INTRODUCTION

Feeding behavior is controlled by both physiological factors, such as metabolites and hormones released during food ingestion and psychological (hedonic or aversive) factors related to the brain reward system and motivation (1). The presence of nutrients in the gastrointestinal tract triggers biochemical changes in the blood and stimulates the secretion of gut hormones (2). Along with biochemical messages, these hormones provide the information on the current alterations in body's energy homeostasis to the nucleus of the solitary tract and hypothalamus via either vagal afferents in the digestive tract or the blood (3). For instance, increased secretion of an orexigenic hormone, ghrelin, precedes food intake and stimulates appetite (3). Other intestinal hormones, secreted after food consumption, e.g., cholecystokinin and GLP-1, inhibit appetite (3). On the other hand, stimulation of a peripheral receptor for somatostatin, a hormone involved in the intestinal hormone release, has been recently found to have no significant effect on food consumption in the rat (4). Interestingly, gastrointestinal hormones, including GLP-1, are likely to be involved in appetite inhibition in obese subjects after the gastric bypass surgery (5).

Both starvation and satiety are associated with changes in brain (6) and intestinal (7) levels of endocannabinoids known to increase food intake by the effect on motivational centers in the limbic system and the hypothalamic feeding centers (8). At the level of hypothalamus endocannabinoids were found to interplay with other neurotransmitters to affect feeding behavior, we have examined whether a stable GLP-1 agonist, exendin-4 and a CB1 receptor antagonist, AM 251, may reciprocally enhance their inhibitory effects on food consumption in the rat. Additionally, we have tested whether the blockade of the GLP-1 receptor by exendin (9-39) modifies AM 251-dependent effects on energy balance. In a dose-response study, male Wistar rats were injected intraperitoneally with either 1.5-6.0 µg/kg exendin-4, 0.5-2 mg/kg AM 251, 80-320 µg/kg exendin (9-39) or their vehicle and the daily food and water intake as well as body weight changes were monitored two days before and two days after the injection. Exendin-4 at a dose of 3.0 and 6.0 µg/kg and AM 251 at a dose 2 mg/kg decreased significantly 24-hour food intake and body weight. Therefore, in the next study, the effects of lower doses of exendin-4 (1.5 µg/kg) and AM 251 (1.0 mg/kg) administered alone or together on food consumption were compared. As opposed to being injected alone, the co-administration of the two resulted in a marked decrease in both daily food intake and body weight. Exendin (9-39) did not modify the suppressory effect of the highest AM 251 dose on food consumption. Apparently, the effect of AM 251 on the appetite is not mediated by GLP-1. The concomitant stimulation of GLP-1 receptor and blockade of CB1 receptor, however, may act synergistically to inhibit appetite in the rat.

Key words: Cannabinoid, body weight, exendin-4, exendin (9-39), CB1 receptor antagonist, food intake, glucagon-like peptide-1
reported to produce undesirable and potentially dangerous psychiatric side effects when used at doses sufficient to inhibit appetite (23). This prompted scientists to look for safer methods for obesity treatment using cannabinoid antagonists (24). One of these methods might involve a polydrug therapy employing low doses of drugs that act synergistically to decrease food consumption. The aim of this study was, therefore, to investigate the effects of a cannabinoid CB1 receptor antagonist, AM 251, and a stable GLP-1 agonist, exendin-4, administered together at low doses on food intake and body weight in the rat. Additionally, we have tested the effect of GLP-1 receptor blockade by exendin (9-39) on AM 251-induced changes in the energy balance.

MATERIAL AND METHODS

Animals

Experiments were carried out on male Wistar rats weighing between 290 and 370 g. During the experiment, the animals were housed in individual, plastic cages and maintained on a 12:12 hour light-dark cycle, at 20-22°C with free access to standard, pelleted rat chow (Motycz, Poland) and water. Each rat received a preweighed amount (100 g) of food and water in a calibrated bottle every day throughout the experiment. The rats were handled every day for 4 days before the injection to accustom them to experimental conditions and to attenuate the possible effect of stress on their feeding behavior. Each group consisted of 6–9 animals. All the experimental procedures were approved by the Local Ethical Committee at the Medical University of Lodz.

