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Pituitary Adenylate Cyclase-Activating Polypeptide Inhibits Food Intake in Mice Through Activation of the Hypothalamic Melanocortin System

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Pituitary adenylate cyclase-activating polypeptide (PACAP) and the proopiomelanocortin (POMC)-derived peptide, α-melanocyte-stimulating hormone (α-MSH), exert anorexigenic activities. While α-MSH is known to inhibit food intake and stimulate catabolism *via* activation of the central melanocortin-receptor MC4-R, little is known regarding the mechanism by which PACAP inhibits food consumption. We have recently found that, in the arcuate nucleus of the hypothalamus, a high proportion of POMC neurons express PACAP receptors. This observation led us to investigate whether PACAP may inhibit food intake through a POMC-dependent mechanism. In mice deprived of food for 18 h, intracerebroventricular administration of PACAP significantly reduced food intake after 30 min, and this effect was reversed by the PACAP antagonist PACAP6-38. In contrast, vasoactive intestinal polypeptide did not affect feeding behavior. Pretreatment with the MC3-R/MC4-R antagonist SHU9119 significantly reduced the effect of PACAP on food consumption. Central administration of PACAP induced c-Fos mRNA expression and increased the proportion of POMC neuron-expressing c-Fos mRNA in the arcuate nucleus. Furthermore, PACAP provoked an increase in POMC and MC4-R mRNA expression in the hypothalamus, while MC3-R mRNA level was not affected. POMC mRNA level in the arcuate nucleus of PACAP-specific receptor (PAC1-R) knock-out mice was reduced as compared with wild-type animals. Finally, i.c.v. injection of PACAP provoked a significant increase in plasma glucose level. Altogether, these results indicate that PACAP, acting through PAC1-R, may inhibit food intake *via* a melanocortin-dependent pathway. These data also suggest a central action of PACAP in the control of glucose metabolism.
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INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP) has been initially isolated from the ovine hypothalamus by virtue of its ability to stimulate adenylyl cyclase activity in anterior pituitary cells (Miyata *et al*, 1989, 1990). PACAP is a member of the vasoactive intestinal polypeptide (VIP)/secretin/glucagon/growth hormone-releasing hormone peptide family (Sherwood *et al*, 2000). PACAP exerts a wide range of biological effects notably in the brain where the peptide acts as a neuromodulator, a neurohormone and a neuroprotective agent (Vaudry *et al*, 2000). The multiple actions of PACAP are mediated through interaction with three types of receptors that is the PACAP-specific receptor (PAC1-R), and the PACAP/VIP mutual receptors VPAC1-R and VPAC2-R (Ishihara *et al*, 1992; Lutz *et al*, 1993; Spengler *et al*, 1993).

PACAP and its receptors are actively expressed in the hypothalamus, which plays a pivotal role in the control of appetite and energy homeostasis (Masuo *et al*, 1993;

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Usdin *et al*, 1994; Hannibal *et al*, 1995; Sheward *et al*, 1995; Piggins *et al*, 1996; Shioda *et al*, 1997). In fact, it has been previously shown that intracerebroventricular (i.c.v.) injection of PACAP reduces food intake in vertebrates (Morley *et al*, 1992; Chance *et al*, 1995; Mizuno *et al*, 1998; Tachibana *et al*, 2004; Matsuda *et al*, 2005). Notably, in rat, PACAP provokes a long-lasting reduction of food consumption, resulting in a marked decrease in body weight (Mizuno *et al*, 1998). There is now clear evidence that α-melanocyte-stimulating hormone (α-MSH), one of the major neuropeptides produced by proopiomelanocortin (POMC) neurons of the arcuate nucleus, exerts a potent anorexigenic activity (Jégou *et al*, 2006). Central administration of α-MSH reduces food intake and body weight through activation of the hypothalamic melanocortin MC3 and MC4 receptor subtypes (MC3-R and MC4-R) (Fan *et al*, 1997; Huszar *et al*, 1997; Yaswen *et al*, 1999; Butler *et al*, 2000; Chen *et al*, 2000). Consistent with a pivotal role of the melanocortin system in the control of food consumption and energy homeostasis, disruption of the MC4-R gene in mice causes hyperphagia and obesity (Huszar *et al*, 1997), while MC3-R gene-deficient mice have normal food consumption but accumulate fat (Chen *et al*, 2000). Indeed, obesity is more severe in mice lacking both MC3-R and MC4-R than in MC4-R-deficient animals (Chen *et al*, 2000), indicating that the two receptors play complementary roles in the control of energy balance.

We have recently found that in the arcuate nucleus of the hypothalamus, POMC neurons express PAC1-R and VPAC2-R mRNAs, and we have shown that, *ex vivo*, PACAP stimulates the expression of the POMC gene and triggers α-MSH release from mediobasal hypothalamic explants (Mounien *et al*, 2006a). However, the *in vivo* effects of PACAP on the hypothalamic melanocortin system have never been assessed. The aim of the present study was thus to investigate the possible implication of the central melanocortin system in the anorexigenic action of PACAP.

MATERIALS AND METHODS

Animals

Experiments were carried out on different types of mice: Swiss albinos CD1 mice (Charles Rivers/IFFA-CREDO, Saint-Germain sur l'Arbresle, France), C57BL/6 control PAC1-$R^{+/+}$ mice and C57BL/6 PAC1-$R^{-/-}$ mice (from Institute of Functional Genomics, CNRS UMR 5203, Montpellier, France). Generation of PAC1-R knockout (PAC1-$R^{-/-}$) mice has been described earlier (Jamen *et al*, 2000). Male Swiss albinos CD1 mice and male C57BL/6 PAC1-$R^{+/+}$ and PAC1-$R^{-/-}$ mice, weighing 25–30 g, were housed in Makrolon cages (L: 40 cm, W: 25 cm, H: 18 cm), with free access to water and food (UAR, Villemoisson-sur-Orge, France) and kept in a well ventilated room, at a temperature of $22 \pm 1^{\circ}C$, under a 12-h light:12-h dark cycle. Animal manipulations were performed according to the European Communities Council Directive of November 24, 1986 (86:609:EEC), were approved by the local Ethical Committee (authorization numbers: N/10-04-04-12 and N/13-04-04-15) and were conducted by authorized investigators.

