Dose combinations of exendin-4 and salmon calcitonin produce additive and synergistic reductions in food intake in nonhuman primates

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Bello NT, Kemm MH, Ofeldt EM, Moran TH. Dose combinations of exendin-4 and salmon calcitonin produce additive and synergistic reductions in food intake in nonhuman primates. Am J Physiol Regul Integr Comp Physiol 299: R945–R952, 2010. First published June 16, 2010; doi:10.1152/ajpregu.00275.2010.—Glucagon-like peptide-1 (GLP-1) and amylin mediate the feedback control of eating by seemingly separate, but overlapping mechanisms. This study examined the effects of combined doses of the GLP-1 agonist, exendin-4 (Ex-4), and the amylin analog, salmon calcitonin (sCT), on food intake and meal patterns in adult male rhesus monkeys. Monkeys received intramuscular injections of Ex-4 (0, 0.1, 0.32, or 0.56 µg/kg), sCT (0, 0.1, or 0.32 µg/kg), or combinations thereof before a 6-h daily access to food. Dose combinations produced reductions in food intake that were significantly greater than those produced by the individual doses. Surface plots of the hourly intake indicated a synergistic interaction at lower doses of Ex-4 and sCT during the first 4 h of feeding and additive effects at hours 5 and 6. Meal pattern analysis revealed the combinational doses reduced average meal size and meal frequency by additive interactions, whereas infra-additive effects were apparent at lower doses for first meal size. Combinational doses were further characterized by administration of repeated daily injections of 0.56 µg/kg Ex-4 + 0.32 µg/kg sCT for 5 days. This resulted in sustained reductions in daily food intake (>70% from saline baseline) for 5 days with residual reductions (~48% from saline baseline) persisting on day 1 following the injections. In contrast, when pair-fed an identical amount of daily food, there was a compensatory food intake increase on day 1 following the pair-feeding (~132% of saline baseline). Such data suggest Ex-4 and sCT interact in an overall additive fashion to reduce food intake and further the understanding of how GLP-1 and amylin agonist combinations influence feeding behavior.

islet amyloid polypeptide; exenatide; gut peptides; obesity

Peripheral glucagon-like peptide 1 (GLP-1) is an incretin primarily released from L-cells of the distal ileum and large intestine in response to intraluminal nutrient contact (4, 27). The insulino tropic actions of GLP-1 agonists have received considerable attention, and several compounds that mimic the action of incretins are effective in the management of non-insulin-dependent diabetes mellitus (NIDDM) (1, 10, 38). One such Food and Drug Administration-approved drug is Byetta (Exenatide), which is a synthetic product of exendin-4 (Ex-4), a naturally occurring 39-amino acid peptide extracted from the venom of the Gila monster (Heloderma suspectum) (35, 39). In addition to a 53% structural homology with mammalian GLP-1 and a high affinity for GLP-1 receptors, Ex-4 has a considerably longer biological active half-life than exogenously administered GLP-1 (33, 39). Separate from its role as an incretin, GLP-1 is considered a meal-induced inhibitory signal (30, 52, 57). In particular, GLP-1 and related agonists, such as exendin-4, have been demonstrated to reduce food intake by slowing gastric emptying, reducing meal size, and promoting feelings of satiety (14, 18, 23, 32, 54, 57). Despite that different GLP-1 compounds readily cross the blood-brain barrier and have central binding and release sites in feeding-related brain structures, the reductions in food intake by these compounds appear to be peripherally mediated, as they are dependent on intact vagal afferent signaling (12, 13, 17, 32, 51).

Amylin is another peptide that is involved in the control of eating. Amylin or islet amyloid polypeptide (IAPP) is a 37-amino acid pancreatic peptide that is cosecreted with insulin and is involved in meal satiety signaling (16, 19, 20). As such, amylin and related compounds also inhibit gastric emptying, reduce meal size, and promote feelings of satiety (31, 50, 59). Synthetic or naturally occurring amylin agonists have been shown to be more potent and have a longer duration of feeding suppression than amylin itself (8, 50, 56). Amylin is a member of the calcitonin family of peptides, and one such potent anorectic analog in humans, primates, and rodents is calcitonin of salmon origin (sCT) (28, 34, 37). This compound irreversibly binds to amylin receptors to produce sustained anorectic responses (15). In contrast to GLP-1, the anorectic potency of amylin agonists is not dependent on intact vagal afferent signaling (31, 40, 55).

