whether bombesin and amylin alter dose-dependently the orexigenic effect of ghrelin. Furthermore, after simultaneous injection of ghrelin and bombesin, we investigated the pattern of c-fos expression in the PVN, ARC, and NTS. Brain nuclei were selected based on the presence of growth hormone secretagogue receptors, the cognate receptor for ghrelin (ARC, PVN) (22), and GRP (BB2)-receptors (NTS, PVN) for bombesin-induced suppression of food intake (43). Additionally, the neuropeptideergic phenotype of Fos-positive neurons in the PVN was analyzed after simultaneous administration of ghrelin and bombesin in relation with the activation of CRF neurons.

**Materials and Methods**

**Animals**

Male Sprague-Dawley rats (Harlan-Winkelmann, Borchen, Germany) weighing 250–300 g were housed in groups of four rats per cage under conditions of controlled illumination (12:12-h light-dark cycle, lights on/off: 6:30 AM/6:30 PM), humidity, and temperature (22 ± 2°C). Animals were fed with a standard rat diet (Altromin, Lage, Germany) and tap water ad libitum. All animals were trained daily to be accustomed to the experimental conditions for 14 days before starting the experiments. During the handling phase, the back position was practiced to make animals familiar with receiving an intraperitoneal injection. Furthermore, every day, animals were removed from their social group for 2 h and set in single cages. This period of social isolation mimics the time schedule of the experiments. Animals used for neuronal mapping of FLI were handled daily for 5 days before the start of the experiment and similarly trained to be held in the back position for intraperitoneal injection.

Animal care and experimental procedures followed institutional ethical guidelines and conformed to the requirements of the state authority for animal research conduct under the protocol G0089/02.

**Pepitide Preparation**

Rat ghrelin and bombesin (Bachem, Heidelberg, Germany) were dissolved in distilled water and stored at −20°C. Rat amylin (Bachem) was dissolved in 0.1% trifluoroacetic acid (Merck, Darmstadt, Germany). Immediately before starting the experiments, peptides were diluted in vehicle solution consisting of sterile 0.15 M NaCl (Braun, Melsungen, Germany) to reach the final concentration of 13 μg/kg (~4 nmol/kg) for ghrelin, 4 or 8 μg/kg for bombesin (~2.5 or 5 nmol/kg), and 1 or 5 μg/kg (~0.3 or 1.5 nmol/kg) for amylin. Peptide solutions were kept on ice for the duration of the experiments.

**Experimental Protocols**

If not otherwise stated, all experiments were performed at the same time of the day (between 8:00 and 11:00 AM), at 1.5 to 4.5 h after the start of the light cycle in freely fed rats to achieve maximum consistency. At that time period freely fed rats have their lowest food intake.

Effects of ghrelin and bombesin injected intraperitoneally singly or in combination on food intake. Rats were injected simultaneously (final volume: 0.5 ml ip) with vehicle plus vehicle (0.15 M NaCl + 0.15 M NaCl, n = 20), ghrelin plus vehicle (13 μg/kg + 0.15 M NaCl, n = 20), vehicle plus bombesin [0.15 M NaCl + 4 (n = 15) or 8 (n = 15) μg/kg], or ghrelin plus bombesin [13 μg/kg + 4 (n = 15) or 8 (n = 15) μg/kg] and returned to single housing cages immediately after the injection. Thereafter, preweighed rat chow was made available to the animals. Food intake was determined by measuring the difference between the preweighed standard chow and the weight of chow at the end of the first 30-min, 1-h, and 2-h periods of food exposure. Doses of peptides selected were based on previous studies (30, 42).