Chemicals

PACAP38 was synthesized by solid-phase methodology as previously described (Chartrel *et al*, 1991). The synthetic peptide was purified by RP-HPLC and characterized by MALDI-TOF MS. VIP, the peptidergic PACAP receptor antagonist PACAP6-38 and the melanocortin receptor antagonist SHU9119 (Ac-Nle^4-c[Asp^5, D-2′Nal^7, Lys^{10}]α-MSH-4-10)-NH_2) were purchased from NeoMPS (Strasbourg, France). The peptides were dissolved in saline (0.9% NaCl) just before i.c.v. injection.

Collection of Tissues

Mice were killed by decapitation. For quantitative reverse transcription-polymerase chain reaction (RT-PCR), the brains were removed and sliced by means of an ice-chilled plexiglass holder, and the hypothalami were dissected out. The hypothalamic explants were excised with fine scissors from immediately behind the optic chiasma to the anterior limit of the mammilary bodies and laterally at the level of the hypothalamic sulcus. Dissected tissues were immediately frozen on dry ice and stored at $-80^{\circ}C$ until homogenization. For *in situ* hybridization, brains were collected, frozen immediately in isopentane ($-30^{\circ}C$) and stored at $-80^{\circ}C$ until use.

Intracerebroventricular Injection

Central administration (10 μl per mouse) of saline or peptide solutions (VIP, PACAP alone or in combination with PACAP6-38) was made, free hand, in the ventricle of Swiss albinos CD1 mice, according to the procedure of Haley and McCormick (1957), similar to that employed by Fichna *et al* (2007) and Do Rego *et al* (2007). To evaluate the effect of SHU9119 on PACAP-induced inhibition of food intake, mice were injected i.c.v. (5 μl) in the left ventricle with saline or with SHU9119 10 min before i.c.v. injection (5 μl) in the right ventricle of saline or PACAP. Control mice were injected with saline (5 μl) in each ventricle 10 min apart. The injection was made with a 50-μl microsyringe (Hamilton, Bonaduz, Switzerland) connected to a needle (external diameter 0.5 mm), of which the bevel protruded only 3.5 mm from a guard limiting its penetration into the brain. The injection in manually immobilized mice lasted approximately 5 s. Intracerebroventricular injections were performed by an experienced investigator. The accuracy of the i.c.v. injection was controlled regularly by using a methylene blue dye and verifying on frontal brain sections the site of injection. In more than 95% of these controls, the ventricle was labeled with the dye. It was also verified on several animals, after killing, that the mark of the needle puncture on the parietal bone was located at least 1.5 mm behind bregma and at least 2.5 mm before lambda, with a laterality between 1 and 2 mm relatively to the brain median line. This corresponds to stereotaxic coordinates of the left lateral ventricle in the Lehmann's atlas for mice: anteriority between 2.95 and 4.15 mm; laterality between 0.95 and 2 mm; and depth between 3 and 4.5 mm.

Food Consumption Experiments in Food-Deprived Mice

Two days before the experiments, Swiss albinos CD1 mice were isolated in individual cages (L, 24 cm; W, 10 cm; H,

10 cm), with free access to water, and the food was directly deposited on the floor of the cages, to accustom the animals to the tests. Eighteen hours before the experiments (1500–0900 h), mice were totally deprived of food but had access to tap water *ad libitum*. Ten minutes after i.c.v. administration of either vehicle or peptide solution, each mouse had access to a weighed food pellet (5 g). The pellet was briefly (< 20 s) removed and weighed every 30 min during the test period.

Quantitative Reverse Transcription-polymerase Chain Reaction Analysis

Total hypothalamic RNAs were extracted by the acid guanidinium-thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) using Tri-Reagent (Sigma-Aldrich, Saint-Quentin Fallavier, France). Contaminating DNA was removed by treatment with RNase-free DNase I (Promega, Charbonnières, France), and cDNA was synthesized with an ImProm-II Reverse Transcription System (Promega) from 1 μg of total RNA. To check for DNA contamination, controls without reverse transcriptase were included in each series of samples. Quantitative RT-PCR was performed on 25 ng of total cDNA with 1 × SYBR Green universal PCR Master mix (Applied Biosystem, Courtaboeuf, France) containing dNTPs, $MgCl_2$, AmpliTaq Gold DNA polymerase, and 300 nM of forward and reverse primers (Proligo, Paris, France). PCR parameters were 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min, using the ABI Prism 7000 sequence detection system (Applied Biosystem). Gene-specific forward and reverse primers were chosen using the Primer Express 2 software (Applied Biosystems). Primer sequences used to detect the mRNA levels of agouti-related protein (AgRP), cocaine- and amphetamine-regulated transcript (CART), corticotropin-releasing hormone (CRH), GAPDH, MC3-R, MC4-R, melanin-concentrating hormone (MCH), neuropeptide Y (NPY), oxytocin (OT), orexins (OX), PACAP, POMC and thyrotropin-releasing hormone (TRH) are listed in Table 1. The efficacy of the PCR amplification was assessed for each primer set from the slope of a standard curve generated with serial dilutions of hypothalamic cDNA. In all cases, the slope of the standard curves was close to −3.3 indicating maximal PCR amplification efficiency. The purity of the PCR product was verified by dissociation curves and agarose gel analysis. The amount of each transcript was evaluated 15, 30 and 60 min after the injection. The amount of cDNA in each sample was determined by the comparative threshold cycle (C_t) method as outlined in User Bulletin no. 2 (Applied Biosystems) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The ΔC_t value was obtained by subtracting the average GAPDH C_t value from the average C_t value of each target. The average ΔC_t of mice normally fed or saline-treated animals at time of injection was used as the calibrator. The fold-change was calculated according to the formula $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ is the difference between ΔC_t and the ΔC_t calibrator value. The C_t values for neuropeptides mRNA expression ranged from 18 to 28 cycles while C_t values for receptors never exceeded 32 cycles. Expression of GAPDH mRNA was not affected by fasting or PACAP treatment and the ratio of the ΔC_t value did not vary with the amount of cDNA.

