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PACAP intraperitoneal treatment suppresses appetite and food intake via PAC1 receptor in mice by inhibiting ghrelin and increasing GLP-1 and leptin

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[1]*Research Service, Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California;* [2]*Division of Digestive Diseases, David Geffen School of Medicine, University of California, Los Angeles, California;* [3]*Molecular, Cellular & Integrative Physiology Program, University of California, Los Angeles, California;* [4]*Division of Gastroenterology, Hepatology and Parenteral Nutrition, Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California;* [5]*CURE/Digestive Diseases Research Center, Department of Medicine, University of California, Los Angeles, California; and* [6]*Division of Pulmonary and Critical Care, Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California*

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Vu JP, Goyal D, Luong L, Oh S, Sandhu R, Norris J, Parsons W, Pisegna JR, Germano PM. PACAP intraperitoneal treatment suppresses appetite and food intake via PAC1 receptor in mice by inhibiting ghrelin and increasing GLP-1 and leptin. *Am J Physiol Gastrointest Liver Physiol* 309: G816–G825, 2015. First published September 3, 2015; doi:10.1152/ajpgi.00190.2015.—Pituitary adenylate cyclase-activating peptide (PACAP) is expressed within the gastroenteric system, where it has profound physiological effects. PACAP was shown to regulate food intake and thermogenesis centrally; however, PACAP peripheral regulation of appetite and feeding behavior is unknown. Therefore, we studied PACAP's effect on appetite and food intake control by analyzing feeding behavior and metabolic hormones in PAC1-deficient (PAC1−/−) and age-matched wild-type (WT) mice intraperitoneally injected with $PACAP_{1–38}$ or $PACAP_{1–27}$ before the dark phase of feeding. Food intake and feeding behavior were analyzed using the BioDAQ system. Active ghrelin, glucagon-like peptide-1 (GLP-1), leptin, peptide YY, pancreatic polypeptide, and insulin were measured following $PACAP_{1–38}$ administration in fasted WT mice. $PACAP_{1–38}$/$PACAP_{1–27}$ injected into WT mice significantly decreased in a dose-dependent manner cumulative food intake and reduced bout and meal feeding parameters. Conversely, $PACAP_{1–38}$ injected into PAC1−/− mice failed to significantly change food intake. Importantly, $PACAP_{1–38}$ reduced plasma levels of active ghrelin compared with vehicle in WT mice. In PAC1−/− mice, fasting levels of active ghrelin, GLP-1, insulin, and leptin and postprandial levels of active ghrelin and insulin were significantly altered compared with levels in WT mice. Therefore, PAC1 is a novel regulator of appetite/satiety. $PACAP_{1–38}$/$PACAP_{1–27}$ significantly reduced appetite and food intake through PAC1. In PAC1−/− mice, the regulation of anorexigenic/orexigenic hormones was abolished, whereas active ghrelin remained elevated even postprandially. PACAP significantly reduced active ghrelin in fasting conditions. These results establish a role for PACAP via PAC1 in the peripheral regulation of appetite/satiety and suggest future studies to explore a therapeutic use of PACAP or PAC1 agonists for obesity treatment.

pituitary adenylate cyclase-activating peptide; PAC1 receptor; appetite; ghrelin; GLP-1; leptin

PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE (PACAP) was originally isolated from the ovine hypothalamus and is highly conserved among vertebrates (1). PACAP, because of its protein structure, particularly in the first 27 amino acids, belongs to the secretin-glucagon superfamily of hormones, which includes vasoactive intestinal peptide (VIP), gastric inhibitory peptide, glucagon-like peptide (GLP)-1 and GLP-2, growth hormone-releasing hormone, peptide histidine methionine, peptide histidine isoleucine, and exendins (34). PACAP exists in two variant forms: $PACAP_{1–27}$ and the COOH-terminally extended form $PACAP_{1–38}$ that originates from the same precursor and stimulates adenylate cyclase activity in pituitary cells (23, 35). PACAP functions through its PAC1 receptor and two VIP receptors, VPAC1 and VPAC2; however, PAC1 receptor is PACAP specific, exhibiting 1,000-fold greater affinity for PACAP than VIP (38). Localization studies in vertebrates have shown that PACAP and PAC1 are highly localized in the gastrointestinal (GI) tract and in the peripheral and central nervous systems (33, 42). In the GI tract, PACAP was found expressed within the enteric nervous system and gastric mucosa (33), where it regulates gastrointestinal motility and gastric acid secretion (14, 30).

Recently, PACAP has been found to play an important role in the regulation of metabolism. Centrally, PACAP was discovered to suppress appetite and feeding by intracerebroventricular (ICV) injection in vertebrate animals, such as goldfishes, chicks, rats, and mice (6, 32, 36, 37, 50, 51). PACAP injected in the hypothalamic ventromedial nucleus (VMN) reduced food intake and increased core body temperature and locomotor activity (44). Together, these data indicate a very relevant role for PACAP in the central nervous system as a mediator of energy homeostasis. Murine models with PACAP-targeted disruptions have a complex phenotype with several altered metabolic functions. PACAP knockout mice have been reported to have a greater loss of core body temperature, causing a higher mortality rate in newborn mice, thus suggesting an important role for PACAP in thermoregulation (13). However, the role of the PACAP high-affinity receptor PAC1 in appetite and energy homeostasis has not yet been clearly elucidated. PAC1-deficient (PAC1−/−) mice have been previously described to have pulmonary hypertension and heart failure (17), as well as hyperinsulinemia in fed conditions (21). The PAC1−/− mouse model has allowed us to better characterize the role of PAC1 in appetite, feeding behavior, and energy homeostasis.

Therefore, in the present study, to fully analyze the peripheral effects of PAC1 and PACAP on food intake, feeding behavior, and metabolic hormones secretion, we have investi-

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gated the effects of a PACAP intraperitoneal (IP) treatment before the start of the dark phase feeding cycle in wild-type (WT) and PAC1−/− mice. PACAP effects on food intake were studied in undisturbed mice fed ad libitum. The changes in meal patterns were analyzed using an automated food intake monitoring system. Finally, to elucidate the peripheral mechanism by which PACAP affects food intake, we measured plasma levels of orexigenic and anorexigenic hormones following PACAP injection in WT and PAC1−/− mice during fasting as well as in postprandial conditions.

MATERIALS AND METHODS

Mice. PAC1 receptor-deficient mice and WT littermate controls were developed as previously described (21) and backcrossed for >10 generations on the C57BL/6 background. Mice were identified by ear clipping, samples were extracted for genomic DNA, and PCR was performed as previously described to establish the mouse genotype (21). All research and procedures involving mice were approved by the Department of Veterans Affairs Institutional Animal Care and Use Committee (protocol no. 05026-03).

Determination of food intake behavior. Microstructure analysis of food intake was performed using the BioDAQ episodic food intake monitor for mice (Research Diets, New Brunswick, NJ). This system allows the continuous monitoring of meal patterns in undisturbed mice with minimal human interference as described in previously published studies (49). The system weighs the food hopper (±0.01 g) second by second and detects not eating as weight stable, and eating as weight unstable. Meals consist of one or more bouts (changes in stable weight before and after a bout) separated by an inter-meal interval (IMI ≥ 5 min). The minimum meal amount was defined as 0.02 g, and a feeding bout that occurred ≥5 min from a previous one was considered as a new meal. Therefore, food intake was considered as one meal when the feeding bout amount was ≥0.02 g and occurred within 5 min of the previous bout. Feeding parameters include cumulative food intake, bout/meal frequency, total time spent eating (time in minutes or percentages), bout/meal size, duration, and eating rate.