Combinational doses of some meal-related and adiposity signaling peptides have been shown to reduce food intake by additive and synergistic interactions (7, 42, 45, 46). For example, combined doses of the within-meal satiety peptide, CCK, and leptin have been shown to synergistically reduce food intake (9, 29). Leptin potentiates the satiety actions of CCK (9), and CCK/leptin combinations result in greater long-term feeding suppression than leptin alone (25, 26). Recent work in investigating the utility of combinational therapies for the treatment of obesity has focused on the coadministration of amylin with leptin (45, 46, 53). These experiments have revealed that combinational doses of amylin and leptin elicit a synergistic interaction over a range of doses, reducing food intake and producing a greater body weight loss than the additive effects of each individual compound (45, 53). One key to such interactions is the combination of peptides that affect food intake through different mechanisms. Although both GLP-1 and amylin are released in response to a meal and play roles in meal termination, they do so through seemingly different mechanisms and sites of actions. This raises the possibility that engaging such mechanisms simultaneously may result in similar interactions in affecting food intake, as has been demonstrated by combining meal-related peptides and leptin. The purpose of these experiments was to determine whether combinations of GLP-1 and amylin agonist com-
pounds could result in synergistic inhibitions of food intake and how such combinations would affect meal patterns in nonhuman primates. Previous separate studies in our laboratory have demonstrated that both EX-4 and sCT individually result in dose-related reductions in food intake in nonhuman primates through changes in meal sizes (3, 49).

MATERIALS AND METHODS

Animals. Four individually housed adult male rhesus monkeys (Macaca mulatta), weighing between 8 and 15 kg, were used in this study. Monkeys were maintained on a 14:10-h light-dark cycle (7:00 AM to 9:00 PM) in an environmentally controlled room with ad libitum access to water. Water intake was not measured in these studies. Food in the form of nutritionally complete 1-g pellets (Bio-serv, Frenchtown, NJ) was provided for 6 h/day beginning at 12:00 PM. Food pellets were available in response to lever pressing on a fixed ratio reinforcement (FR) schedule. The FR schedule was individually determined for each animal to prevent cheek pouching of pellets. The FR ratios ranged from FR3 to FR15 lever presses per pellet (i.e., two monkeys were at a FR10, one was at a FR3, and the other monkey had a FR15). The monkeys were weighed every 2 wk. All procedures were reviewed and approved by the Johns Hopkins University Animal Care and Use Committee.

Acute injections. Monkeys received an intramuscular injection of saline, salmon calcitonin (Bachem, King of Prussia, PA), exendin-4 (Bachem), or a combination of exendin 4 (Ex-4) and salmon calcitonin (sCT). Doses of sCT were 0, 0.1, or 0.32 μg/kg (0, 29.13, or 93.24 pmol/kg), whereas Ex-4 doses were 0, 0.1, 0.32, or 0.56 μg/kg (0, 23.8, 76.4, or 133.7 pmol/kg). The individual parent doses of Ex-4 and sCT used in this study have been demonstrated previously to reduce daily food intake, reduce meal sizes, or both (3, 49). Injections were administered once daily 15 min before the onset of food access (11:45 AM). The injections were randomized within a series. At the time of injection, monkeys had been fasted for ~18 h (end of the feeding program on the previous day). Approximately 5 days were allowed between injections to eliminate any carryover or compensatory effects from previous administrations. Intakes were computer monitored on all days as a time stamp of each pellet delivery.

Consecutive 5-day injections. In a second series of experiments, combination doses of Ex-4 (0.056 μg/kg) and sCT (0.32 μg/kg) were given for 5 consecutive days to determine whether this dose combination sustained reductions in food intake without tachyphylaxis. Again, doses were administered 15 min before onset of the feeding program. To verify whether the combinational doses had any residual effects on food intake, intakes were monitored for three additional (i.e., postinjection) days following the 5-day injection series. In addition, a pair-feeding experiment was conducted a month later. For this, animals received their respective individual daily intake of each of the 5 days that matched their intakes during the experimental combinational doses of Ex-4 + sCT. Ad libitum intake was again monitored for the following 3 days.