Experimental procedure

1. Dose-response study

The aim of this study was to investigate the dose-response effect of increasing doses of exendin-4 (BACHEM, Switzerland), exendin (9-39) (BACHEM, Switzerland), and AM 251 (1-(2,4-dichlorophenyl)-5-(4-methoxyphenyl)-4-methyl-N-(1-piperidinyl)-1H-pyrazole-3-carboxamide; SIGMA, USA) on feeding behavior as well as to identify the lowest dose of each drug that affects significantly food intake. In experiment 1, each rat received a single intraperitoneal (i.p.) injection of 1.5, 3.0 or 6.0 µg/kg body weight (bw) exendin-4 or its vehicle (0.9% sterile saline). In experiment 2, rats were injected i.p. with 0.5, 1.0 or 2.0 mg/kg bw. AM 251. AM 251 was initially dissolved in dimethylsulphoxide (DMSO, SIGMA, USA) and then diluted with saline to obtain the required concentrations. The maximum DMSO concentration was 2% v/v and this vehicle solution was injected to control rats. In experiment 3, the animals were given an i.p. injection of 80, 160 or 320 µg/kg bw. exendin (9-39) or 0.9% sterile saline. All solutions were administered in a volume of 0.1 ml/100 g bw. All injections were made 1-1.5 hour before lights off. Daily food and water intake as well as body weight were recorded two days before and two days after the injection.

2. Combined-treatment study

In the first experiment, rats were injected i.p. with either 1.5 µg/kg exendin-4, 1 mg/kg AM 251, 1 mg/kg AM 251 followed 15 min later by 1.5 µg/kg exendin-4, or saline. The doses of the drugs were selected based on results obtained in series 1. For both drugs, doses lower than those found to be sufficient for the significant food intake inhibition were administered. Daily food/water consumption and body weight were measured as described previously.

In the second experiment, rats were injected ip. with either 160 µg/kg exendin (9-39), 2 mg/kg AM 251, 160 µg/kg exendin (9-39) followed 15 min later by 2 mg/kg AM 251, or saline. Body weight as well as food and water intake were measured as indicated above.

Statistical analysis

The data from both studies were analyzed by one-way repeated measures ANOVA followed by the Fisher’s post-hoc test using STATISTICA 8 (Statsoft, Poland). The differences were considered to be significant when $p \leq 0.05$.

RESULTS

There were no significant differences between rats in all groups in both studies with respect to the initial body weight as well as food and water intake.

Dose-response study

Exendin-4 significantly ($p<0.001$) reduced food consumption 24 h after the injection (Fig. 1A) when administered at a dose of 3.0 or 6.0 µg/kg bw. Therefore, we employed the lowest dose of 1.5 µg/kg bw. for further experiments aimed at the examination of possible interactions between exendin-4 and a CB1 receptor antagonist. Exendin-4 had no effect on the daily water intake (Fig. 1B) in either group. Only the highest dose of exendin-4 (6.0 µg/kg) diminished significantly body weight both 24 ($p<0.001$) and 48 hours ($p<0.05$) after the injection as shown in Fig. 1C.

A significant ($p<0.01$) decrease in the daily food intake was shown only when AM 251 was used at a dose of 2 mg/kg bw. (Fig. 2A) and, therefore, we employed a lower dose of 1 mg/kg bw. in the combined-treatment study. AM 251 diminished remarkably ($p<0.05$) daily water intake at a dose of 2 mg/kg only (Fig. 2B). Although body weight tended to decrease in rats treated with 2 mg/kg AM 251 (Fig. 2C), these changes were not significant ($p=0.060$).

Exendin (9-39) at any dose did not change significantly the daily food and water intake as well as body weight.

Combined-treatment study

Exendin-4 and AM 251 administered alone at low doses, as indicated above, had no significant effects on daily food and water intake as well as body weight changes (Fig. 3). When, however, these drugs were administered together, a clear ($p<0.05$) suppressory effect on the 24-hour food consumption occurred (Fig. 3A). This effect was associated with a significant ($p<0.05$) reduction in body weight (Fig. 3C).