Food intake experiments. PAC1−/− and WT mice (8–12 wk) were single-housed under controlled illumination (0600–1800 h), temperature (21–23°C), and humidity (40–60%). The BioDAQ cages were used for continuous monitoring of meal patterns in undisturbed mice that ingested a regular rodent diet (AIN-93M; Research Diets, New Brunswick, NJ). Mice were habituated to these cages and to feeding from the hopper after 5–7 days, as indicated by normal food intake and regular body weight gain. $PACAP_{1–38}$ was administered by IP injection at concentrations of 1 nM, 10 nM, 100 nM, or 1 μM in 200 μl of saline before the start of the dark-phase feeding period (1800 h) in both PAC1−/− and WT mice. Similarly, $PACAP_{1–27}$ was administered by IP injection at 1 nM, 10 nM, 100 nM, or 1 μM in 200 μl of saline before the start of the dark-phase feeding period (1800 h) in both PAC1−/− and WT mice. Simultaneously in each experiment, control groups of the same genotype mice were injected with 200 μl of saline only.

Plasma blood samples. $PACAP_{1–38}$ was administered by IP injection at a concentration of 100 nM in 200 μl of saline into both PAC1−/− and WT mice. Mice were fasted overnight before they were injected. Whole blood samples were collected 30 min postinjection. Another group of overnight fasted mice was injected with $PACAP_{1–38,}$ or saline as control, and then allowed to feed ad libitum for 30 min before whole blood samples were collected. Plasma samples were obtained from the whole blood collection, and a cocktail of protease inhibitors containing protease inhibitor cocktail tablets with EDTA (Roche, Indianapolis, IN), aprotinin (Pittsburgh, PA), and dipeptidyl peptidase IV (DPP-IV) inhibitor (Millipore, Billerica, MA) was added.

Measurement of plasma hormone levels. Plasma levels of selected metabolic hormones were measured using a mouse metabolic hormone magnetic assay kit (Millipore) according to the manufacturer's instructions. Plasma samples were collected from both PAC1−/− and WT mice groups after either overnight fasting or, in the post prandial group, after overnight fasting followed by ad libitum access to food for 30 min before whole blood samples were collected. The hormones included in the panel were active ghrelin, GLP-1, glucagon, insulin, leptin, and peptide YY (PYY). Each sample was assayed in duplicate on a 96-well plate. Total plasma ghrelin levels were measured using an ELISA kit (Millipore). Analysis of quality control standards provided in the kit matched expectations, and the assay had an inter-assay precision of <25% and an intra-assay precision of <7%.

Statistical analysis. To study dose-dependent effects on cumulative food intake of both $PACAP_{1–38}$ and $PACAP_{1–27}$, IP injected into WT or PAC1−/− mice, compared with vehicle during each hour postinjection, we used a two-way ANOVA model with dose (100 nM, 1 μM, 10 μM, or vehicle), time (hour), and a two-way interaction between dose and time. Similarly, we used a two-way ANOVA model to analyze the 4-h period of food intake. The 24-h cumulative food intake was analyzed using a one-way ANOVA model comparing dose (100 nM, 1 μM, or 10 μM) with vehicle.

The metabolic hormone parameters in $PACAP_{1–38}$ IP injected into overnight-fasted WT mice were analyzed using an unpaired *t*-test. Metabolic hormone parameters in PAC1−/− vs. WT mice were analyzed using a two-way ANOVA model with mouse type, condition (fasted vs. postprandial), and a two-way interaction term between mouse type and condition. All analyses and graphs were conducted using GraphPad Prism 6 Software (La Jolla, CA).

RESULTS

$PACAP_{1–38}$ and $PACAP_{1–27}$ injected IP reduce food intake through the high-affinity PAC1 receptor. $PACAP_{1–38}$ and $PACAP_{1–27}$ IP injected in WT mice, at a dose of 100 nM, 1 μM, or 10 μM in 200 μl of saline, induced a dose-dependent decrease of cumulative food intake during the dark phase of feeding. The time course of PACAP effects on cumulative food intake is illustrated in Fig. 1. In WT mice, cumulative food intake after $PACAP_{1–38}$ injection was significantly reduced in a dose-dependent manner, as assessed using the BioDAQ automated food intake monitoring system for mice. Both $PACAP_{1–38}$ (Fig. 1*A*) and $PACAP_{1–27}$ (Fig. 1*B*) significantly reduced cumulative food intake postinjection, beginning at 3 h with a 10 μM dose, at 4 h with a 1 μM dose, and at 8 h with a 100 nM dose. However, in PAC1−/− mice, no significant reduction in food intake was observed following $PACAP_{1–38}$ at a dose of 100 nM, 1 μM, or 10 μM (Fig. 1*C*). The 24-h total food consumption was reduced following administration of $PACAP_{1–38}$ at 10 μM (1.80 ± 0.20 g; $P < 0.0001$), 1 μM (2.51 ± 0.10 g; $P = 0.0001$) and 100 nM dose (2.72 ± 0.13 g; $P = 0.004$) compared with vehicle (3.25 ± 0.11 g; Fig. 2*A*). Similarly, $PACAP_{1–27}$ treatment reduced the 24-h food consumption in a dose-dependent manner at 10 μM (1.79 ± 0.20 g; $P < 0.0001$), 1 μM (2.23 ± 0.08 g; $P < 0.0001$), and 100 nM (2.66 ± 0.13 g; $P = 0.04$) compared with vehicle (3.10 ± 0.15 g; Fig. 2*B*). In PAC1−/− mice, $PACAP_{1–38}$ treatment at any used dose did not reduce 24-h food intake, and the 24-h food intake was nearly identical to that for vehicle in WT mice (Fig. 2*C*).

The analysis of the murine food intake data subdivided into 4-h time periods revealed a dose-dependent reduction of food intake only in $PACAP_{1–38}$ or $PACAP_{1–27}$ IP injected in WT but not in PAC1−/− mice. $PACAP_{1–38}$ injections induced a re-