**Effects of ghrelin and amylin injected intraperitoneally singly or in combination on food intake.** The experimental protocol was performed as described in the first experiment. Freely fed rats were simultaneously injected intraperitoneally (final volume: 0.5 ml) with vehicle plus vehicle (0.15 M NaCl + 0.15 M NaCl, n = 10), ghrelin plus vehicle (13 μg/kg + 0.15 M NaCl, n = 10), vehicle plus amylin [0.15 M NaCl + 1 (n = 10) or 5 (n = 10) μg/kg], or ghrelin plus amylin [13 μg/kg + 1 (n = 10) or 5 (n = 10) μg/kg] and returned to single housing cages immediately after the injection. Thereafter, preweighed rat chow was made available to the animals. Food intake was determined by measuring the difference between the preweighed standard chow and the weight of chow at the end of the first 30 min, 1 h, and 2 h of food exposure. Doses of amylin were based on previous studies (35, 36, 40).
Effects of 5 μg amylin on food intake injected intraperitoneally at the beginning of the dark phase. This control experiment was done to demonstrate the positive action of 5 μg amylin/kg ip to reduce food intake in freely fed rats under standard experimental conditions. Freely fed rats were injected intraperitoneally (final volume: 0.5 ml) with 5 μg amylin/kg (n = 9) or with vehicle solution (0.15 M NaCl, n = 9) 10 min before the dark phase started. Immediately after the injection, animals were returned to single housing cages. Thereafter, preweighed rat chow was made available to the animals. Food intake was determined by measuring the difference between the preweighed standard chow and the weight of chow at the end of the first 30 min and 1 h food exposure.

Effects of ghrelin and bombesin injected singly or in combination on c-Fos-like-immunoreactivity in hypothalamic and brain stem nuclei. Freely fed rats were injected intraperitoneally (final volume 0.5 ml) with vehicle plus vehicle (0.15 M NaCl + 0.15 M NaCl, n = 5), ghrelin plus vehicle (13 μg/kg + 0.15 M NaCl, n = 5), vehicle plus bombesin (0.15 M NaCl + 4 (n = 5) or 8 (n = 5) μg/kg), or ghrelin plus bombesin (13 μg/kg + 4 (n = 5) or 8 (n = 5) μg/kg). Immediately after injection, animals were deprived of food to avoid an influence of ghrelin-induced increase in food intake on Fos expression in the brain but had ad libitum access to water (67). At 90 min after the intraperitoneal injection, animals were deeply anesthetized with intraperitoneal injections of 100 mg/kg ketamine (Ketanest; Curamed, Karlsruhe, Germany) and 10 mg/kg xylazine (Rompun 2%; Bayer, Leverkusen, Germany) and heparinized with 2.500 U heparin ip (Liquemin; Hoffmann-La Roche, Grenzach-Whylen, Germany). Transcardial perfusion, performed as described before (29), consisted of a 10-s flush of a plasma substitute (Longasteril 70; Fresenius, Bad Homburg, Germany) followed by a mixture of 4% wt/vol paraformaldehyde, 0.05% vol/vol glutaraldehyde, and 0.2% vol/vol picric acid in 0.1 M phosphate buffer, pH 7.4, for 30 min and ended with a 5% wt/vol sucrose solution for 5 min. After dissection, brains were kept in a 5% wt/vol sucrose solution overnight and then cut into 1.0 to 4.5 mm coronal blocks enclosing the respective hypothalamic and brain stem regions using a Plexiglas brain matrix. For cryoprotection, blocks were moved through a sucrose gradient (15% wt/vol and 27.3% wt/vol), then shock-frozen in hexane at −70°C, and stored at −80°C until further processing.

**Immunohistochemistry**

Staining for FLI. First, 25 μm free-floating brain sections were pretreated with 1% wt/vol sodium borohydride (in PBS) for 15 min. Subsequently, sections were incubated in a solution containing 5% wt/vol BSA and 0.3% vol/vol Triton X-100 in PBS for 60 min for blockade of unspecific antibody binding. Thereafter, the diluted primary antibody solution (rabbit anti-rat Fos protein, Oncogene Research Products, Boston, MA; 1:4,000 in a solution of 5% wt/vol BSA, 0.3% vol/vol Triton X-100, and 0.1% wt/vol sodium azide in PBS) was applied for 36 h at room temperature. After rinsing sections in PBS three times and incubation in a solution containing 5% wt/vol BSA and 0.3% vol/vol Triton X-100 for 60 min, FITC-labeled goat-anti-rabbit IgG (Sigma, St. Louis, MO) was applied for 12 h at room temperature in an appropriate dilution (1:800 in 5% wt/vol BSA in PBS). Sections were rinsed again three times in PBS and incubated in a solution containing 5% wt/vol BSA, 3% wt/vol rabbit normal serum, and 0.3% vol/vol Triton X-100 for 5 h. Then, tetramethylrhodamine isothiocyanate-labeled rabbit-anti-guinea pig IgG (Sigma) was applied for 12 h at room temperature (1:200 in 5% wt/vol BSA, 3% wt/vol rabbit normal serum, 0.3% vol/vol Triton X-100, and 0.1% vol/vol sodium azide in PBS).