Behavioral assessments. All monkeys were observed behaviorally 15 min, 30 min, 1 h, and 3 h after each injection. In particular, monkeys were assessed for changes in alertness or activity and the presence of excessive salivation, gagging, vomiting, or evidence of vomiting.

Meal patterns. Pellet data were analyzed for total daily intake, hourly interval intake, and meal patterns (i.e., first meal size, average meal size, and meal frequency). Meals were defined as the acquisition of at least five pellets preceding and followed by a period of at least 10 min without feeding. These meal parameters accounted for >95% of the pellets received. To facilitate comparisons across monkeys with different levels of baseline intake, daily intakes in response to peptide agonist treatments were expressed as a percentage of their saline baseline intake (percent saline) measured as the mean intake of the 3 days prior to beginning a new series of doses on which IM saline was administered. Meal patterns from the time-stamped output data were generated using a customized computer program as previously described (3).

Statistical analyses. Meal patterns, hourly intake, and total daily intake were analyzed using Statistica 7.1 (Tulsa, OK). Total intakes (i.e., cumulative 6-h intake) and meal pattern data were analyzed using mixed-model repeated-measures ANOVA (doses of compounds as within factors). Individual hourly intakes were analyzed by two-way repeated-measures ANOVA (doses and hourly intake as the within factors). Meal sizes and frequencies were recorded as “0” for the 5-day repeated injection experiment in instances in which individual monkeys failed to eat during the 6-h daily access. Post hoc comparisons were made with Neuman-Keuls tests. To determine whether the range of combinational doses resulted in an additive or synergistic effect, a response surface regression was performed on each dependent variable. The response surface method was a quadratic surface design to determine the intercept (which was significant in all analyses) and linear effects of Ex-4, sCT, and the interaction between Ex-4 + sCT. Similar to what has been defined by others using combinational interactions and response surface methodology (11, 42), the recognized relationship between sCT and Ex-4 was determined by the interaction term. Infra-additivity or synergy was suggested by a P value of <0.05, whereas additivity was suggested by a P value ≥0.05. The shape of the quadratic-fit surface responses plots are presented where appropriate to further distinguish the infraadditive, additive, or synergic relationship between Ex-4 and sCT. For the 5-day repeated administration study, body weights before and after administrations were analyzed by using a dependent t-test. Data are expressed as means ± SE.

RESULTS

Behavioral assessments. Observation of the monkeys following each injection did not reveal any outward signs of nausea or malaise. During the observed period, monkeys displayed normal activity and were found either sitting quietly upright or lever pressing for pellets.

Total daily and hourly food intake following acute injections.

From their respective saline baselines, there was a dose-dependent reduction in total daily food intake (i.e., 6-h cumulative total daily intake) for Ex-4 [F (3, 9) = 8.0, P < 0.01], sCT [F (2, 6) = 8.9, P < 0.05], and the combination doses of Ex-4 + sCT [F (6, 18) = 8.0, P < 0.001]. Table 1 presents the hourly interval cumulative intake. Values are expressed as a percentage of each monkey’s saline intake for each hourly interval. There was an overall effect for dose [F (10, 30) = 23.0, P < 0.001], hour [F (5, 15) = 26.5, P < 0.001], and the interaction between hour × dose [F (50, 150) = 1.45, P < 0.05]. Post hoc comparisons revealed reductions in corresponding hourly food intake between single doses of each compound and the combinational doses as indicated in Table 1. There was no evidence to suggest that the magnitude of the suppressive effects of the compounds or their combinations were related to the individual FR ratios for each monkey. The response surface analysis for each hourly interval was suggestive of synergy for Ex-4 + sCT on intake from hours 1 through 4, and an additivity effect on intake at hours 5 and 6. For hours 1 through 3, the P values were <0.01 for the Ex-4 and the sCT terms and the P value was <0.001 for the Ex-4 + sCT interaction term. At hour 4, however, the effects were P < 0.01 for the individual Ex-4 term and the Ex-4 + sCT interaction term, whereas the sCT term was P > 0.05. At hours 5 and 6 (i.e., total daily intake), only the effect for the Ex-4 term generated a P < 0.05 on the cumulative hourly intake, see Fig. 1 for
surface plots for each hour. The surface plots revealed a more complex pattern of interaction for hours 1 through 4. The low-dose combinations of Ex-4 + sCT resulted in synergistic (greater than additive) suppressions of food intake through hours 1–4, whereas the relationship between Ex-4 and sCT was more characteristically additive for hours 5 and 6. The data for the cumulative hourly intake for hour 6 represents the daily total intake. As such, the total daily intake for the dose combination produced an additive interaction between Ex-4 and sCT.