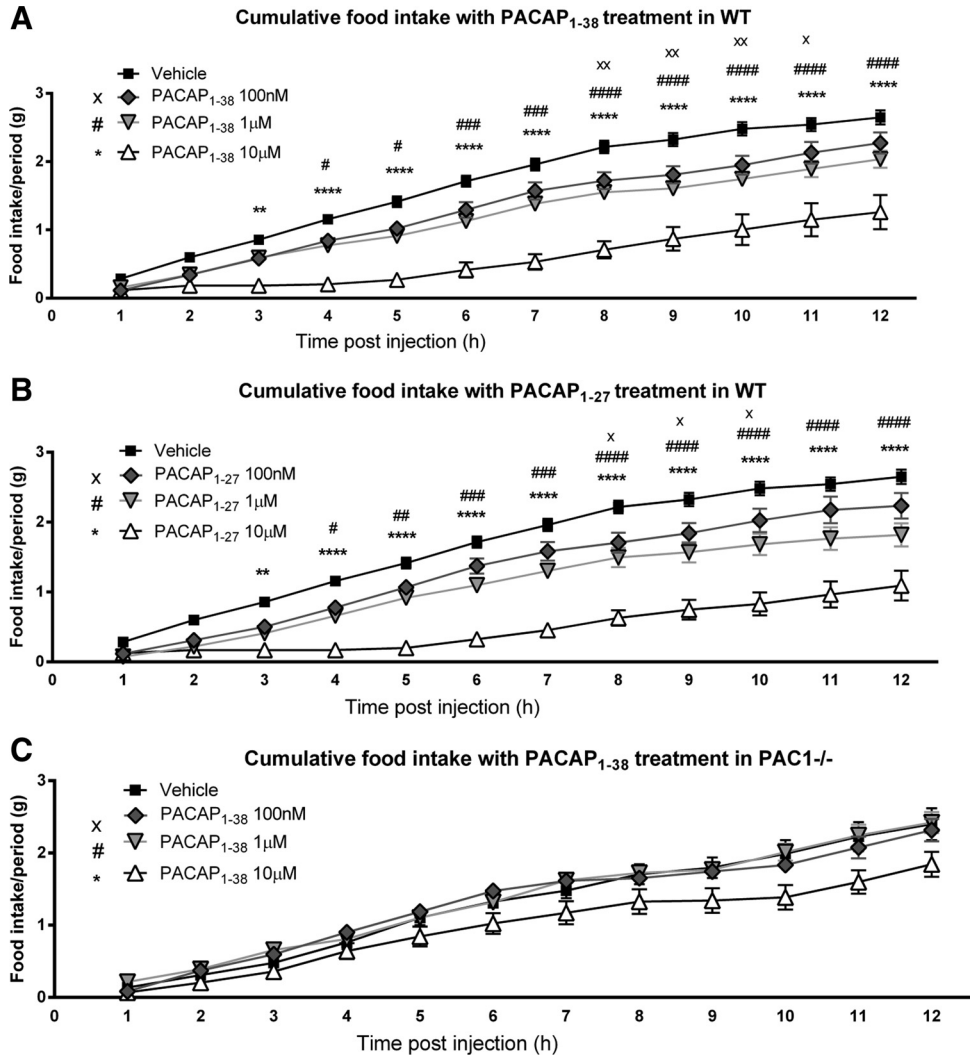

Fig. 1. Pituitary adenylate cyclase-activating peptide (PACAP) variants $PACAP_{1–38}$ and $PACAP_{1–27}$ intraperitoneally (IP) injected at a dose of 100 nM, 1 μM, or 10 μM in 200 μl of saline induced a decrease in cumulative food intake in a dose-dependent manner during the dark phase of feeding in wild-type (WT) but not PAC1-deficient (PAC1−/−) mice. *A*: $PACAP_{1–38}$ injection in WT mice significantly reduced cumulative food intake in a dose-dependent manner, beginning at 3 h with a 10 μM dose, at 4 h with a 1 μM dose, and at 8 h with a 100 nM dose. *B*: $PACAP_{1–27}$ injection in WT mice significantly reduced manner cumulative food intake in a dose-dependent, beginning at 3 h with a 10 μM dose, at 4 h with a 1 μM dose, and at 8 h with a 100 nM dose. *C*: no significant reduction in food intake was observed following $PACAP_{1–38}$ injection at 100 nM, 1 μM, or 10 μM dose in PAC1−/− mice. Data are means ± SE of 16 mice/group. $^{**}P < 0.01$; $^{****}P < 0.0001$, 10 μM vs. vehicle. $\#P < 0.05$; $\#\#P < 0.01$; $\#\#\#P < 0.001$; $\#\#\#\#P < 0.0001$, 1 μM vs. vehicle. $\times P < 0.05$; $\times\times P < 0.01$, 100 nM vs. vehicle.

duction in food intake after 0–4 h postinjection at 100 nM (0.85 ± 0.08 g; $P < 0.0001$), 1 μM (0.92 ± 0.07 g; $P < 0.0001$), and 10 μM (0.14 ± 0.04 g; $P = 0.005$) compared with vehicle (1.16 ± 0.05 g; Fig. 3*A*). WT mice, injected at a higher dose of 10 μM $PACAP_{1–38}$, continued to show a reduced food intake for additional 4–8 h postinjection compared with vehicle (0.44 ± 0.09 vs. 1.00 ± 0.10 g; $P < 0.0001$). $PACAP_{1–27}$ injection induced a reduction in food intake beginning at 0–4 h postinjection at doses of 1 μM (0.63 ± 0.08 g; $P < 0.0001$) and 10 μM (0.38 ± 0.07 g; $P = 0.03$) compared with vehicle (0.95 ± 0.05 g; Fig. 3*B*). A $PACAP_{1–27}$-induced effect of food intake reduction was also observed 4–8 h postinjection at 1 μM (0.85 ± 0.11 g; $P = 0.05$) and 10 μM (0.62 ± 0.07 g; $P < 0.0001$) compared with vehicle (1.15 ± 0.09 g). In PAC1−/− mice, IP injections of $PACAP_{1–38}$ failed to cause significant alteration of food intake (Fig. 3*C*).

Analysis of the feeding microstructure in the studied mice, using an automated episodic feeding monitoring system, showed that $PACAP_{1–38}$ altered all the examined feeding behavior parameters. $PACAP_{1–38}$ significantly reduced bout duration during the first 0–4 h postinjection at 1 μM (80.87 ± 7.35 min; $P = 0.01$) and 10 μM (22.47 ± 6.16 min; $P < 0.0001$) compared with vehicle (108.10 ± 9.40 min) and also 4–8 h postinjection at a 10 μM dose (45.43 ± 9.06 min; $P < 0.0001$) compared with vehicle (102.03 ± 8.26 min; Fig. 4*A*). Similarly, $PACAP_{1–38}$ significantly reduced bout frequency during the first 0–4 h postinjection at 10 μM (15.13 ± 3.72 bouts; $P < 0.0001$) compared with vehicle (41.56 ± 2.67 bouts) and 4–8 h postinjection (15.13 ± 2.77 bouts; $P < 0.0001$) compared with vehicle (36.40 ± 1.97 bouts; Fig. 4*B*). The percentage of time spent feeding was also significantly reduced in the first 0–4 h post $PACAP_{1–38}$ injection at 1 μM (33.69 ± 3.06%; $P = 0.01$) and 10 μM (9.36 ± 2.57%; $P < 0.0001$) compared with vehicle (45.04 ± 3.92%) and also 4–8 h postinjection at 10 μM (18.92 ± 3.78%; $P < 0.0001$) compared with vehicle (42.52 ± 3.44%; Fig. 4*C*). Meal structure and pattern were therefore significantly reduced in $PACAP_{1–38}$ IP injected mice. Meal size was significantly decreased in the first 0–4 h postinjection at 1 μM (823.36 ± 53.56 mg; $P < 0.0001$) and 10 μM (202.50 ± 47.24 mg; $P < 0.0001$) compared with vehicle (1,310.00 ± 90.74 mg) and 4–8 h postinjection at 10 μM (496.25 ± 92.66 mg; $P = 0.01$) compared with vehicle (951.20 ± 105.74 mg; Fig. 4*D*). The percentage of time spent in meals was significantly reduced 0–4 h postinjection at 1 μM (50.64 ± 4.69%; $P = 0.0002$) and 10 μM (14.26 ± 3.77%; $P < 0.0001$) compared with vehicle