Table 1 Sequence of the oligonucleotides used for quantitative RT-PCR amplification

Primer	Sequence 5′ → 3′	Accession number
AgRP	AGCTTTGGCGGAGGTGCT GCGACGCGGAGAACGA	NM 007427
CART	GCCAAGGCGGCAACTTC TCTTGCAACGCTTCGATCTG	NM 013732
CRH	CCCGCAGCCCTTGAATT TTCACCCATGCGGATCAGA	NM 205769
MC3-R	ATGATCGCCGTGATCAACAG AATCATAGAGTCGAAGATATTATCCATGTG	NM 008561
GAPDH	ACTCCACTCACGGCAAATTCA TCTCGCTTCTGGAAGATGGT	BC 083080
MC4-R	AAGCTGCCCAGATACAACTTATGA ACGCGCTCCAGTACCATAACA	NM 016977
MCH	ACCGCTCTCGTCGTTTTTGTA TTCAGAAGGAGGATACTGCAGAAAGA	NM 029971
NPY	TCCGCTCTGCGACACTACAT TGCTTTCCTTCATTAAGAGGTCTG	NM 023456
OT	CGACCCTGAGTCTGCCTTCTC GCGCTAAAGGTATTCCCAGAAA	NM 011025
OX	GTTCCTGCCGTCTCTACGAACT CGCTTTCCCAGAGTCAGGATA	NM 010410
PACAP	TCCCTGGGATCAGACCAGAA GAAGTCTTGCAGCGGGTTTC	NM 009625
POMC	TGAACATCTTTGTCCCCAGAGA TGCAGAGGCAAACAAGATTGG	NM 008895
TRH	CCCAGCCAGTTTGCACTCTT AGATCAAAGCCAGAGCCATCA	BC 053493

In Situ Hybridization

c-Fos riboprobes were prepared by *in vitro* transcription of a 896-bp cDNA fragment (position 133–1028 of the rat c-Fos sequence, GenBank accession number NM_022197; this sequence exhibits 96% identity with the mouse nucleotide sequence) subcloned into the pGEM-T cloning vector (Promega). A 409-bp DNA fragment (position 221–629 of the rat POMC gene exon III, GenBank accession number J00759; this sequence exhibits 92% identity with the mouse nucleotide sequence) was amplified by PCR using as a

template a plasmid containing the full length exon III cDNA and subcloned into PCR^{tm}II (Invitrogen, Groningen, The Netherlands). Antisense and sense riboprobes were generated with T7 or SP6 polymerases in the presence of ^{35}S-UTP or digoxigenin (DIG)-11-UTP.

Frozen frontal brain sections (14-μm thick) were cut through the entire length of the arcuate nucleus. All tissue sections were mounted on gelatine and polylysine-coated slides and stored at −80°C. Single *in situ* hybridization with S^{35}-labeled c-Fos or POMC probes was performed as previously described (Mounien *et al*, 2006b). Briefly, sections were initially fixed in 4% paraformaldehyde, acetylated, treated with Triton X-100 (0.2%) and covered with prehybridization buffer (50% formamide, 0.6 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.02% Ficoll, 0.02% polyvinylpyrollidone, 0.1% bovine serum albumin, 1 mM EDTA, pH 8.0, 550 μg/ml denatured salmon sperm DNA, 50 μg/ml yeast tRNA). Hybridization was performed overnight at 55°C in the same buffer (except for salmon sperm DNA, the concentration of which was lowered to 60 μg/ml) supplemented with 10 mM dithiothreitol, 10% dextran sulfate and heat-denatured riboprobes (10^7 c.p.m./ml). Sections were washed in 2 × SSC at 60°C and treated with RNase A (50 μg/ml) at 37°C for 1 hour. Five high-stringency washes were performed in 0.01 × SSC containing 14 mM β-mercaptoethanol and 0.05% sodium pyrophosphate. Brain slices were dehydrated in graded alcohols containing 0.3 M ammonium acetate and air-dried. The brain slices were dipped into photographic NTB2-emulsion (Kodak, Rochester, NY, USA), exposed at 4°C for 14 or 25 days for c-Fos or POMC *in situ* hybridization, respectively, and then counterstained with hematoxylin. The specificity of each hybridization procedure was verified by incubating serial sections with the sense riboprobes. Very low background labeling was observed on control sections. For dual *in situ* hybridization with the radiolabeled c-Fos and cold POMC probes, a DIG-labeled POMC probe was added to the hybridization buffer (1 : 100; vol/vol). Specific labeling was visualized by incubation with an antidigoxigenin Fab fragments conjugated to alkaline phosphatase (Roche Diagnostics) followed by staining with 4-nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate which gives rise to a purple end product. The reaction was stopped by rinsing for 5 h in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8). Slices were then dried, dipped into autoradiographic K5 emulsion (Ilford, Saint-Priest, France) and exposed for 20 days.

Quantitative Analysis of *In Situ* Hybridization

The density of autoradiographic silver grains was assessed by using the Mercator software (Exploranova, La Rochelle, France) interfaced to an Eclipe E-600 microscope (Nikon, Tokyo, Japan) equipped with a CCD Sony DXC950 camera. To compare c-Fos mRNA expression in the arcuate nucleus from mice injected with saline or PACAP, the arcuate nucleus was delimited and the number of grains per surface unit (100 μm^2) was quantified under a × 20 darkfield objective. The grain density was then corrected for the average background signal that was determined on an adjacent equivalent surface. Three slices anatomically matched across animals were analyzed for each mouse. Then, for each animal, the grain density was averaged and the mean (± SEM) from three mice was calculated for each group. In the case of dual *in situ* hybridization, POMC mRNA-expressing cells were first identified under bright-field illumination, and the density of grains atop each POMC neuron was determined under epi-illumination. Cells were identified as labeled with the c-Fos probe when the number of silver grains over POMC perikarya was at least five times higher than the background. The number of single or double-labeled POMC cells in the arcuate nucleus was counted for each tissue section (three sections/animal). Then, the proportion of POMC neurons expressing simultaneously c-Fos mRNA was determined for each animal and the mean percentage of co-labeled cells (± SEM) from three mice was calculated for each group. To compare POMC mRNA expression in hypothalamus from PAC1-R$^{+/+}$ and PAC1-R$^{-/-}$ mice, sixteen frontal sections from each brain were anatomically matched across animals to measure relative cellular POMC mRNA content in the arcuate nucleus. First, POMC mRNA-expressing cells were identified under darkfield illumination using a × 20 objective. Then, the grain density (number of grain per 100 μm^2) for each cell was measured with a × 100 epi-illumination darkfield objective. The grain density was then corrected for the average background signal that was determined on adjacent negative cell bodies. Cells were considered labeled when the number of silver grains overlying the cytoplasm exceeded five times the corrected background. All labeled cells were included for counting. Then, for each animal, the grain density/cell was averaged and the mean (± SEM) from four mice was calculated.