First meal size following acute injections. An examination of the meal patterns indicated a dose-dependent reduction in first meal size from saline baselines for Ex-4 \([F(3, 9) = 7.7, P < 0.01]\), sCT \([F(2, 6) = 7.3, P < 0.05]\), and the combination doses of Ex-4 + sCT \([F(6, 18) = 9.3, P < 0.001]\). Expressed as a percentage of saline baseline, individual and combina-

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**Table 1. Cumulative hourly intake for exendin 4, salmon calcitonin, and combination doses**

<table>
<thead>
<tr>
<th></th>
<th>Hour 1</th>
<th>Hour 2</th>
<th>Hour 3</th>
<th>Hour 4</th>
<th>Hour 5</th>
<th>Hour 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 µg/kg Ex4</td>
<td>95.0 ± 8.5</td>
<td>80.6 ± 10.0</td>
<td>91.5 ± 7.7</td>
<td>90.4 ± 9.3</td>
<td>85.8 ± 16.4</td>
<td>89.9 ± 9.7</td>
</tr>
<tr>
<td>0.32 µg/kg Ex4</td>
<td>34.1 ± 12.6</td>
<td>38.0 ± 16.8</td>
<td>54.6 ± 17.7</td>
<td>68.9 ± 9.3</td>
<td>68.2 ± 8.2</td>
<td>76.4 ± 2.8</td>
</tr>
<tr>
<td>0.56 µg/kg Ex4</td>
<td>20.0 ± 10.1</td>
<td>26.3 ± 15</td>
<td>24.1 ± 13.3</td>
<td>36.0 ± 12</td>
<td>40.2 ± 9.7</td>
<td>52.4 ± 3.6</td>
</tr>
<tr>
<td>0.1 µg/kg sCT</td>
<td>83.3 ± 17.1</td>
<td>79.1 ± 7.7</td>
<td>80.0 ± 10.0</td>
<td>86.9 ± 6.6</td>
<td>80.7 ± 8.1</td>
<td>80.5 ± 5.9</td>
</tr>
<tr>
<td>0.32 µg/kg sCT</td>
<td>22.5 ± 8.4</td>
<td>30.7 ± 12</td>
<td>40.4 ± 11.6</td>
<td>50.9 ± 9.1</td>
<td>62.6 ± 8.3</td>
<td>72.1 ± 5.7</td>
</tr>
<tr>
<td>0.1 µg/kg Ex4 + 0.1 µg/kg sCT</td>
<td>53.9 ± 6.8</td>
<td>57.5 ± 9.0</td>
<td>73.4 ± 2.7</td>
<td>83.1 ± 5.2</td>
<td>83.1 ± 5.2</td>
<td>82.8 ± 3.9</td>
</tr>
<tr>
<td>0.32 µg/kg Ex4 + 0.1 µg/kg sCT</td>
<td>29.2 ± 11.2</td>
<td>23.2 ± 4.6</td>
<td>38.6 ± 9.8</td>
<td>53.6 ± 11.8</td>
<td>59.7 ± 5.7</td>
<td>64.9 ± 6.3</td>
</tr>
<tr>
<td>0.56 µg/kg Ex4 + 0.1 µg/kg sCT</td>
<td>12.3 ± 8.4</td>
<td>13.4 ± 5.1</td>
<td>18.7 ± 1.7</td>
<td>23.8 ± 4.1</td>
<td>34.4 ± 2.5</td>
<td>47.3 ± 2.7</td>
</tr>
<tr>
<td>0.1 µg/kg Ex4 + 0.32 µg/kg sCT</td>
<td>29.6 ± 10.5</td>
<td>26.6 ± 9.0</td>
<td>37.8 ± 8.2</td>
<td>21.7 ± 2.5</td>
<td>25.1 ± 3.1</td>
<td>36.2 ± 5.8</td>
</tr>
<tr>
<td>0.32 µg/kg Ex4 + 0.32 µg/kg sCT</td>
<td>15.3 ± 2.7</td>
<td>15.9 ± 3.1</td>
<td>18.6 ± 1.8</td>
<td>21.7 ± 2.5</td>
<td>25.1 ± 3.1</td>
<td>36.2 ± 5.8</td>
</tr>
<tr>
<td>0.56 µg/kg Ex4 + 0.32 µg/kg sCT</td>
<td>10.5 ± 5.8</td>
<td>15.9 ± 5.7</td>
<td>13.4 ± 4.5</td>
<td>13.8 ± 2.9</td>
<td>25.4 ± 5.6</td>
<td>36.7 ± 7.8</td>
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Comparisons are made down the hour columns. Similar letters indicate significance from each corresponding parent dose of exendin 4 (EX4) or salmon calcitonin (sCT).