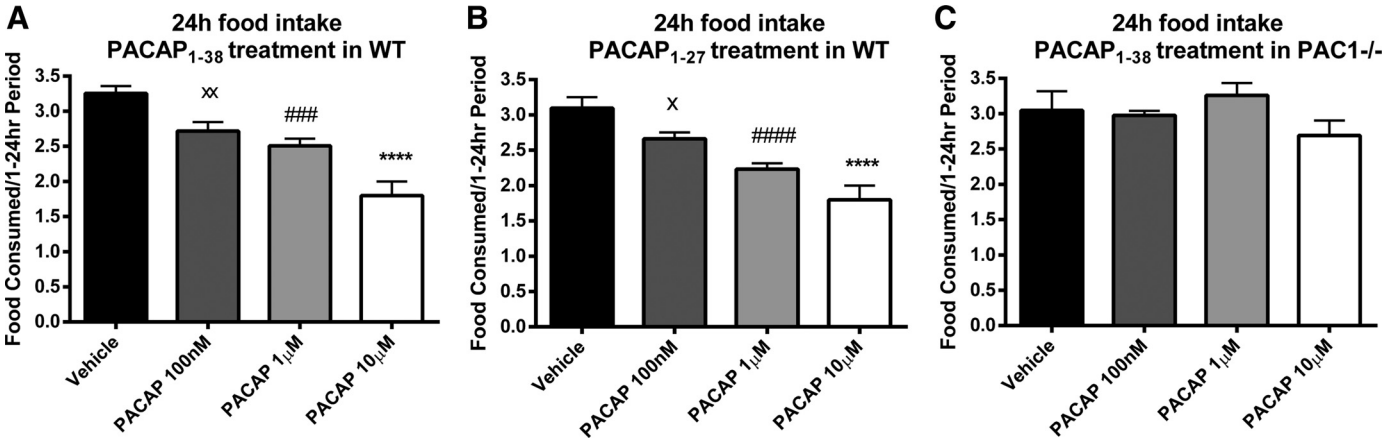

Fig. 2. Twenty-four-hour total food consumption was reduced in a dose-dependent manner following IP injection of either $PACAP_{1–38}$ or $PACAP_{1–27}$ before the dark phase in WT but not in PAC1−/− mice. *A*: $PACAP_{1–38}$ injected in WT mice compared with vehicle. *B*: $PACAP_{1–27}$ injected in WT mice compared with vehicle. *C*: $PACAP_{1–38}$ injected in PAC1−/− mice compared with vehicle. Data are means ± SE of 16 mice/group. ****$P < 0.0001$, 10 μM vs. vehicle. ###$P < 0.001$; ####$P < 0.0001$, 1 μM vs. vehicle. ×$P < 0.05$; ××$P < 0.01$, 100 nM vs. vehicle.

(75.24 ± 7.79%; Fig. 4*E*). Similarly, the total meal time was significantly reduced 0–4 h post injection at 1 μM (121.54 ± 11.25 min; $P < 0.0001$) and 10 μM (34.23 ± 9.04 min; $P = 0.0002$) compared with vehicle (180.59 ± 18.71 min; Fig. 4*F*). The meal duration was also significantly reduced 0–4 h postinjection at 1 μM (39.11 ± 8.21 min; $P = 0.0002$) and 10 μM (19.64 ± 4.58 min; $P < 0.0001$) compared with vehicle (90.43 ± 19.16 min; Fig. 4*G*). Finally, the eating rate was significantly reduced 0–4 h postinjection at 100 nM (2.61 ± 0.55 mg/min; $P < 0.0001$), 1 μM (3.53 ± 0.23 mg/min; $P < 0.0001$), and 10 μM (0.84 ± 0.20 mg/min; $P < 0.0001$) compared with vehicle (5.47 ± 0.35 mg/min) and also 4–8 h postinjection at 100 nM (2.19 ± 0.44 mg/min; $P < 0.0001$), 1 μM (2.98 ± 0.22 mg/min; $P = 0.02$), and 10 μM (2.22 ± 0.42 mg/min; $P = 0.002$) compared with vehicle (4.16 ± 0.36 mg/min; Fig. 4*H*).

$PACAP_{1–38}$ IP injection suppressed active ghrelin and active ghrelin/total ghrelin levels in overnight fasting WT mice. $PACAP_{1–38}$ at 1 μM dose significantly reduced active ghrelin plasma levels compared with vehicle-injected controls (306.90 ± 66.78 vs. 533.40 ± 79.81 pg/ml; $P = 0.04$; Fig. 5*A*). Accordingly, the percentage of active ghrelin/total ghrelin was significantly lower following PACAP injection compared with vehicle (7.22 ± 1.41 vs. 16.59 ± 3.44%; $P = 0.03$; Fig. 5*B*). However, $PACAP_{1–38}$ did not affect significantly the plasma levels of the following metabolic hormones: GLP-1 (89.49 ± 5.94 vs. 82.61 ± 9.35 pg/ml; Fig. 5*C*), insulin (725.10 ± 161.30 vs. 650.60 ± 139.40 pg/ml; Fig. 5*D*), glucagon (169.0 ± 8.32 vs. 172.0 ± 14.8 pg/ml; Fig. 5*E*), and PYY (198.30 ± 8.53 vs. 177.00 ± 17.66 pg/ml; Fig. 5*F*).

PAC1−/− mice had altered plasma levels of metabolic hormones. PAC1−/− mice had significantly elevated levels of active ghrelin during both fasting (711.60 ± 140.90 vs. 384.60 ± 61.68 pg/ml; $P = 0.04$) and postprandial conditions (410.90 ± 96.02 vs. 90.28 ± 43.98 pg/ml; $P = 0.006$; Fig. 6*A*). Accordingly, the percentage of active ghrelin/total ghrelin was significantly elevated during both fasting (24.70 ± 3.62 vs. 14.62 ± 1.61%; $P = 0.04$) and postprandial conditions (19.21 ± 3.43 vs. 6.48 ± 3.62%; $P = 0.02$) in PAC1−/− mice (Fig. 6*B*). GLP-1 levels were significantly lower in PAC1−/− compared with WT mice during postprandial conditions (18.87 ± 5.99 *vs.* 50.62 ± 11.93 pg/ml; $P = 0.03$) but not during fasting conditions (Fig. 6*C*). Strikingly, plasma insulin levels were significantly lower in PAC1−/− compared with WT mice in both fasting conditions (75.92 ± 24.89 vs. 404.00 ± 65.45 pg/ml; $P = 0.0002$) and, more importantly, in postprandial conditions (150.00 ± 24.01 vs. 2,835.00 ± 479.60 pg/ml; $P < 0.0001$; Fig. 6*D*). Furthermore, plasma leptin levels were significantly lower in PAC1−/− compared with WT mice in both fasting (304.80 ± 176.4 vs. 3,012 ± 697.3 pg/ml; $P = 0.005$) and postprandial conditions (304.8 ± 176.4 vs. 3,012 ± 697.3 pg/ml; $P = 0.005$; Fig. 6*E*). Plasma levels of glucagon and PYY did not present any significant difference between PAC1−/− and WT mice during fasting or postprandial conditions (Fig. 6, *F* and *G*).

DISCUSSION

This study addresses the peripheral role of PACAP and its high-affinity receptor PAC1 in appetite control, feeding behavior, and metabolic hormone regulation. Our data show that both $PACAP_{1–38}$ and $PACAP_{1–27}$, IP injected in WT mice before the beginning of the dark phase, produced a significant long-lasting and dose-dependent decrease in food intake and meal patterns for 24 h postinjection. Although PACAP binds to different receptors, its peripheral anorexigenic effects appear to be mediated primarily by PAC1 receptor activation. In fact, $PACAP_{1–38}$ IP injected in PAC1−/− mice failed to induce a decrease of food intake during the dark phase of feeding. Notably, our data support the hypothesis that the mechanisms underlying PACAP/PAC1 suppression of appetite and feeding behavior are linked to the inhibition of active ghrelin release and the regulation of GLP-1, insulin, and leptin hormone secretion.