Measurement of Plasma Corticosterone and Glucose Levels

Trunk blood was collected into polystyrene tubes chilled on ice. After centrifugation (1500 *g*, 5 min), plasma was collected and stored at −25°C. Plasma corticosterone concentration was determined on dried ethanol extracts in duplicate by radioimmunoassay, as previously described (Leboulenger *et al*, 1982). The corticosterone antibodies exhibited weak cross-reactivity with aldosterone (0.6%) and testosterone (1.8%) and very low cross-reactivity with other circulating steroid hormones. Plasma glucose level was measured with a LifeScan glucose analyzer (LifeScan, Issy-les-Moulineaux, France).

Statistical Analysis

Statistical analyses were performed using PRISM (Graphpad, San Diego, CA, USA). All data are expressed as mean ± SEM. Differences between groups were assessed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by a *post hoc* multiple comparison Dunnett's test depending on the experimental design. Antagonistic effects were analyzed using two-way ANOVA, and *post hoc* multiple comparison Student–Newman–Keuls test was used for multiple comparisons between groups. Normal distribution and equality of variance were assessed before analysis. For each test, a *p*-value of 0.05 or less was considered as statistically significant.

RESULTS

Dose–Response and Time Course Effects of PACAP and VIP on Food Intake

In mice deprived of food for 18 h, i.c.v. injection of graded doses of PACAP (0.11–0.66 nmol) provoked a significant dose-dependent inhibition of food intake during the first 30-min period of testing (one-way ANOVA, $F(5,66) = 20.97$; $p < 0.001$) and during the whole 3-h observation period ($F(5,66) = 24.94$; $p < 0.001$) (Figure 1a). In contrast, i.c.v. injection of graded doses of VIP (0.3–0.6 nmol) had no significant effect (one-way ANOVA, $F(3,44) = 1.38$; $p = 0.263$) on food consumption in food-deprived mice (Figure 1b).

Effect of PACAP6-38 or SHU9119 on PACAP-Induced Inhibition of Food Intake

i.c.v. injection of the PACAP receptor antagonist PACAP6-38 (2.4 nmol) alone did not significantly ($p > 0.05$) affect spontaneous food intake in mice deprived of food for 18 h, but significantly ($p < 0.001$) attenuated the inhibitory effect of PACAP (0.22 nmol) on food consumption during the whole 60-min observation period (Figure 2a). Similarly, i.c.v. administration of the MC3-R/MC4-R antagonist SHU9119 (18 pmol) alone had no significant ($p > 0.05$) effect on food intake but significantly ($p < 0.001$) reduced PACAP-evoked inhibition of food consumption (Figure 2b). A two-way ANOVA revealed a significant interaction: between PACAP6-38 and PACAP $F(1,44) = 5.73$, $p < 0.05$; and between SHU9119 and PACAP $F(1,36) = 6.55$, $p < 0.05$.

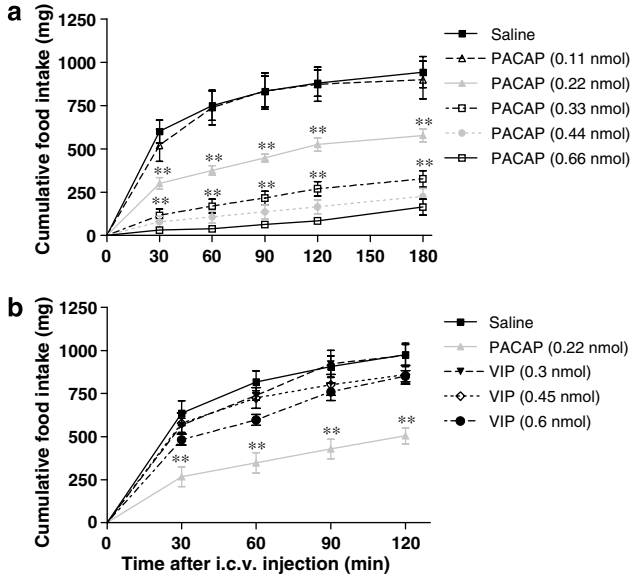

Figure 1 Time course of the effect of PACAP (a) and VIP (b) on food intake. Mice deprived of food during 18 h were injected i.c.v. (10 µl) with saline, or with graded doses of PACAP (0.11–0.66 nmol) or VIP (0.3–0.6 nmol). Ten minutes after i.c.v. injection, each animal had access to a weighed food pellet. Cumulative food intake was then measured every 30 min during 3 h (a) or 2 h (b). Each value is the mean (± SEM) from 12 animals. $^{**}p < 0.01$ *vs* saline-injected mice (Dunnett's *post hoc* test).

Effect of Food Deprivation on PACAP, POMC and NPY mRNA Expression

The levels of PACAP, POMC and NPY mRNAs in the hypothalamus of mice normally fed or mice deprived of food for 48 h were determined by quantitative RT-PCR. Food deprivation resulted in a significant decrease in POMC and PACAP mRNA (−29 and −41%, respectively; $p < 0.05$) (Figure 3). In contrast, food deprivation significantly increased NPY mRNA levels (+172%; $p < 0.001$) (Figure 3).

Effect of PACAP on c-Fos mRNA Expression in the Arcuate Nucleus

Only few c-Fos mRNA-expressing cells were visualized by *in situ* hybridization in the arcuate nucleus after i.c.v. administration of saline (Figure 4a). In contrast, i.c.v. administration of PACAP (0.22 nmol) caused a marked induction of c-Fos mRNA expression in the arcuate nucleus 30 min (+314%; $p < 0.05$) and 60 min (+432%; $p < 0.001$) after injection (Figure 4b and c).