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**Fig. 1. Quadratic-fit surface plots for effects of peripheral doses of exendin-4 (Ex-4), salmon calcitonin (sCT), and Ex-4 + sCT on cumulative hourly food intake of rhesus monkeys (n = 4) consuming 1-g pellets. Data are expressed as hourly intake for the corresponding hour of the saline intake (percent saline) from Table 1. The surface plots revealed a synergistic shape for low doses of each compound for hours 1 through 4 demonstrated by the pattern of the surface partitions that are not equal in size. For hours 5 and 6, the surface plots demonstrated an additive relationship. Note the surface partitions are equal in size and size for hours 5 and 6. Daily food access was for 6 h represents the total daily intake.**
tional doses reduced first meal size $[F(10, 30) = 6.8, P < 0.01]$. Post hoc testing, however, did not reveal any differences between the single parent doses of Ex-4 and sCT and the combinational doses; see Fig. 2A. Response surface analysis for first meal size demonstrated a $P < 0.05$ for the Ex-4 and Ex-4 + sCT terms, and a $P > 0.05$ for the sCT term. As shown in Fig. 2B, the surface plot displays an infra-additive effect for 0.1 $\mu$g/kg of Ex-4 and sCT doses, while a ceiling effect was evident in the flattening out of the surface plot at the higher doses of both compounds.

**Average meal size following acute injections.** For average meal size from the respective saline baselines, there was a dose-dependent response for Ex-4 $[F(3, 9) = 7.9, P < 0.01]$ and the combination doses of Ex-4 + sCT $[F(6, 18) = 3.5, P < 0.05]$, but not for sCT. Expressed as a percentage of saline baseline, there was an overall significant effect of treatment on average meal size $[F(10, 30) = 6.8, P < 0.01]$. The combinational doses produced significant reductions from sCT only. In particular, reduction from 0.1 $\mu$g/kg sCT was demonstrated at 0.1 $\mu$g/kg Ex-4 + 0.1 $\mu$g/kg sCT, 0.32 $\mu$g/kg Ex-4 + 0.1 $\mu$g/kg sCT, and 0.56 $\mu$g/kg Ex-4 + 0.1 $\mu$g/kg sCT ($P < 0.05$ for all). Reductions from the 0.32 $\mu$g/kg sCT were also observed at 0.56 $\mu$g/kg Ex-4 + 0.32 $\mu$g/kg sCT ($P < 0.05$); see Fig. 3A. The response surface analysis and surface plot for average meal size suggested additivity with a $P < 0.05$ for the Ex-4 term, and a $P > 0.05$ for both the sCT and Ex-4 + sCT terms; see Fig. 3B. 