Sections were rinsed in PBS three times again, then embedded in anti-fading solution and analyzed using a confocal laser scanning microscope.

**Data and Statistical Analysis**

**Immunohistochemistry.** Semiquantitative assessment of FLI was achieved by counting the number of FLI-positive cells as described before (30). Cells with green nuclear staining were considered FLI-positive. Every second of all consecutive coronal 25-μm sections was counted for FLI-positive staining bilaterally in the ARC, PVN, and NTS throughout their rostrocaudal extent. Anatomic correlations were made according to landmarks given in Paxinos and Watson’s stereotaxic atlas (48). FLI-positive cells were counted in 10 sections per rat of the PVN, and 15 sections per rat of the ARC and NTS. The investigator counting the number of Fos-positive cells was blinded to treatments received by the animals. The average number of FLI-positive cells per section for the brain nuclei mentioned above was calculated for each rat. Data are expressed as median and interquartile ranges of the average number of cells/section. Differences between groups were evaluated by the nonparametric Kruskal-Wallis-ANOVA median test and Mann-Whitney U-tests; P < 0.05 was considered significant.

Semiquantitative assessment of FLI and CRF-like immunoreactivity double staining was achieved by counting the total number of Fos and CRF-positive neurons in the PVN. Thereafter, the percentage of activated, i.e., Fos-positive, CRF neurons and the percentage of CRF-positive Fos neurons for all treatment groups (n = 3 per group) was calculated. Data are presented as means ± SE.

**Feeding experiments.** The cumulative food intake monitored at the end of the first 30-min, 1-h, and 2-h periods after peptide or vehicle injection was expressed as food intake g/body wt (30). Data are expressed as means ± SE and analyzed by ANOVA. Differences between groups were evaluated by the least significant difference test; P < 0.05 was considered significant.

The cumulative food intake in the control experiment with 5 μg amylin/kg ip monitored at the end of the first 30-min and 1-h periods after peptide administration in the dark phase was also expressed as food intake (g/body wt) (kg). Data are expressed as means ± SE, and differences between both groups were evaluated by Student’s t-test; P < 0.05 was considered significant.

**RESULTS**

**Effects of Ghrelin and Bombesin Injected Intraperitoneally Singly or in Combination on Food Intake**

Within the first half hour, ghrelin (13 μg /kg ip) significantly increased food intake compared with the intraperitoneal vehicle plus vehicle group (g/kg: 3.66 ± 0.80 vs. 1.68 ± 0.42; P <
BOMBSIN BLOCKS THE OREXIGENIC EFFECT OF GHRELIN

Fig. 1. Effects of ghrelin, bombesin, and ghrelin plus bombesin on 2-h food intakes. Freely fed rats were injected intraperitoneally with vehicle, bombesin (4 or 8 μg/kg), ghrelin (13 μg/kg) plus bombesin (4 or 8 μg/kg), or ghrelin (13 μg/kg) and cumulative food intake (expressed as g/kg body wt) was measured at 0.5, 1, and 2 h. Data are expressed as means ± SE of number of rats indicated in parenthesis. *P < 0.05 compared with vehicle + vehicle or as represented.

Fig. 2. Effects of ghrelin, amylin, and ghrelin plus amylin on 2-h food intakes. Freely fed rats were injected intraperitoneally with vehicle, amylin (1 or 5 μg/kg), ghrelin (1 or 10 μg/kg), or ghrelin (13 μg/kg) and cumulative food intake (expressed as g/kg body wt) was measured at 0.5, 1, and 2 h. Data are expressed as means ± SE of number of rats indicated in parenthesis. *P < 0.05.