Co-Localization of POMC and c-Fos mRNA in the Arcuate Nucleus

Double staining of sections with the DIG-labeled POMC probe and the S^{35}-labeled c-Fos probe revealed that i.c.v. injection of PACAP (0.22 nmol) significantly increased the proportion of POMC neurons-expressing c-Fos mRNA 30 and 60 min after the injection (22.8 ± 7.1%; $p < 0.05$ and

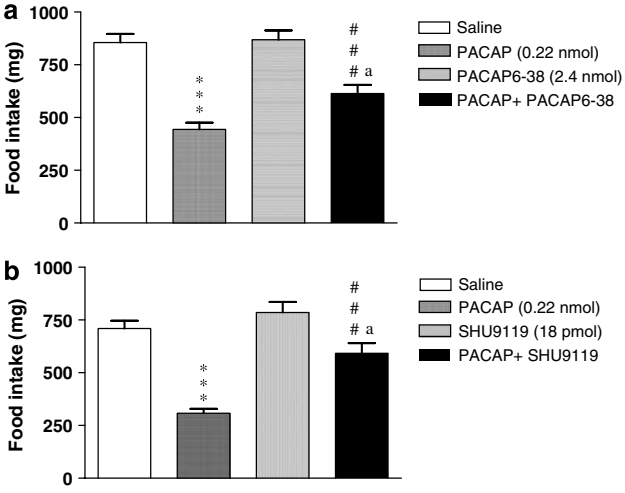

Figure 2 Effect of PACAP6-38 and SHU9119 on PACAP-induced inhibition of food intake. (a) Mice deprived of food during 18 h were injected i.c.v. (10 µl) with saline, or with 0.22 nmol PACAP alone or in combination with 2.4 nmol PACA6-38. (b) Mice deprived of food during 18 h were injected i.c.v. (5 µl) in the left ventricle with saline or with SHU9119 (18 pmol) 10 min before i.c.v. injection (5 µl) in the right ventricle of saline or PACAP (0.22 nmol). Ten minutes after i.c.v. injection, each animal had access to a weighed food pellet. Cumulative food intake was then measured during 60 min. Each value is the mean (± SEM) from 10 (b) –12 (a) animals. $^{***}p < 0.001$ *vs* saline-injected mice; $^{\#\#\#}p < 0.001$ *vs* PACAP-injected mice (Student–Newman–Keuls test). A two-way ANOVA revealed a significant interaction between PACAP6-38 and PACAP $F(1,44) = 5.73$, $^{a}p < 0.05$, and between SHU9119 and PACAP $F(1,36) = 6.55$, $^{a}p < 0.05$.

25.6 ± 1.2%; $p < 0.001$, respectively), when compared with the vehicle-treated groups (2.2 ± 0.9 and 3.8 ± 0.8%, respectively) (Figure 5).

Effect of PACAP on Hypothalamic Gene Expression

The effect of PACAP on POMC and MC-R mRNA levels was determined by quantitative RT-PCR. Time course experiments revealed that i.c.v. administration of PACAP (0.22 nmol) significantly increased POMC mRNA level within 30 min (+44%; $p < 0.05$) and MC4-R mRNA level within 15 min (+47%; $p < 0.05$) after injection (Figure 6a and c). In contrast, PACAP did not affect MC3-R mRNA level (Figure 6b).

Concurrently, the effect of PACAP on mRNA levels of several orexigenic and anorexigenic neuropeptides were evaluated. Time course experiments showed that i.c.v. administration of PACAP (0.22 nmol) had no effect on AgRP, CART, NPY, OT and TRH mRNA levels whatever the time after injection (Figure 7). In contrast, PACAP significantly decreased CRH, MCH and OX mRNA contents in the hypothalamus within 60 min after injection (−26, −27 and −20%, respectively; $p < 0.05$–0.01; Figure 7).

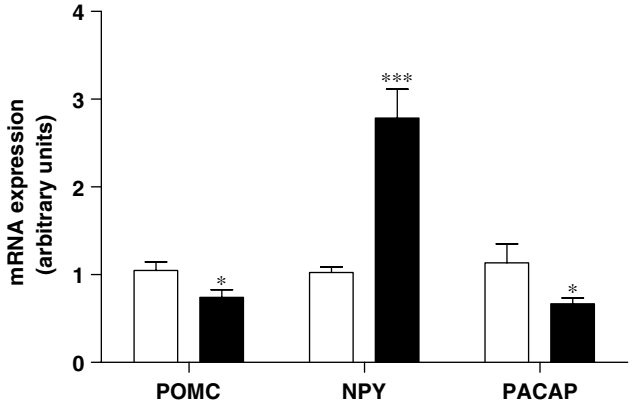

Figure 3 Effect of food deprivation on mRNA levels of POMC, NPY and PACAP in the hypothalamus. Mice were normally fed (open bars) or deprived of food during 48 h (solid bars). The relative concentrations of POMC, NPY and PACAP mRNAs were determined by quantitative RT-PCR. The average ΔC_t of mice normally fed was used as calibrator. Each value is the mean (± SEM) from 10 animals. *$p < 0.05$, ***$p < 0.001$ *vs* controls (Student's *t*-test).

Effect of PAC1-R Disruption on POMC mRNA Levels

In situ hybridization histochemistry of POMC gene expression in the arcuate nucleus revealed that the labeling intensity was lower in PAC1-$R^{-/-}$ mice than in the PAC1-$R^{+/+}$ controls (Figure 8a and b). Quantification of silver grains over POMC mRNA-containing neurons showed a significant difference in grain density between the PAC1-$R^{-/-}$ and PAC1-$R^{+/+}$ groups (−40%; $p < 0.01$; Figure 8c).

Effect of PACAP on Plasma Corticosterone and Glucose Levels

As glucocorticoids and carbohydrates can profoundly affect energy homeostasis, plasma corticosterone and glucose