**Meal frequency following acute injections.** Meal frequency was reduced from saline for Ex-4 $[F(3, 9) = 3.9, P < 0.05]$ and Ex-4 + sCT $[F(6, 18) = 3.9, P < 0.05]$, but not for sCT. The largest suppressions from saline baseline resulted from the 0.56 $\mu$g/kg dose of Ex-4 or the combination of this dose with sCT. When taken as a percentage of saline, however, the effect of dose on meal frequency only approached significance $[F(10, 30) = 2.1, P = 0.052]$. Response surface analysis for meal frequency demonstrated a $P > 0.05$ for the Ex-4 and Ex-4 + sCT terms, and a $P < 0.05$ for the sCT term.

**Consecutive 5-day injections.** As illustrated in Fig. 4A, daily repeated injections of 0.56 $\mu$g/kg Ex-4 + 0.32 $\mu$g/kg sCT produced sustained reductions in total daily intake $[F(8, 24) = 17.35, P < 0.001]$. Reductions from the saline baseline were

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**Fig. 2. Effects of peripheral doses of Ex-4, sCT, and Ex4 + sCT on first meal size in rhesus monkeys consuming 1-g pellets. First meal sizes are expressed as a percentage of the 3-day saline average meal size (means ± SE).**

**A:** although there were overall significance differences for single and combinational doses, post hoc, there were no significant difference between single doses and the combinational doses. **B:** An infra-additive relationship for the 0.1 $\mu$g/kg of Ex-4 and the sCT was apparent, while a ceiling effect was evident in the flattening out of the surface plot at the higher doses of both compounds.

**Fig. 3. Effects of peripheral doses of Ex-4, sCT, and Ex4 + sCT on average meal size in rhesus monkeys consuming 1-g pellets. Average meal sizes are expressed as a percentage of the 3-day saline average meal size (means ± SE).**

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**Fig. 4.**
observed at injection day 1 (~72% reduction) through day 5 (~81% reduction) and postinjection day 1 (~48% reduction; \( P < 0.001 \) for all). There were also significant reductions in first meal size \([F(8, 24) = 4.8, P < 0.01]\), average meal size \([F(8, 24) = 3.0, P < 0.05]\), and meal number \([F(8, 24) = 5.3, P < 0.01]\). Post hoc testing revealed meal number was reduced from saline baseline on all injection days \((P < 0.05)\) for all. There was also a significant reduction in first meal size on injection days 3 and 4 \((P < 0.01)\) and near-significant reductions on days 1 and 5 \((P = 0.06)\), and day 2 \((P = 0.05)\) from the saline baseline. Two of the four monkeys failed to eat on injection days 3 and 4, but they resumed eating on injection day 5. On the basis of the 2-wk weighing before and after the experiment, body weights were significantly reduced by 2.7 \(\pm 0.2\%\) following the 5-day repeated injections and 3-day postinjection days refeeding \((t = 23.1, P < 0.001)\). For the pair-feeding experiment, the daily allotment of food was restricted to the amount consumed during 5-day repeated-injection experiment and, naturally resulted in a significant effect on total daily intake \([F(8, 24) = 49.2, P < 0.001]\). Post hoc testing revealed a significant increase in daily food intake on refeeding day 1 \((P < 0.01)\) from the saline baseline, see Fig. 4B. On the basis of the 2-wk weighing, body weights were significantly reduced by 1.4 \(\pm 0.2\%\) following the 5-day pair-feeding and 3-day postinjection days refeeding period \((t = 7.3, P < 0.01)\). There were no significant differences in 2-wk body weights between the 5-day consecutive injections and the pair-feeding experiments.