Effects of Ghrelin and Amylin Injected Intraperitoneally Singly or in Combination on Food Intake

Within the first half hour, ghrelin (13 μg/kg ip) significantly increased food intake compared with the intraperitoneal vehicle plus vehicle group (g/kg: 3.96 ± 0.56 vs. 0.82 ± 0.59; P < 0.004; Fig. 2). Amylin injected intraperitoneally at 1 or 5 μg/kg simultaneously with ghrelin (13 μg/kg) did not alter the hyperphagia response to ghrelin (g/kg: 4.37 ± 1.12, P > 0.69 and 3.01 ± 0.78, P > 0.37, respectively, Fig. 2). Amylin injected intraperitoneally alone at 1 or 5 μg/kg did not significantly alter food intake during this time period compared with the vehicle plus vehicle group (g/kg: 1.07 ± 0.63, P > 0.81 and 0.93 ± 0.58, respectively, P > 0.91; Fig. 2). At 1 and 2 h after ghrelin intraperitoneal administration, we observed significant changes in cumulative food intake only between the ghrelin group and both doses of amylin alone (Fig. 2). Furthermore, both doses of amylin alone showed a nonsignificant trend to decrease food intake during these time intervals compared with the vehicle plus vehicle group (Fig. 2).

Effect of 5 μg Amylin on Food Intake Injected Intraperitoneally at the Beginning of the Dark Phase

Amylin injected intraperitoneally alone at 5 μg/kg resulted in a statistically nonsignificant reduction in food intake during the first half hour compared with the vehicle-treated group (7.01 ± 0.89 vs. 9.25 ± 0.8 g/kg, P > 0.09; Fig. 3). One hour after amylin injection, the cumulative food intake was significantly decreased in the amylin group compared with the control group (7.01 ± 0.89 vs. 10.06 ± 0.6 g/kg, P < 0.016; Fig. 3).

Effects of Ghrelin and Bombesin Injected Intraperitoneally Singly or in Combination on c-fos Expression in Hypothalamic and Medullary Nuclei

ARC. Ghrelin injected intraperitoneally induced a significant increase of FLI in ARC neurons compared with the vehicle plus vehicle group (median of FLI-positive neurons per section: 42 vs. 19, P < 0.008; Fig. 4). Bombesin injected alone (4 or 8 μg/kg ip) did not modify Fos expression in ARC neurons compared with vehicle group (Fig. 4).
Fig. 3. Effect of amylin on 1-h food intake. Freely fed rats were injected intraperitoneally with 5 μg amylin/kg or vehicle solution immediately at the beginning of the dark phase. Amylin induced a significant decrease in cumulative food intake (expressed as g/kg body wt) after 1 hour compared with vehicle. Data are expressed as means ± SE of number of rats indicated in parenthesis. *P < 0.05 compared with vehicle.

At both doses of bombesin (4 and 8 μg/kg) injected simultaneously, ghrelin (13 μg/kg) also induced a significant rise in the number of Fos-labeled cells in ARC neurons compared with the vehicle group (median: 36, P < 0.008 and median: 31, P < 0.008, respectively; Fig. 4).

Paraventricular nucleus. A combination of bombesin at a dose of 8 μg/kg ip, and ghrelin significantly increased the number of FLI-positive neurons in the PVN 4.2-fold compared with the vehicle group (median: 113 vs. 28, respectively, P < 0.008; Figs. 5 and 6), and all other groups including ghrelin plus vehicle (Fig. 6). Ghrelin injected intraperitoneally alone significantly increased the number of FLI-positive neurons in the PVN ~0.6-fold compared with vehicle (median: 45, P < 0.008; Fig. 6). Interestingly, simultaneous intraperitoneal injection of ghrelin and bombesin at 4 μg/kg also increased neuronal activity significantly ~0.7-fold compared with the vehicle group (median: 49, P < 0.008; Fig. 6). Bombesin injected alone (4 or 8 μg/kg ip) did not have a significant effect on neuronal activation in the PVN (median: 31, P > 0.31; and median: 33, respectively P > 0.55; Fig. 6).