The hypothalamus, GI tract, and peripheral organs such as the liver, pancreas, and adipose tissue are parts of an integrated system that regulates appetite and food intake. In the central nervous system (CNS), the greatest density of PACAP and PAC1 occurs in the hypothalamus supraoptic nuclei, VMN, and periventricular nuclei (PVN), areas known to play a critical role in appetite regulation and energy homeostasis, thus sup-

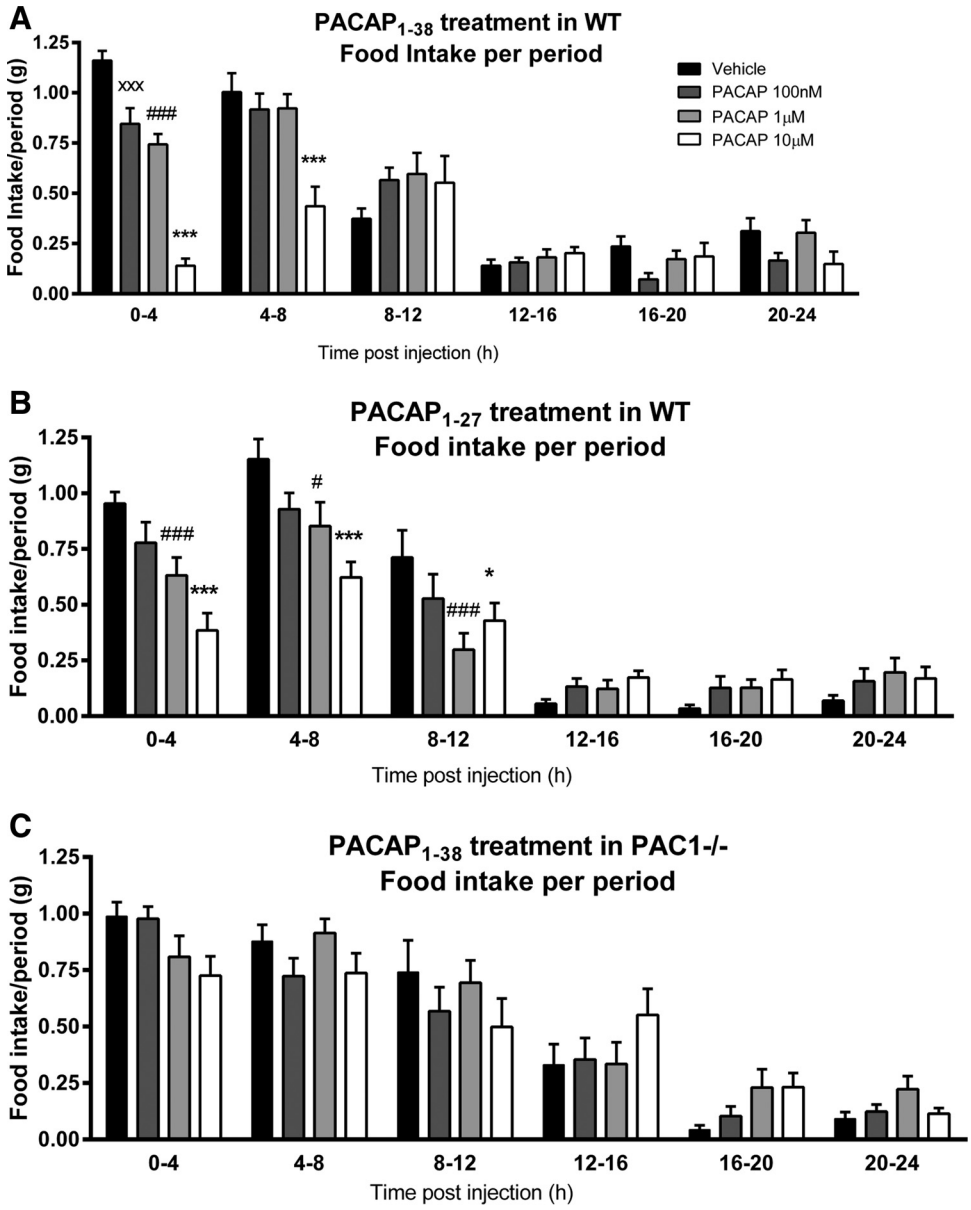

Fig. 3. $PACAP_{1–38}$ and $PACAP_{1–27}$ reduced food intake in a dose-dependent manner following IP injection before the dark phase of feeding in WT but not in PAC1−/− mice. *A*: $PACAP_{1–38}$ injection in WT mice at 100 nM, 1 μM, and 10 μM reduced food intake in a dose-dependent manner 0–4 h postinjection compared with vehicle, and $PACAP_{1–38}$ injected at the higher dose of 10 μM continued to show a reduced food intake for an additional 4–8 h postinjection. *B*: $PACAP_{1–27}$ injection in WT mice at 1 and 10 μM reduced food intake in a dose-dependent manner 0–4 h and 4–8 h postinjection compared with vehicle. *C*: in PAC1−/− mice, IP injections of $PACAP_{1–38}$ failed to cause significant alteration of food intake. Data are means ± SE of 16 mice/group. $****P < 0.0001$, 10 μM vs. vehicle. $\#P < 0.05$; $\#\#\#\#P < 0.0001$, 1 μM vs. vehicle. $\times\times P < 0.01$, 100 nM vs. vehicle.

porting the hypothesis that PACAP/PAC1 pathway is central in the regulation of metabolism and energy balance (18, 44, 47, 54). In the CNS, several studies demonstrated that ICV injected PACAP suppresses appetite and food intake in mice and rats (6, 31, 36, 37, 44) as well as in chicks (50) and goldfishes (32). In the CNS, the hypothalamus has two sites, the PVN and the VMN, which regulate energy homeostasis (28, 52). PACAP and PAC1 receptor mRNA is highly expressed in the hypothalamus, specifically in the arcuate nucleus (ARC), VMN, and dorsomedial nuclei (16, 17). It also has been reported that in the CNS, PACAP and PAC1 mRNA expression are significantly increased by excessive feeding (31). In the VMN, an area of the hypothalamus that upon stimulation enhances satiety and promotes metabolism through the sympathetic nervous system, PACAP mRNA expression decreases during fasting conditions, but it is increased following a high-fat diet (52, 59). The expression of both PACAP and its PAC1 receptor in the hypothalamus suggests that they play a crucial role in the central regulation of feeding behavior via the hypothalamic melanocortin system (39). The central physiological effects of PACAP are mediated through its PAC1 receptor, as confirmed by the use of the PAC1-specific antagonist $PACAP_{6–38}$, which blocks the inhibitory central effects of PACAP on food intake (37, 39, 44, 45). Furthermore, these enhancing satiety effects were replicated by using the PAC1 receptor-specific agonist maxadilan (38), which was shown to significantly suppress food intake in mice (60). Previous studies showed that a single 1-μg dose of PACAP, injected centrally into the VMN before the nocturnal phase of feeding, produced a significant body weight loss 24 h postinjection (18). Similarly, when PACAP was administered centrally in the posterior region of the stria terminalis bed nucleus, weight loss was observed for 24 h postinjection in a dose-dependent manner (24). Recently, PACAP and thyrotropin-releasing hormone (TRH) were shown to be crucial in the ARC to the excitatory drive of the agouti-related peptide-expressing neurons that control hunger (27).