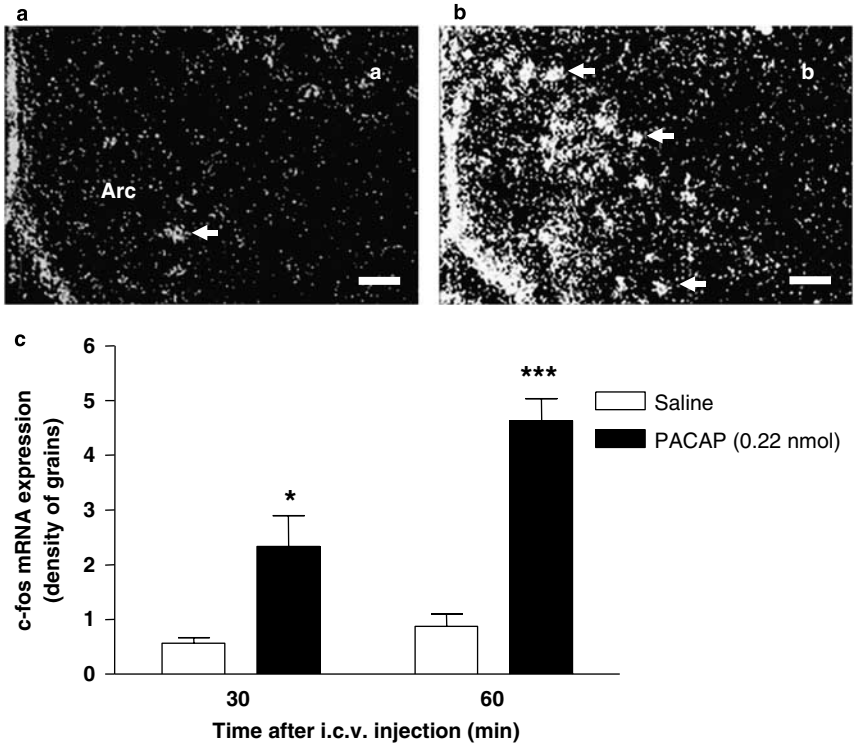

Figure 4 Effect of PACAP on c-Fos mRNA levels in the arcuate nucleus. Mice deprived of food during 18 h were injected i.c.v. (10 µl) with saline, or with PACAP (0.22 nmol). (a, b) *In situ* hybridization darkfield photomicrographs illustrating c-Fos mRNA expression in frontal sections at the level of the mid-anterior posterior region of the arcuate nucleus (Arc) from saline- (a) or PACAP-injected mice (b) 60 min after the injection. Arrows indicate cells expressing c-Fos mRNA. Scale bars = 50 µm. (c) Quantitative analysis of c-Fos mRNA levels in the Arc from saline- or PACAP-injected mice 30 and 60 min after the injection. Each value is the mean (± SEM) from three animals (three tissue sections each). *$p < 0.05$, ***$p < 0.001$ *vs* respective controls (Student's *t*-test).

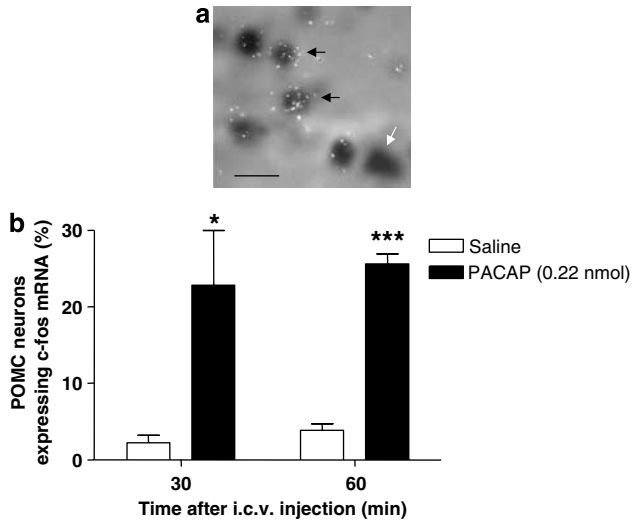

Figure 5 Effect of PACAP on POMC neurons expressing c-Fos mRNA in the arcuate nucleus. Mice deprived of food during 18 h were injected i.c.v. (10 μl) with saline or with PACAP (0.22 nmol). (a) Bright-field micrograph with epi-illumination of frontal section at the level of the arcuate nucleus illustrating dual *in situ* hybridization with the S^{35}-labeled antisense probe to c-Fos mRNA (silver grains) and DIG-labeled antisense probe to POMC mRNA (dark precipitate). Black arrows point to POMC neurons expressing c-Fos mRNA. The white arrow points to a neuron expressing POMC mRNA but not c-Fos mRNA. Scale bars = 20 μm. (b) Quantitative analysis of the percentage of POMC-positive neurons expressing c-Fos mRNA in the arcuate nucleus from saline- or PACAP-injected mice 30 and 60 min after the injection. Each value is the mean (± SEM) from three animals (three tissue sections each). $^{*}p<0.05$, $^{***}p<0.001$ *vs* respective controls (Student's *t*-test).

levels were measured. i.c.v. administration of PACAP (0.22 nmol) did not significantly affect plasma corticosterone level during the 60-min period following injection (Figure 9a). In contrast, PACAP induced a significant increase in plasma glucose concentration within 30 min (+40%; $p<0.05$) and 60 min (+95%; $p<0.01$) after injection (Figure 9b).

DISCUSSION

Previous studies have shown that PACAP exerts a marked inhibitory effect on food consumption (Morley *et al*, 1992; Chance *et al*, 1995; Mizuno *et al*, 1998; Tachibana *et al*, 2004; Matsuda *et al*, 2005). However, the neuronal pathways involved in the anorexigenic action of PACAP remain largely unknown. The present study demonstrates that the effect of PACAP on feeding behavior is mediated at least in part through modulation of the hypothalamic melanocortin system.

The anorexigenic effect of PACAP has been evidenced in various vertebrate species including rat (Chance *et al*, 1995; Mizuno *et al*, 1998), mouse (Morley *et al*, 1992), chicken (Tachibana *et al*, 2004) and goldfish (Matsuda *et al*, 2005), but the type of receptor involved has not been characterized. We confirmed that i.c.v. administration of PACAP to food-deprived mice causes a dose-dependent reduction of food consumption during at least 3 h after the injection. In addition, we found that the effect of PACAP was attenuated by the PACAP antagonist PACAP6-38 and was not mimicked by VIP. These observations indicate that PACAP