Double immunohistochemical staining for Fos and CRF in ghrelin (13 μg/kg ip)- plus bombesin (8 μg/kg ip)-treated rats revealed that the majority of FLI-positive neurons in the PVN were positive for CRF-like immunoreactivity (59.3 ± 4.4%; Figs. 7 and 8A). The simultaneous peripheral administration of bombesin and ghrelin at this doses induced Fos expression in 22.4 ± 0.8% of CRF-immunoreactive neurons in the PVN (Fig. 8B). Furthermore, we observed that 45.4 ± 10.6% of Fos neurons in the PVN induced by ghrelin (13 μg/kg ip) alone were also CRF positive (Fig. 8A). Intraperitoneal ghrelin at this dose induced Fos expression in 10.3 ± 1.9% of CRF-immunoreactive neurons in the PVN (Fig. 8B).

Nucleus of the solitary tract. In the NTS, bombesin (8 μg/kg) injected intraperitoneally alone or with ghrelin (13 μg/kg) increased the number of FLI-positive or neuronal ~1.5-fold compared with the vehicle group (Fig. 9). All other treatments did not have any significant effect on c-fos expression in the NTS (Fig. 9).

Fig. 4. Effects of ghrelin, bombesin, and ghrelin plus bombesin on the number of Fos-like immunoactivity (FLI)-positive neurons in the arcuate nucleus (ARC) of the hypothalamus. Freely fed rats (n = 5) were injected intraperitoneally with vehicle (0.15 M NaCl), bombesin (4 or 8 μg/kg), ghrelin (13 μg/kg), or ghrelin plus bombesin (13 μg/kg plus 4 or 8 μg/kg), and were euthanized 90 min later to process brains for c-Fos-like immunoreactivity (c-FLI). Data are presented by using box and whiskers plots. The upper hinge of the box represents the 75th and the lower one the 25th percentile. The median is shown as a line across the box. The whiskers above and below the boxes represent the largest and smallest observed scores. *P < 0.05 vs. vehicle, bombesin 4 or 8 μg/kg. n, number of rats.

Multiple GI peptides are involved in the regulation of ingestive behavior in mammals (62). Current knowledge of the interactions between peripheral peptide signals is limited, although synergistic interaction has been demonstrated between gut signals inhibiting food intake (6). In the present study, we provide evidence that peripheral bombesin, when administered simultaneously with ghrelin, blocks the orexigenic action of intraperitoneal-administered ghrelin in freely fed rats previously accustomed to the experimental conditions. In contrast, peripheral amylin did not influence the orexigenic effect of ghrelin under the same conditions. These findings suggest that interactions between GI peptides involved in the regulation of food intake are peptide specific. In parallel with these behavioral observations, intraperitoneal ghrelin plus bombesin, but not intraperitoneal amylin, affects neuronal activity in the hypothalamus. In particular, we found that the increase of FLI in the PVN was 1.5-fold more pronounced after coadministration of ghrelin plus bombesin compared with that of ghrelin alone, while bombesin injected singly had no effect on neuronal activity in this brain nucleus.

Ghrelin (13 μg/kg ip) stimulated feeding behavior in freely fed rats resulted in a more than 100% increase in the amount of food ingested during the first 30 min after peripheral injection of the peptide. The response lasted for 1 h and was no longer observed after 2 h, consistent with the rapid and short-live orexigenic action of peripherally injected ghrelin in rodents and humans and its function in short-term regulation of feeding (30, 46, 66, 67, 71, 72). In addition, we observed that coinjection of bombesin (8 μg/kg ip) inhibited ghrelin-induced increase in food intake within the first 30 min. The inhibitory effect was long lasting, as shown by significantly reduced food
intake in the bombesin plus ghrelin group compared with vehicle plus ghrelin throughout the 2-h experimental period.