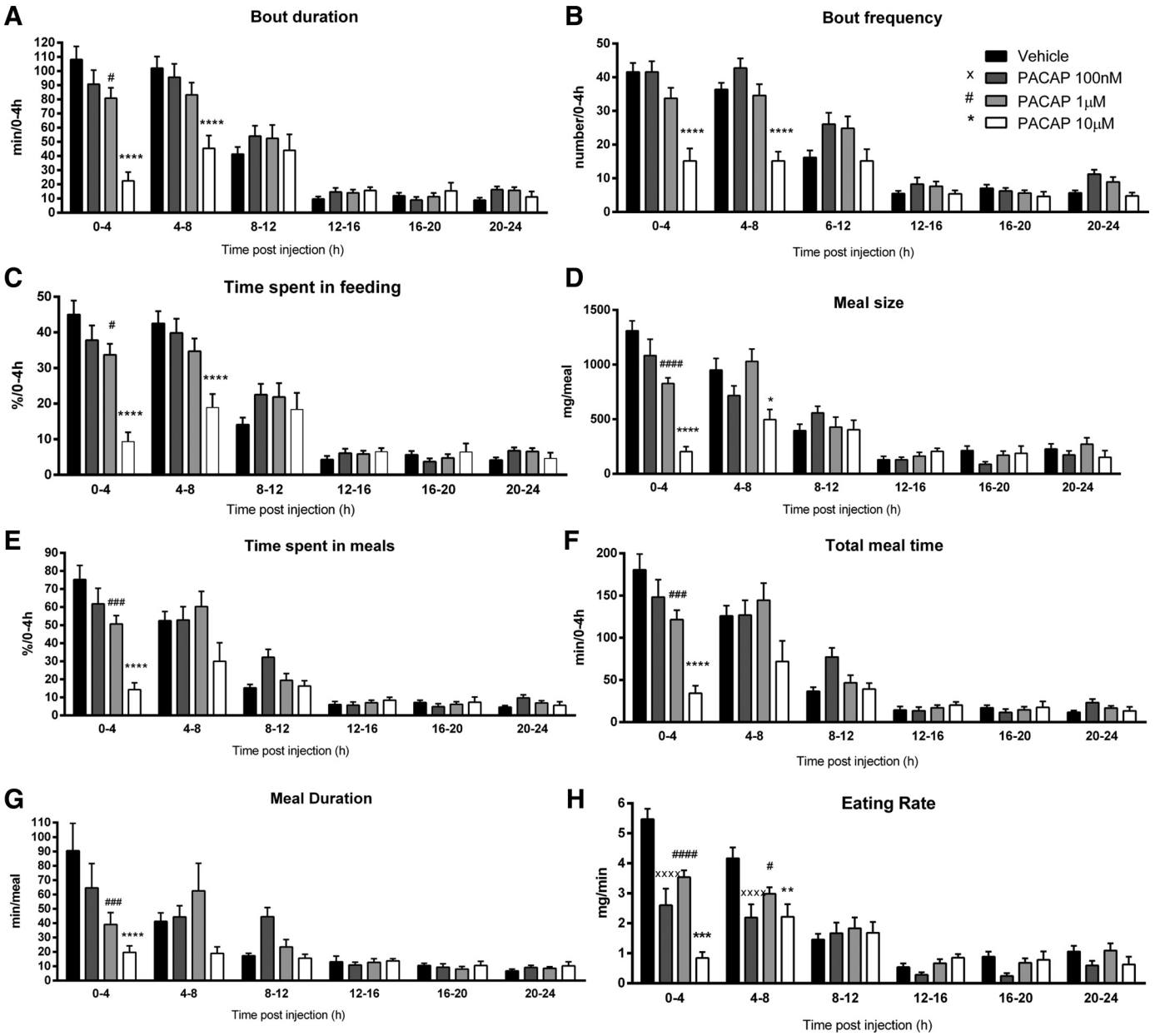

Fig. 4. Analysis of the feeding microstructure in the studied mice, using an automated episodic feeding monitoring system, showed that $PACAP_{1–38}$ injection significantly altered all the examined feeding behavior parameters compared with vehicle. A: bout duration was reduced during the first 0–4 h and 4–8 h post $PACAP_{1–38}$ injection. B: bout frequency was reduced during the first 0–4 h and 4–8 h post $PACAP_{1–38}$ injection. *C*: time spent in feeding was also reduced during the first 0–4 h and 4–8 h post $PACAP_{1–38}$ injection. *D*: meal size was decreased in the first0-4 h and 4–8 h post $PACAP_{1–38}$ injection. *E*: time spent in meals was reduced 0–4 h post $PACAP_{1–38}$ injection. *F*: total meal time was reduced 0–4 h post $PACAP_{1–38}$ injection. *G*: meal duration was also reduced 0–4 h post $PACAP_{1–38}$ injection. *H*: eating rate was reduced 0–4 h and 4–8 h post $PACAP_{1–38}$ injection. Data are means ± SE of 16 mice/group. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$, 10 μM vs. vehicle. $\#P < 0.05$; $\#\#\#P < 0.001$; $\#\#\#\#P < 0.0001$, 1 μM vs. vehicle. $\times\times\times\times P < 0.0001$, 100 nM vs. vehicle.

In the GI tract, PACAP and its PAC1 receptor have been shown to play major physiological effects in the GI tract. PACAP and PAC1 immunoreactivity was localized in the myenteric ganglia and nerve fibers in the longitudinal smooth muscle layers of the esophagus, stomach, and small and large intestines (3, 26, 33, 46). In this study, we demonstrate that peripherally administered PACAP affects food intake up to 24 h.

Our results demonstrate that PACAP is a novel peripheral nutrient sensor that regulates food intake and satiety through its high-affinity PAC1 receptor, which inhibits active ghrelin levels. Previously, PACAP and PAC1 were found expressed on gastric nerves (33), and PAC1, localized on gastric enterochromaffin-like (ECL) and parietal cells in the gastric mucosa, was shown to regulate acid secretion (30, 61) through somatostatin release, activation of somatostatin receptor 2 (41), and stimulation of pepsinogen release from chief cells (7). In this study, we demonstrated that PACAP IP administration inhibits active ghrelin secretion in overnight-fasted mice. Additionally, we found in PAC1−/− mice a dysregulation in active ghrelin secretion during both fasting and postprandial conditions. In PAC1−/− mice, ghrelin levels remained elevated even after