**DISCUSSION**

These experiments set out to determine the interaction of acute injections of the GLP-1 agonist Ex-4 and the amylin agonist sCT on the food intake and meal patterns in nonhuman primates. Although others have examined the effects of peptide combinational therapies on food intake \((42, 45, 53)\), this is the first study to examine the feeding inhibitory action of a combination of a GLP-1 agonist and an amylin analog. For these studies, monkeys were maintained on a schedule of 6-h daily access to food, and animals were trained to lever press during this time for their daily ration of nutritionally complete 1-g pellets. To discern the pharmacological relationship between EX-4 and sCT, we used response surface methodology, which allows a characterization of the nature of potential interactions over the dose ranges. This method has been commonly used to characterize drug interactions in a variety of different experimental designs \((11, 48)\). Although sCT has been demonstrated to irreversibly bind to amylin receptors \[which explains its longer duration of action compared with other amylin agonists (22)]\, it was chosen for use in this study primarily on the basis of findings from our previous investigation \((3)\). In that study, sCT dose-dependently decreased daily food intake, reduced meal sizes, and produced sustained reductions with repeated administration in nonhuman primates. The present study is an extension of those and other findings that examined the feeding-suppressive effects of Ex-4 in nonhuman primates from our laboratory \((49)\). To avoid using a dose range that was either too narrow or too broad for measuring effects on feeding behavior, we limited the range of doses for Ex-4 and sCT to those that have been previously shown to reduce daily food intake in nonhuman primates \((3, 49)\). Those earlier findings of dose-dependent decreases in total daily food intake with individual doses of EX-4 and sCT were replicated in this study. The response surface analysis demonstrated that interactions of Ex-4 and sCT on total daily food intake were additive, and a similar additive pattern was illustrated in the quadratic-fit surface plot. For hourly cumulative food intake intervals, the interactions were more complex with low-dose combinations producing synergistic effects at hours 1 through 4, but an additive interaction on hours 5 and 6. For higher-dose combinations where the individual doses resulted in marked suppression of food intake, a ceiling effect was essentially obtained. During this time, monkeys did not display any outward signs of malaise or nausea.

Examination of the patterns of dose interactions on meal patterns revealed overall additive interactions for average meal size and meal frequency. The pattern of effects on first meal size produced a more mixed interaction. There was an infra-additive response for the low doses of Ex-4 and sCT, but this approached a ceiling effect at the higher doses. Overall, the findings from the response surface analyses revealed that acute injections Ex-4 and sCT had a synergistic pattern for low doses in the early part of the 6-h cumulative feeding \((i.e., \text{hours 1 through 4})\), but this relationship waned toward the end of the feeding session to produce a more additive pattern of influence. The suppressive effects of the compounds also are maintained considerably longer than the reported peak plasma concentrations of the individual compounds. It has been reported, for instance, the half-life of intravenous and subcutaneous administered Ex-4 \((3 \mu g/kg)\) in rhesus monkeys is 1.33 \(\pm 0.06\) h and 0.48 \(\pm 0.02\) h, respectively \((2)\). Along the same lines, 100 IU \((20 \mu g)\) intramuscular injections of sCT had a half-life of 0.93 \(\pm 0.3\) h in humans \((41)\). Further experiments are needed...
to determine whether this inhibition of feeding by Ex4 + sCT is mediated by a single or multiple converging downstream signals that are involved in the control of feeding behavior.

Another goal of the study was to determine whether the anorectic potency of the combinational doses would attenuate with repeated injections for 5 days. The combinational dose of 0.56 μg/kg Ex4 + 0.32 μg/kg sCT produced sustained reductions, ~75% from saline baseline, in total daily food intake over the injection series. While individual doses of 0.56 μg/kg Ex4 and 0.32 μg/kg sCT produced a similar degree suppression, the 0.56 μg/kg Ex4 + 0.32 μg/kg sCT was chosen for the 5-day consecutive experiment because average meal sizes with this combinational dose were significantly reduced from the 0.32 μg/kg sCT parent dose. In addition, we previously demonstrated in nonhuman primates that 5-day repeated injections of 0.56 μg/kg Ex4 resulted in an ~65% sustained reduction in daily food intake (49), and in another separate study, we demonstrated that a similar sustained magnitude of suppression was also demonstrated with 5-day repeated injections of 0.32 μg/kg sCT (3). The present findings with 5-day repeated injections of 0.56 μg/kg Ex4 + 0.32 μg/kg sCT also demonstrated a reduction in total daily food intake, but unlike those studies, the reduction in intake resulted predominantly from changes in meal number, not meal sizes, on all injection days. In the present study, first meal size was only significantly reduced on days 3 and 4. The changes in meal number and not meal size with this Ex4 + sCT combination may represent a convergence of mechanisms that is dependent on the dose combinations and repeated administrations or may simply be due to the magnitude of the reduction in food intake.