We previously demonstrated a comparable long-lasting inhibition of ghrelin-induced food intake by simultaneous peripheral injection of CCK (30) that was recently confirmed by other investigators (18). The specificity of the inhibitory action of bombesin on ghrelin-induced stimulation of feeding is suggested by the present finding that amylin (1 and 5 μg/kg ip) also administered simultaneously with ghrelin, did not diminish the rise in food intake. The failure to detect interaction between the peptides’ effects on food intake and neuronal activity by combined intraperitoneal injection of ghrelin and amylin indicates that peripheral bombesin and amylin unfold their satiety effects via different pathways and neuronal brain networks.

Convergent evidence suggests that amylin acts predominantly via the AP/NTS to reduce food intake, without involvement of peripheral afferent neural transmission (50). This is supported by the high density of amylin receptors in this hindbrain area (58) and increased c-fos expression in the AP

Fig. 5. Effects of ghrelin, bombesin, and ghrelin plus bombesin on Fos-like immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN). Animals injected intraperitoneally with vehicle plus vehicle (A and D), vehicle plus bombesin 4 μg/kg (B and E), or vehicle plus bombesin 8 μg/kg (C and F) showed a moderate induction of Fos expression (green staining) in the PVN. In contrast, the density of Fos expression was increased after simultaneous treatment with 13 μg ghrelin/kg plus vehicle (G and J), 13 μg ghrelin/kg plus bombesin 4 μg/kg (H and K), and with ghrelin plus 8 μg/kg ip bombesin (I and L). Cell nuclei are stained red as a result of the counterstaining with propidium iodide. The white line delineates the area of the PVN in accordance with landmarks from in the Paxinos and Watson rat brain atlas (48). Bar = 100 μm. 3V, third ventricle.
and NTS induced by peripheral injection of amylin (49, 51). It has also been found that peripheral injection of bombesin, as well as of CCK and amylin induces c-fos expression in lateral parabrachial nucleus neurons (26, 49). In contrast to bombesin or CCK, amylin does not induce any increase in c-fos expression in the PVN (51), although this hypothalamic nucleus receives direct projections from the NTS (55). Therefore, it can be speculated that bombesin and CCK stimulate other cell groups in the NTS besides those stimulated by amylin. This might explain why, in the present study, amylin did not influence ghrelin-induced food intake at simultaneous peripheral administration of both peptides.

The doses of amylin (1 and 5 μg/kg) used in the present study did not significantly alter food intake in nonfasted rats under these experimental conditions. However, these doses were reported to be efficient to decrease food intake in food-deprived rats (35, 36). Interestingly, the experimental study with amylin (5 μg/kg ip) alone injected immediately before the dark phase started revealed a significant reduction of food intake. Therefore, it is possible that the time point of peptide injection is of importance for the action of amylin to reduce food intake in rats. Morley et al. (44) have shown that a dose of 25 μg amylin/kg ip is required to reduce food intake in

Fig. 6. Effects of ghrelin, bombesin, and ghrelin plus bombesin on the number of FLI-positive neurons in the PVN. Freely fed rats (n = 5) were injected intraperitoneally with vehicle (NaCl), bombesin (4 or 8 μg/kg), ghrelin (13 μg/kg), or ghrelin plus bombesin (13 μg/kg plus 4 or 8 μg/kg) and were euthanized 90 min later to process brains for c-FLI. The data are presented by using box and whiskers plots, as in Fig. 4. *P < 0.05 vs. vehicle plus bombesin (4 or 8 μg/kg), and vs. ghrelin plus bombesin (4 or 8 μg/kg); #P < 0.05 vs. vehicle; &P < 0.05 vs. vehicle.