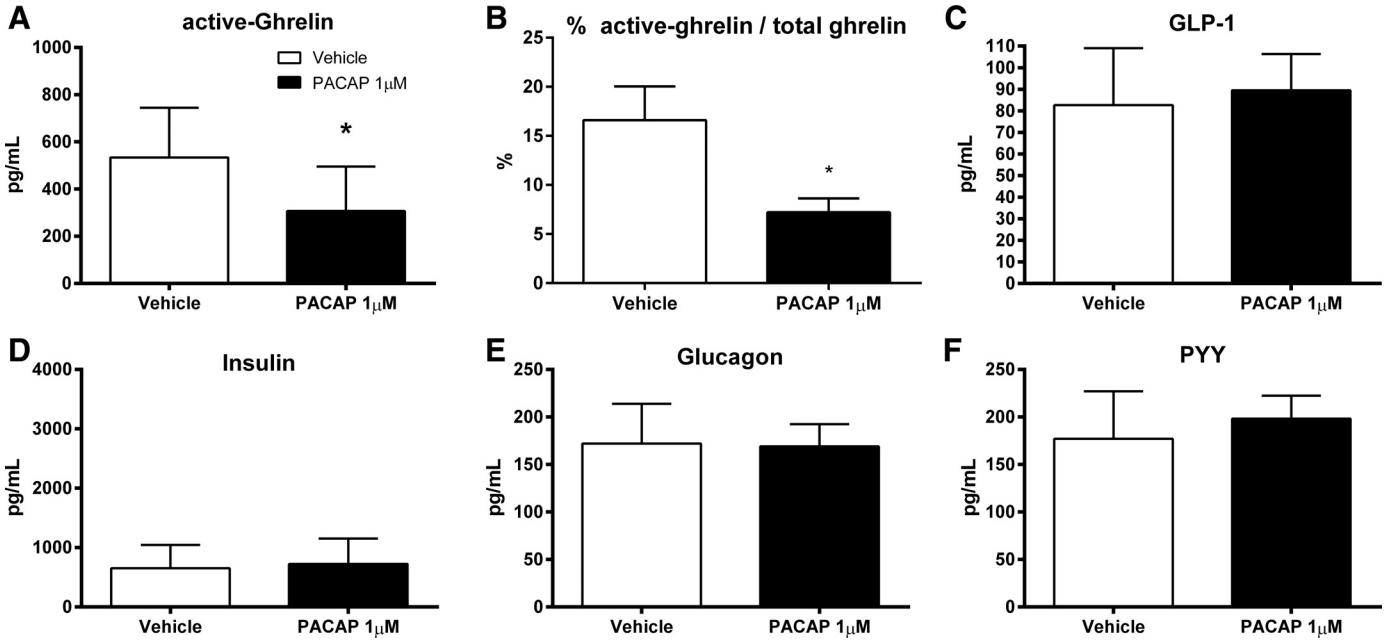

Fig. 5. $PACAP_{1–38}$ reduced plasma active ghrelin levels in fasted WT mice. Mice were fasted overnight before 1 μM of $PACAP_{1–38}$ in 200 μl of saline was injected. Blood plasma was withdrawn 30 min postinjection. *A*: $PACAP_{1–38}$ injected into fasted WT mice significantly reduced plasma active ghrelin compared with vehicle. *B*: percentage of ratio of plasma active ghrelin to total ghrelin. *C–F*: plasma levels of glucagon-like peptide-1 (GLP-1; *C*), insulin (*D*), glucagon (*E*), and peptide YY (PYY; *F*) were not significantly altered compared with vehicle. Data are means ± SE of 8 mice/group. $*P < 0.05$.

feeding. In the GI tract, ghrelin is predominantly found in the stomach, where it is mainly produced by the PD/1 endocrine cells of the gastric oxyntic mucosa and, by binding to the GHS-R1a receptor, stimulates food intake (25, 58). In the gastrointestinal tract, the primary role of ghrelin is to stimulate food intake and modulate energy expenditure (58). In both rodents and in humans, plasma ghrelin levels are elevated during fasting and decreased after feeding (54). There are two known forms of ghrelin: a 28-amino acid peptide with an *n*-octanoylated serine in position 3, acyl-ghrelin (active ghrelin), and a des-acylated [des-(Gln14)] ghrelin. Desacyl ghrelin is considered the inactive form of ghrelin because it does not activate the ghrelin receptor (GHS-R1a) and does not induce the same endocrine effects of acyl ghrelin (19). Ghrelin has

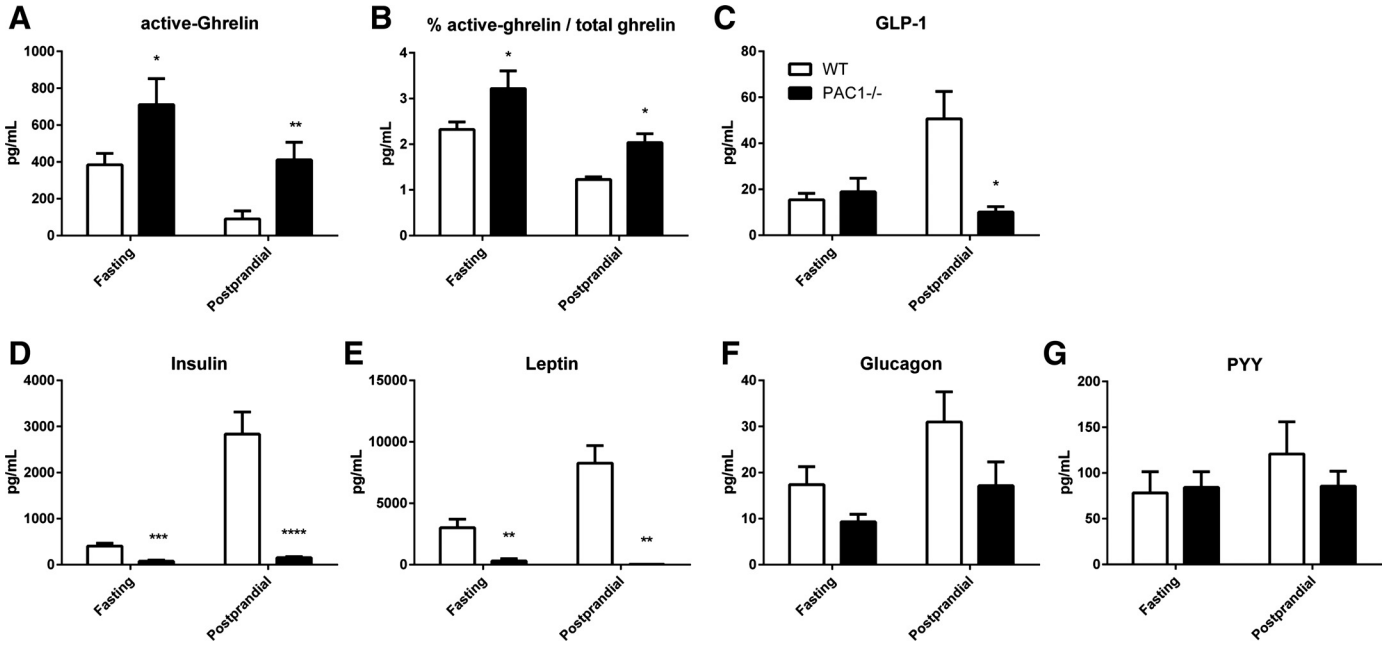

Fig. 6. Differences in metabolic hormones in fasting and postprandial conditions between PAC1−/− and WT mice. In fasting conditions, mice were fasted overnight and plasma was withdrawn. In postprandial conditions, mice were fasted overnight and refed for 30 min before plasma was withdrawn. Plasma levels of active ghrelin (*A*), percentage of active ghrelin/total ghrelin (*B*), GLP-1 (*C*), insulin (*D*), leptin (*E*), glucagon (*F*), and PYY (*G*) were assessed in PAC1−/− compared with WT mice. Data are means ± SE of 8 mice/group. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

also been detected in the small intestine, hypothalamus, pituitary gland, pancreas, heart, and adipose tissue (11, 19, 25, 55, 57). Currently, the mechanisms regulating ghrelin secretion are not fully understood; however, peptide hormones such as oxytocin and dopamine have been reported to regulate in vitro ghrelin secretion (20). Dopamine, which is substantially produced in the GI tract, was demonstrated to stimulate ghrelin secretion via its D1A receptors (20). Although PACAP and PAC1 expression has not yet been identified on PD/1 cells of the gastric fundus, PAC1 receptors have been characterized on ECL cells (61), which have been shown to secrete ghrelin (48). Further knowledge on ghrelin secretion processes is currently limited due to the difficulty of pure gastric mucosa cells isolation techniques (22).