Both AM 251 2 mg alone and in combination with exendin (9-39) significantly (both $p<0.05$) diminished food intake when compared to controls (Fig. 4A). There was, however, no difference in the reduction in food intake between rats treated with AM 251 only and those given AM 251 together with exendin (9-39). AM 251 administered alone reduced markedly ($p<0.050$) body weight. However, previous exendin (9-39) injection abolished AM 251-induced body weight changes (Fig. 4C).

DISCUSSION

The main finding of this study is that simultaneous administration of two anorexigenic drugs, exendin-4 and AM 251, at subanorectic doses results in a decrease in 24-hour food
ingestion and body weight in the rat. Exendin-4 is a potent and selective agonist of the GLP-1 receptor whilst AM 251 is an antagonist of the CB1 receptor. Since both activation of the GLP-1 receptor and blockade of the CB1 receptor are known to reduce appetite in animals and humans (25, 26), we have compared the effects of exendin-4 and AM251 administered alone or in combination on daily food intake in the rat.

In the first study, we evaluated the effects of various doses of each drug on 24-hour food intake and body weight changes. Based on the results of previous studies from our laboratory (21) and those reported by other authors (14, 27, 28) we selected drug dosages that could be supposed to affect the daily food intake in the rat under the conditions of our experiments. As expected, both exendin-4 and AM 251 at a dose of, respectively, 3.0 µg/kg and 2 mg/kg bw . reduced significantly daily food intake when injected separately. Therefore, in the next series, where possible synergistic effects of both pharmaceuticals were investigated, we used a half-size dose of each drug that did not affect significantly the daily food ingestion and body weight.

In the combined-treatment study, we have demonstrated that the concurrent subthreshold stimulation of the GLP-1 receptor with exendin-4 and blockade of the CB1 receptor with a low dose of AM 251 caused a clear decrease in food consumption and a resultant body weight reduction, the events that could not be seen when each drug was administered alone in the same dose. This finding indicates that these drugs may reciprocally enhance their effects on energy balance of the body. On the other hand, water intake in rats treated with both exendin-4 and AM 251 was comparable to that in the control group. This indicates that the synergistic interaction of both drugs does not involve their effects on water balance. It is of interest that exendin-4 was found previously to exert similar, synergistic effects with another anorectic gut hormone PYY (29) and an amylin analog, calcitonin (30).

To date, there have been no reports on possible crosstalk between the endocannabinoid system and GLP-1 with regard to their effects on energy balance. However, blockade of the cannabinoid CB1 receptor and concomitant inactivation of an

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Fig. 1. Effects of various doses of exendin-4 on daily food intake (panel A), water intake (panel B) and body weight changes (panel C) in the rat. The initial value in panels A and B is mean food/water consumption during two consecutive days before the injection of the drug. The subsequent measurements were made 24 and 48 hours after the injection. Body weight was measured on the day of injection and compared to results obtained 24 and 48 hours after the injection. Data are mean ±S.E.M. * p<0.05, ** p<0.001.
opioid receptor were shown to have synergistic effects on food intake and body weight changes (31). An opioid antagonist, naloxone, and AM 251, when given collectively, inhibited food intake to a greater extent than each drug injected alone (31). Similarly, a CB1 receptor antagonist, SR 141716 (rimonabant) and fenfluramine (32) as well as SR 141716 and cholecystokinin (33) were shown to have additive, suppressory effects on food intake in rats. On the other hand, such additive effects on food consumption did not occur when rimonabant was injected along with another anorectic drug, sibutramine (34). Although, in view of the known adverse effects of CB1 receptor antagonists, the conclusions from our study should be drawn carefully, the finding that AM 251 and exendin-4 may reciprocally augment their anorectic effects indicates potential utility of both drugs in polydrug therapy for obesity. Moreover, the fact that the significant reduction in body weight could be achieved at low doses of the drugs as well as the lack of their effects on water intake may prove the safety of such a combination therapy.