Fig. 7. Effects of immunohistochemical staining for Fos and corticotropin-releasing factor after treatment with ghrelin, bombesin, and ghrelin plus bombesin-injected intraperitoneally in the PVN. A: at treatment with ghrelin (13 μg/kg ip) plus bombesin at a dose of 8 μg/kg ip, 59.3% of Fos neurons were CRF positive. At injection of ghrelin alone, 45.4% of the Fos-positive neurons were also CRF positive. B: bombesin (8 μg/kg) and ghrelin simultaneously injected intraperitoneally induced Fos expression in 22.4% and ghrelin alone in 10.3% of CRF-immunoreactive neurons in the PVN. Data are expressed as means ± SE of the number (n = 3) of rats.
peripheral ghrelin induces c-fos on food intake (15). In particular, we previously showed that ARC to the PVN in the orexigenic effect of peripheral ghrelin is consistent with previous evidence that NPY projections from the ARC activate CRF neurons (60). It is known that orexin neurons, located in the lateral hypothalamus (8), are activated after intracerebroventricular injection of ghrelin (13 μg/kg, or ghrelin plus bombesin (13 μg/kg plus 4 or 8 μg/kg) and were euthanized 90 min later to process brains for c-FLI. The data are presented by using box and whiskers plots, as in Fig. 4. *P < 0.05 vs. vehicle, vs. ghrelin, and vs. ghrelin plus 4 μg bombesin/kg; #P < 0.05 vs. vehicle, vs. ghrelin, and vs. ghrelin plus 4 μg bombesin/kg.

The observed activation of neurons in the ARC in response to peripheral injection of ghrelin is consistent with previous studies in mice and rats (25, 30, 67). Furthermore, it has been reported that ARC neurons activated by peripheral ghrelin contain NPY, a well-established orexigenic peptide (67). Convergent evidence supports a role of NPY projections from the ARC to the PVN in the orexigenic effect of peripheral ghrelin on food intake (15). In particular, we previously showed that peripheral ghrelin induces c-fos expression in the PVN (52), which was confirmed in the present study. Likewise, desacyl ghrelin induces a change of neuronal activity in the ARC and PVN (2).

Systemic administration of bombesin at a dose of 10 μg/kg induces c-fos expression in the PVN in fed or fasted rats (7, 34). After the intraperitoneal administration of bombesin, at doses of 4 and 8 μg/kg, we observed no changes of c-fos expression in the PVN. This difference in c-fos expression might be explained by differences in peptide doses or in the metabolic status of freely fed vs. fasted rats in our previous study (7). Fasting is known to activate ARC neurons projecting to the PVN (68), which may increase their responsiveness to peripheral bombesin under fasted vs. fed conditions. Nevertheless, compared with control animals, neurons in the NTS expressed significantly higher levels of Fos after bombesin-injected intraperitoneally at 8 μg/kg alone or with ghrelin compared with vehicle group. This is in good agreement with previous reports (7) and may contribute to the higher expression of Fos in the PVN in bombesin plus ghrelin-treated rats since NTS neurons project the PVN (34, 55).

In the present experiments, simultaneous administration of ghrelin and bombesin at 8 μg/kg led to a robust increase in the number of Fos-positive neurons in the PVN. Since combined ghrelin plus bombesin injection did not modulate the ghrelin induced c-fos expression in neurons of the ARC, it can be inferred that bombesin inhibits ghrelin-induced food intake more likely through the enhanced neuronal activity in the PVN. This observation can be clearly distinguished from our previous study where we demonstrated that intraperitoneal CCK-8S inhibited ghrelin induced Fos expression in ARC neurons (30). Recently, it has been shown that pretreatment with CCK inhibits ghrelin transmission on the afferent vagal nerve (18). However, one could speculate that the inhibition of the ghrelin effect on ARC neurons by CCK is mediated by mechanisms involving afferent fibers of the vagal nerve.