Another finding from the repeated injections of the combinational doses of 0.56 μg/kg Ex4 + 0.32 μg/kg sCT was that reductions in total daily food intake persisted for 1 day following the termination of the injections. When pair-fed to the same amount of total daily food consumed during the combinational injections, the monkeys had a pronounced rebound in their total daily intake on post-pair-fed day 1. In either the Ex4 + sCT repeated injections or the pair-feeding protocol, the feeding suppression over the 5 days resulted in a similar decrease in body weight at the 2-wk body weight check. Taken together, this suggested the repeated combinational dose of Ex4 + sCT did not produce tolerance to the anorectic potency, but had a lingering suppressive effect on feeding. A similar sustained suppression of food intake following postinjection day 1 has been observed with sCT, but not with Ex4 (3, 49). This suppressive effect may be maintained by the persistence of amylinergic signaling. The area postrema (AP) is a hindbrain circumventricular organ critical for the feeding-suppressive effects of amylin and related agonists (21, 24). In addition, sustained peripheral infusions (22 day) of amylin in diet-induced obesity (DIO)-prone rats resulted in increased proopiomelanocortin mRNA expression in hypothalamic arcuate nucleus (43), highlighting a potential forebrain involvement for the sustained anorectic effects of amylin.

This is the first study to examine the interaction of a GLP-1 agonist and amylin analog on meal patterns in any species. Other studies have used combinational therapies to measure food intake and body weight changes in rodents and humans. Much of this work has focused on the synergistic interaction of leptin and amylin to reduce food intake and decrease body weight (5, 6, 44, 45, 53). In addition, other studies have shown that chronic (14 day) infusions of single-dose combinations of GLP-1 agonist, AC3174, and leptin reduced cumulative food intake and body weight in DIO rats. However, the relative reductions in food intake and body weight changes were not greater than AC3174 alone (45).

One limitation of this study is that we did not examine the mechanism of action of the observed behavioral responses. In particular, we did not measure plasma levels of amylin or GLP-1 to determine whether circulating levels of these hormones contributed to the effects of sCT, Ex-4, and sCT + Ex-4 on food intake. Nonetheless, combinational doses of sCT and Ex-4 are likely to engage distinct mechanisms for the control of eating, but where they converge on similar neuronal populations is not known. The feeding inhibitory actions of peripheral GLP-1 depend upon vagal afferent mediation, while those of amylin analogs are mediated through the AP. Along these lines, peripheral injections of amylin (>0.5 μg/kg) or Ex-4 (1 μg/kg) in the rat increase neuronal activation, as measured by the presence of immunopositive cells labeled for c-Fos, in similar hindbrain areas, such as the AP and the nucleus of the solitary tract (47, 58). One thought has been that amylin agonists may activate GLP-1-expressing neurons in the nucleus of the solitary tract. However, amylin-induced c-Fos in the nucleus of the solitary tract has not been demonstrated in neurons that express GLP-1 (36). In addition, the anorectic actions of GLP-1 were not blocked by an amylin antagonist (36). The current data do suggest that GLP-1 and amylin agonists activate separate anorectic pathways that converge to reduce food intake. However, further experiments are needed to characterize the site of this convergence.

Perspectives and Significance

Multiple peripheral and central mechanisms interact in the control of feeding behavior. In this experiment, we used a GLP-1 agonist and an amylin analog to determine their combined effects on food intake and meal patterns in nonhuman primates. These compounds interacted with a mix of additive and synergistic mechanisms to reduce food intake. There appears to be early infra-additive or synergistic interactions in the lower dose range, but an additive relationship predominates the feeding response over the broad range of doses. Moreover, combinational therapy of Ex-4 + sCT produced sustained daily food reductions without tolerance, nausea, malaise, or rebound feeding. These findings further support the view that engaging multiple feeding inhibitory pathways to reduce food intake could be a potential strategy for the treatment of obesity.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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