The possible mechanism underlying the synergistic interaction between the GLP-1 agonist and CB1 receptor antagonist remains unclear. The CB1 receptor has been identified in vagal afferents in the digestive tract (35) and the GLP-1 receptor gene has been detected in the nodose ganglion cells (36) indicating that both drugs might directly modify the activity of vagal afferents relaying information from the alimentary tract to feeding centers in the brain. Interestingly, GLP-1 and endocannabinoids use the same intracellular signaling pathway, related to AMP-activated protein kinase (AMPK), to affect the activity of feeding centers in the hypothalamus (37). Since GLP-1 reduces the activity of the enzyme and endocannabinoids produce the opposite effect, it may be supposed that simultaneous activation of the GLP-1 receptor by exendin-4 and blockade of the CB1 receptor by AM 251, as employed in our study, diminish the AMPK activity more than each drug administered alone. Possibly, this could account for the synergism of both drugs.
The CB1 receptor blockade was also found to attenuate the intracellular signal transduction produced by stimulation of the orexin receptor (38) as well as to reduce the orexigenic effect of ghrelin (11). On the other hand, GLP-1 and its agonist were found to attenuate the secretion of ghrelin (39). Hence, AM 251 and exendin-4 might act additively to inactivate orexigenic pathways and decrease appetite. It is also of interest that cholecystokinin, an appetite-reducing gut-brain hormone, diminishes expression of the CB1 receptor in vagal afferent terminals (35). Hypothetically, the similar mechanism might be responsible, at least in part, for the appetite-reducing effect produced by subanorectic doses of exendin-4 and AM 251 seen in our study.

We have also found that the GLP-1 receptor antagonist, exendin (9-39), did not change either food consumption or body weight even when administered peripherally at a high dose of 320 µg/kg bw. In the combined-treatment study, we used a lower dose of exendin (9-39), similar to that reported previously to prevent the action of another anorectic hormone, leptin (21). We found that exendin (9-39) did not modify the AM 251-dependent suppression of food intake indicating that the anorectic action of CB1 receptor antagonist is not mediated by GLP-1. In contrast to rats treated with AM 251 alone, however, there was no significant reduction in body weight in animals injected with both exendin (9-39) and AM 251. Apparently, the blockade of the GLP-1 receptor could prevent the AM 251-treated rats from weight loss through a mechanism other than that related to the control of feeding centers. This mechanism might involve the concomitant reduction in the metabolic rate. Data concerning possible effects of the GLP-1 receptor inhibition on the metabolic rate are very scarce. The lack of the effect of acute (this study) as well as chronic (40) exendin (9-39) treatment on body weight indirectly indicates that the blockade of the peripheral GLP-1 receptor has no effect on metabolism in rats with normal energy balance. Possibly, the hypothetical suppressory effect of GLP-1 receptor blockade on the metabolic rate occurs only under conditions of negative energy balance seen in rats treated with the CB1 receptor inhibitor. This

Fig. 3. Effects of a combination of subanorectic doses (see Results) of exendin-4 (Ex-4) and AM 251 on daily food intake (panel A), water intake (panel B) and body weight changes (panel C) in the rat. The initial value in panels A and B is mean food/water consumption during two consecutive days before the injection of the drug. The subsequent measurements were made 24 and 48 hours after the injection. Body weight was measured on the day of injection and compared to results obtained 24 and 48 hours after the injection. Data are mean ±S.E.M. * p<0.05.
hypothesis, however, needs to be supported by the experimental data that are beyond the scope of the present study.

In summary, we have found that concurrent inhibition of the CB1 receptor and activation of the GLP-1 receptor by AM 251 and exendin-4, respectively, administered at subanorectic doses result in a significant decrease in 24-hour food, but not water, intake as well as in a marked reduction in body weight in the rat. This indicates that there is a synergistic interaction between these drugs in terms of their effects on body energy balance. Since the blockade of the GLP-1 receptor did not alter the AM 251-induced reduction in appetite, we conclude that GLP-1 does not mediate the suppressory effect of the CB1 receptor inhibitor on feeding behavior in the rat.

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Conflict of interests: None declared.

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