It has been suggested that central ghrelin blocked GABA release from neurons in the PVN via NPY projections, and this inhibition may be involved in the activation of CRF neurons (10). One could hypothesize that peripheral ghrelin also causes an inhibition of GABA release in the PVN via NPY-positive projections from the ARC to the PVN. This hypothesis can be supported by the fact that ghrelin alone injected intraperitoneally induces neuronal activation in ~10% of the CRF neurons of the PVN. This ghrelin-induced inhibition of GABA release would enable the PVN to become susceptible to bombesin-related influences. It has been shown in various studies that bombesin stimulates hypothalamic CRF release (27, 28, 47). Double labeling performed in the present study revealed that ~60% of these neurons in the PVN activated by simultaneous administration of ghrelin plus bombesin were CRF-positive neurons. However, simultaneous administration of ghrelin and 8 μg bombesin activated ~22% of the PVN-CRF neurons. These data in conjunction with the fact that intracerebroventricular injection of CRF inhibits food intake (59) support the contention that activation of CRF-positive neurons in the PVN may be part of the underlying mechanisms mediating interactions of central and peripheral bombesin and ghrelin (12, 24). Nevertheless, the question remains, which role ghrelin-activated CRF neurons play in the regulation of food intake. The orexigenic effect of ghrelin could be mediated via NPY/AgRP fibers that project from the ARC to the lateral hypothalamus. NPY/AgRP-positive fibers have been detected in the lateral hypothalamus (8) and AgRP is exclusively being synthesized in ARC neurons (60). It is known that orexin neurons, located in the lateral hypothalamus (53), are activated after intracerebroventricular administration of ghrelin or NPY (65). Furthermore, it has been demonstrated that microinjections of orexin antibodies reduced food intake in rats (65). Therefore, the activation of CRF neurons by ghrelin could point out a regulatory counter mechanism, which terminates food intake. Furthermore, the ghrelin-inducible GABA inhibition in the PVN could facilitate activation of additional CRF neurons (by bombesin for example) and thus block food intake. It is worth noting that this hypothesis may be part of the underlying mechanisms mediating interac-
mentioning that in animals that received simultaneous injections of ghrelin and 4 µg bombesin, no significant activation of CRF neurons in the PVN was found. We expected an increase in activated CRF neurons of ~10% similar to the group that received injection of ghrelin alone. One can speculate that the reason for the different results might be displaced injections (e.g., into the bowel lumen).

Very recently, Date et al. (18) showed that intravenous injection of CCK prevented intravenous ghrelin-induced decrease in gastric vagal afferent activity and neuronal activation in the ARC as shown by Fos expression. The same research group showed in a previous study that afferent projections to the brain are part of the pathways through which peripheral ghrelin influences food intake (17). Peripheral administration of bombesin induces a GRP (BB2) receptor-mediated activation of gastric vagal afferent discharge partly through indirect mechanisms related to the release of CCK and activation of gastric mechanoreceptors, since there are no specific bombesin binding sites on the cervical vagus (57, 74). However, the mechanisms of action of bombesin injected peripherally to suppress food intake are largely dependent on spinal afferent fibers along with vagal afferent mechanisms (42, 63). In addition, while peripheral bombesin-induced delayed gastric emptying is mediated via activation of CCK-A receptors, the suppression of food intake by bombesin is independent from the CCK-A receptor (33). Consequently, we can rule out that bombesin-induced inhibition of ghrelin-induced food intake is primarily mediated by directly influencing the afferent vagal nerve or that bombesin action is secondary to peripheral bombesin-induced CCK release (11).

In a previous study, we observed that CCK alone administered peripherally, without ghrelin, in freely fed animals did not significantly inhibit food intake (30). We observed the same lack of effect on food intake in the present study for bombesin and amylin. Most studies showing an inhibitory effect of peripheral bombesin or amylin on food intake were conducted in fasted animals to assess the termination of hyperphagia induced by a fast in animals (40, 42). Thus the nonfasting state of the animals is very likely the reason for the lack of a significant reduction of food intake during treatment with bombesin or amylin alone in the present study. On the other hand, the time point (light or dark phase) of injection also seems to be important for the physiological action of GI satiety peptides, as demonstrated in the present study for amylin. However, under these experimental conditions it was possible to demonstrate the stimulating effect of ghrelin on food intake as well as its inhibition by GI satiety peptides.

In summary, the present data show that the orexigenic effect of peripheral ghrelin is inhibited by peripheral bombesin in freely fed rats while peripheral amylin does not have such effect. The inhibition of ghrelin’s orexigenic action induced by bombesin is associated with a robust approximately threefold increase in the number of c-Fos-positive neurons in the PVN, and most of the neurons activated by the combined intraperitoneal injection of ghrelin and bombesin in the PVN are CRF positive. These results suggest that enhanced activation of CRF-positive neurons in the PVN may play role in bombesin-induced sustained inhibition of peripheral ghrelin-induced food intake.

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