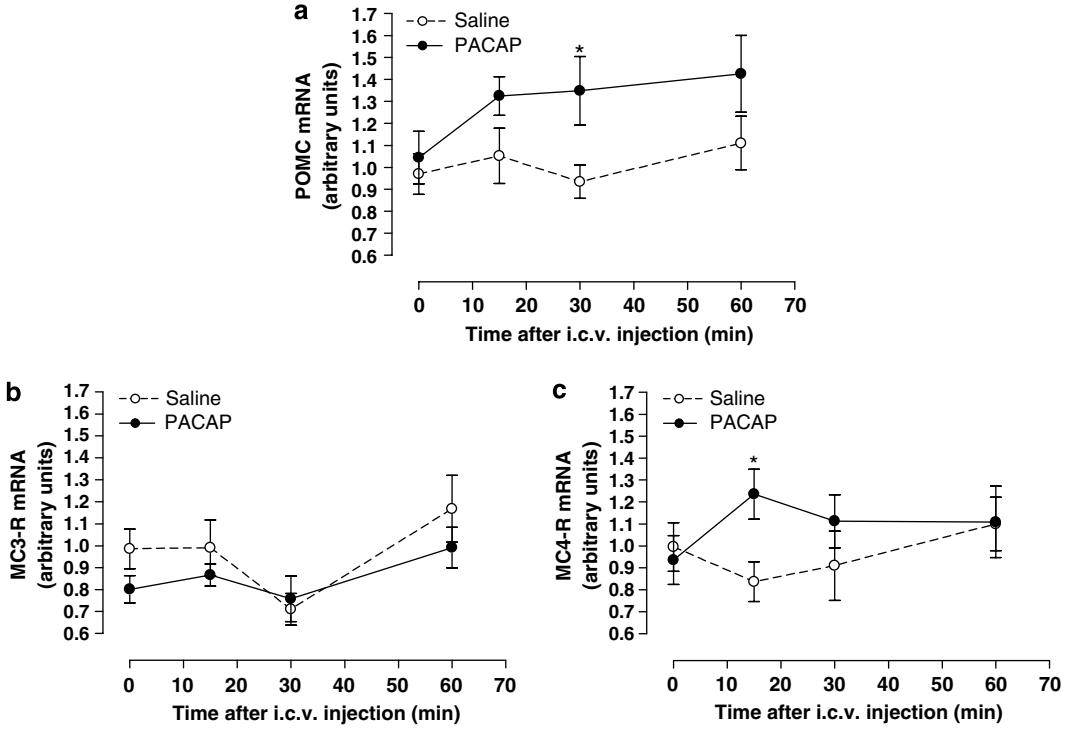

Figure 6 Effect of PACAP on POMC (a), MC3-R (b) and MC4-R (c) mRNA levels in the hypothalamus. Mice were injected i.c.v. (10 μl) with saline or 0.22 nmol PACAP. The relative concentrations of POMC, MC3-R and MC4-R mRNAs were determined by quantitative RT-PCR 15, 30 and 60 min after the injection. The average ΔC_t of saline-treated animals at the time of injection was used as a calibrator. Each value is the mean (± SEM) from nine or 10 animals. $^{*}p<0.05$ *vs* controls (Student's *t*-test).

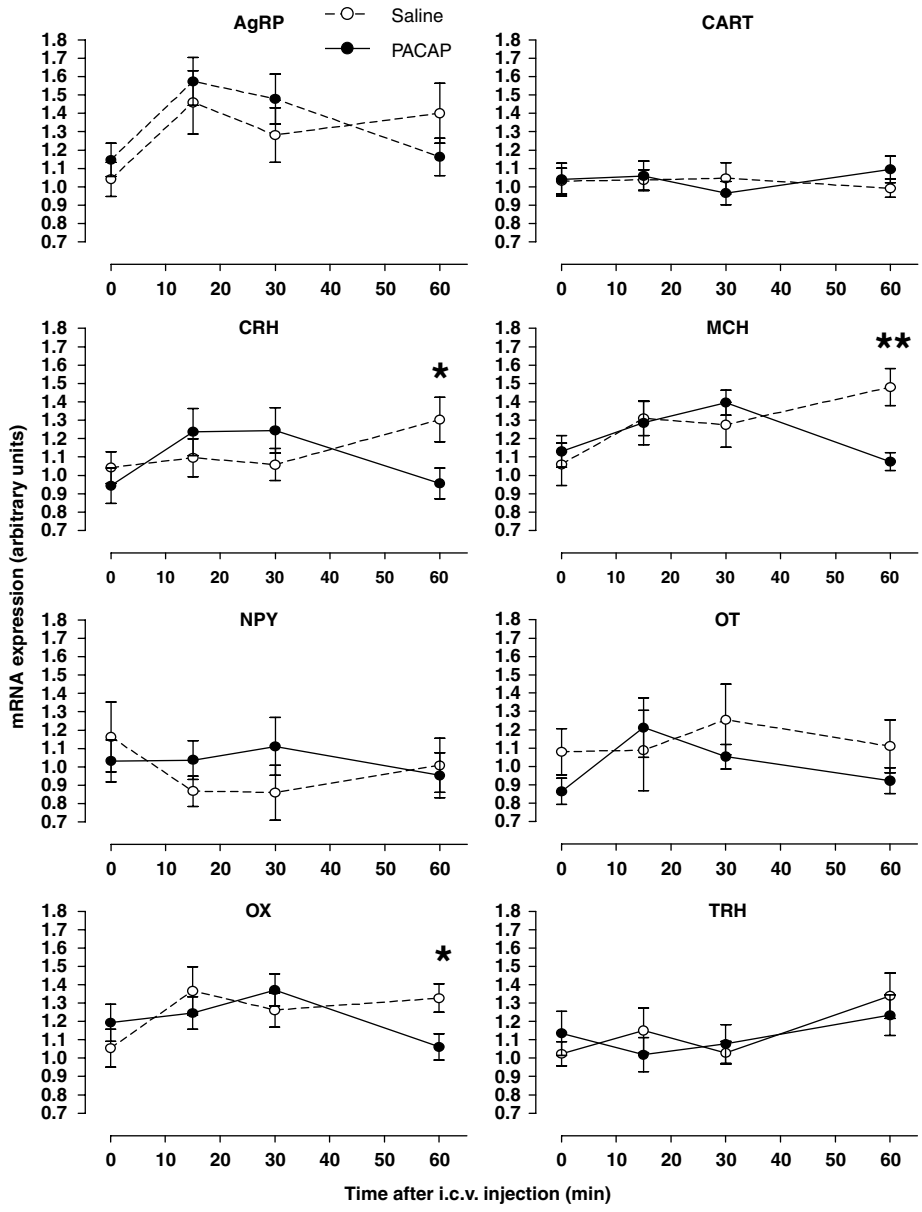

Figure 7 Effect of PACAP on neuropeptide mRNA levels in the hypothalamus. Mice were injected i.c.v. (10 µl) with saline or 0.22 nmol PACAP, and neuropeptide mRNA concentrations were determined by quantitative RT-PCR. The relative concentrations of neuropeptide mRNAs were determined by quantitative RT-PCR 15, 30 and 60 min after the injection. The average ΔC_t of saline-treated animals at the time of injection was used as a calibrator. Each value is the mean (± SEM) from nine or 10 animals. AgRP, agouti-related protein; CART, cocaine- and amphetamine-regulated transcript; CRH, corticotropin-releasing hormone; MCH, melanin-concentrating hormone; NPY neuropeptide Y; OT, oxytocin; OX, orexins; TRH, thyrotropin-releasing hormone. *$p < 0.05$, **$p < 0.01$ *vs* controls (Student's *t*-test).

exerts its anorexigenic action through activation of the PACAP-selective receptor PAC1-R. As previously reported, fasting inhibited the expression of POMC, the precursor of the anorexigenic peptide α-MSH, and stimulated the expression of the orexigenic peptide NPY (White and Kershaw, 1989; Schwartz *et al*, 1997). Consistent with a physiological role of PACAP in the control of food intake, we found that fasting downregulated PACAP gene expression.