In this work we support the hypothesis that PACAP and PAC1 are able to modulate appetite and feeding behavior through the regulation of metabolic hormone pathways. Appetite and feeding behavior are controlled by a series of hormonal and neural signals, which are generated in the GI tract to provide feedback to the CNS on the availability of nutrients. PACAP has been found in vivo to release insulin (9) and glucagon (59) from the pancreas, glucocorticoids from the adrenal cortex (40), growth hormone from the pituitary gland (50), and catecholamine from the adrenal medulla (15). PAC1−/− mice have a dysregulation in insulin secretion, as confirmed in the current study by plasma insulin levels measured in fasting and postprandial conditions (21). It has been reported that PAC1 receptors are expressed on pancreatic β-cells (9, 21) and stimulate insulin secretion; therefore, they can have a protective role in type 1 diabetes. Our results demonstrate that in the PAC1−/− mice, there is a reduction in insulin responsiveness to meal ingestion, thus confirming previously reported studies that showed PACAP insulinotropic effect. In PAC1−/− there was no increase in postprandial plasma insulin levels, in such a way as to confirm that in physiological conditions, PAC1 receptor is responsible for the control of insulin secretion after food intake (8, 10, 21). No significant differences were noted between the studied WT and PAC1−/− mice groups in food intake at baseline. Thus we cannot conclude whether endogenous PACAP has effects on appetite. We also found that PAC1 is involved in the regulation of leptin, which was abolished in PAC1−/− mice during both fasting and postprandial conditions. Leptin exerts its effects on food intake and metabolic rate by acting on hypothalamic neurons (18). PACAP is an important mediator of the leptin effects in the CNS, since PACAP mRNA was significantly reduced in fasting leptin knockout *ob*/*ob* mice but increased following an ICV injection of leptin (18). Furthermore, in PACAP knockout mice (PACAP−/−), an ICV injection of leptin had no significant effect in modulating feeding behavior (53). PAC1 has been shown to be an important player in leptin-induced anorexia, body weight loss, and body temperature modulation (18, 53). Leptin is mainly synthesized by the adipose tissue that expresses PAC1 receptors (56). We showed that exogenous PACAP affected primarily ghrelin plasma levels, whereas in the PAC1−/− mouse there was a decrease in leptin, elevated ghrelin, and reduced GLP-1 and basal insulin levels; PACAP IP treatment reduced only serum ghrelin levels. These results suggest that PACAP may act at other receptors such as VPAC1 or VPAC2, especially in its high-affinity PAC1 receptor-deficient mice. Therefore, future studies targeting the PACAP/PAC1 signaling pathways to block the hypertensive effect of leptin while preserving its metabolic and anorexigenic effects may be a potential novel therapeutic target for treating both obesity and hypertension. Ghrelin, physiologically released during fasting conditions, is known to be the most potent orexigenic hormone. Consequently, on the basis of our data documenting PACAP-induced suppression of ghrelin plasma levels even following fasting conditions, we support the hypothesis that this is the main mechanism underlying PACAP anorexigenic effects. Characterization of PACAP and PAC1 roles in energy homeostasis has provided insights into the widespread and complex physiological role of PACAP. Several studies using PACAP−/− mice have demonstrated that lack of PACAP results in abnormalities of metabolic homeostasis. PACAP is crucial for thermoregulation, because PACAP−/− mice had a greater loss of core body temperature due to insufficient norepinephrine stimulation of brown adipose tissue (13). Also, PACAP−/− mice had an altered thyroid hormone axis leading to lower mRNA levels of TRH and brown adipose tissue type 2 deiodinase (1). Physiologically, PACAP−/− mice have been reported to be leaner than their littermates on a regular chow diet at 21°C due to decreased adiposity; however, this difference was eliminated at 28°C (1, 12). Furthermore, PACAP−/− mice had higher concentrations of serum triglycerides and cholesterol (44). PACAP injections into the VMN of WT mice increased core body temperature and spontaneous locomotor activity (44) as well as brown adipose uncoupling protein 1 mRNA expression, thus confirming PACAP's role in thermogenesis. In vitro, PACAP was able to stimulate adipogenesis in NIH 3T3-L1 preadipocyte cell line by increasing cAMP production and phosphorylation of MAPK (ERK1/2) (4). Therefore, PACAP can mediate brown fat tissue adipogenesis, possibly through its PAC1 receptor, given that PAC1 mRNA and receptors expression were significantly upregulated following PACAP stimulation (4).

In conclusion, our results suggest that peripheral PACAP is a potential novel appetite suppressant and regulator of orexigenic/anorexigenic hormones. The central and peripheral PACAP/PAC1 regulation of appetite/satiety and feeding behavior further supports the emerging concept of the GI tract as an endocrine organ with an essential sensing and signaling role toward the physiological body energy homeostasis. Previously, in prior studies, PACAP administered intravenously was able to cross the blood-brain barrier (5). Therefore, although in our studies we did not specifically investigate whether this would occur following IP injection, we cannot completely exclude a central effect of PACAP. Most importantly, the effects of PAC1 receptor activation that lead to appetite suppression, through inhibition of active ghrelin and regulation of leptin and insulin secretion, point to a novel potential approach for treating obesity and insulin resistance in the future by using PAC1 agonists. The long-lasting satiety-inducing effect of PACAP via PAC1 observed in our study is of particular interest for future clinical studies. PACAP has been reported to have poor metabolic stability and a half-life of minutes in the blood circulation, where it is catabolized by DEPP-IV (29, 62). Future studies should explore the potential use of PACAP in combination with DPP-IV inhibitors, which are already utilized in clinical trials for appetite disorders and obesity treatments (2) to enhance the anorexigenic and metabolic effects of PACAP. With all findings taken together, the current report

supports evidence that the PACAP/PAC1 pathway is a potential regulator of appetite/satiety, body metabolism, and energy expenditure even in the periphery, thus establishing a rationale for a potential use of PACAP/PAC1 agonists as novel pharmacological agents in the treatment of appetite disorders as well as obesity and its associated pathologies.

The discovery that PACAP via PAC1 not only induces satiety but also regulates energy expenditure suggests that PACAP analogs may be promising therapeutic agents for obesity treatment and need to be further explored. In summary, PACAP administered peripherally suppresses ghrelin plasma levels and appetite in such a way as to enhance satiety.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

J.P.V., D.G., J.R.P., and P.G. conception and design of research; J.P.V., D.G., L.L., S.O., R.S., J.R.P., and P.G. performed experiments; J.P.V., D.G., L.L., S.O., R.S., J.N., W.P., J.R.P., and P.G. analyzed data; J.P.V., D.G., L.L., S.O., R.S., J.N., J.R.P., and P.G. interpreted results of experiments; J.P.V., D.G., L.L., S.O., J.N., W.P., J.R.P., and P.G. prepared figures; J.P.V., D.G., R.S., J.R.P., and P.G. drafted manuscript; J.P.V., D.G., L.L., S.O., R.S., J.N., W.P., J.R.P., and P.G. edited and revised manuscript; J.P.V., D.G., L.L., S.O., R.S., J.N., W.P., J.R.P., and P.G. approved final version of manuscript.

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