We have previously shown that POMC neurons of the arcuate nucleus express PACAP receptors and that PACAP provokes a significant increase in biosynthesis and release of α-MSH from hypothalamic explants (Mounien *et al*, 2006a), suggesting that the anorexigenic effect of PACAP may be mediated through the melanocortin system. To test this hypothesis, we have administered the MC3-R/MC4-R antagonist SHU9119 before PACAP treatment. Although it has been reported that central injection of SHU9119 causes a dose-dependent stimulation of feeding (Fan *et al*, 1997; Williams *et al*, 2004), the dose of 18 pmol of SHU9119 applied in the present study had no significant effect by itself on food consumption. However, at this moderate dose, SHU9119 partially blocked the effect of PACAP on food intake. These data provide the first evidence that anorexia induced by central injection of PACAP is mediated, at least in part, through activation of the melanocortin pathway.

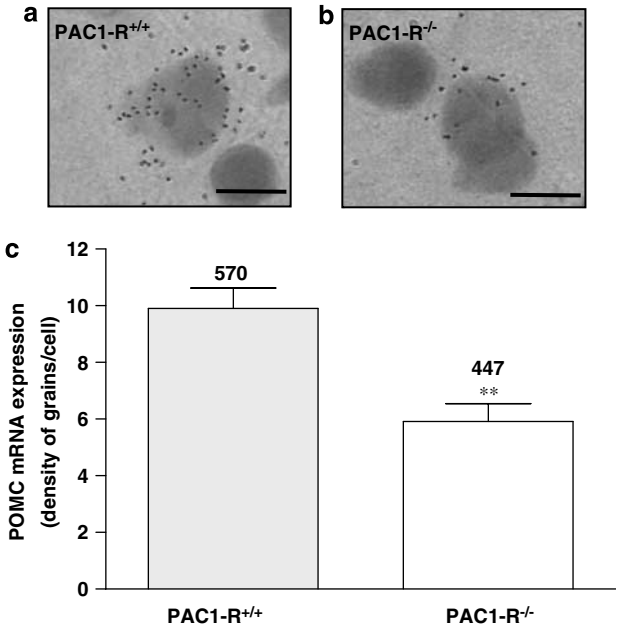

Figure 8 Effect of PAC1-R gene disruption on proopiomelanocortin (POMC) mRNA in the arcuate nucleus (Arc) of the hypothalamus. (a, b) *In situ* hybridization photomicrographs illustrating POMC mRNA expression in frontal sections of the Arc from PAC1-$R^{+/+}$ (a) or PAC1-$R^{-/-}$ mice (b). (c) Quantitative analysis of POMC mRNA levels in PAC1-$R^{+/+}$ and PAC1-$R^{-/-}$ mice. Each value is the mean (±SEM) from four animals (16 tissue sections each). $^{**}p<0.01$ *vs* PAC1-$R^{+/+}$ mice (Student's *t*-test). The total number of POMC-positive neurons in the Arc in which the density of silver grains has been measured is indicated above each column. Scale bar = 10 μm.

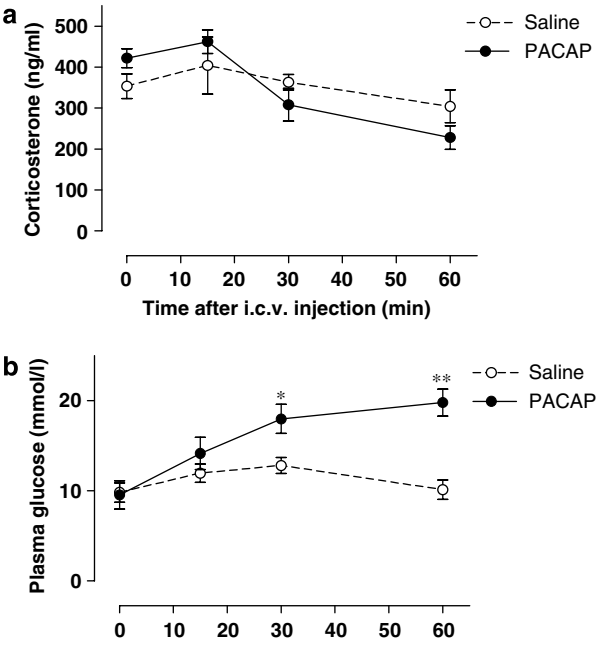

Figure 9 Time course of the effect of PACAP on plasma corticosterone and glucose concentrations. Mice were injected i.c.v. (10 μl) with saline or 0.22 nmol PACAP, and plasma corticosterone (a) and glucose (b) concentrations were determined 15, 30 and 60 min after the injection. Each value is the mean (±SEM) from six animals. $^{*}p<0.05$, $^{**}p<0.01$ *vs* controls (Student's *t*-test).

In support of this notion, i.c.v. administration of PACAP to mice that had been deprived of food for 18 h induced the expression of c-Fos, a marker of cellular activation (Rowland, 1998), in the arcuate nucleus where POMC neurons are located. In addition, PACAP caused an increase of POMC neurons expressing c-Fos mRNA indicating that PACAP can actually stimulate the activity of POMC neurons. I.c.v. injection of PACAP also increased POMC and MC4-R mRNA levels in the hypothalamus, suggesting that PACAP can activate melanocortinergic neurotransmission both at the pre- and post-synaptic levels. In contrast to MC4-R, hypothalamic MC3-R mRNA levels were not affected by PACAP treatment. Consistent with this latter observation, it has been previously shown that melanocortins do not exert their anorexigenic effects through activation of MC3-R (Chen *et al*, 2000; Cummings and Schwartz 2000). We also found that POMC mRNA expression is decreased in C57BL/6 PAC1-R KO mice indicating that, in this particular strain, endogenous PACAP may exert a stimulatory tone on POMC neurons activity through PAC1-R activation. Surprisingly, however, in fasting CD1 mice, i.c.v. administration of the PACAP antagonist PACAP6-38 had no effect by its own on food intake. Although, the use of different mice strains (CD1 *vs* C57BL/6) may account for this discrepancy, other explanations can be proposed. First, several studies have shown that PACAP6-38 is not a pure antagonist in that it retains some agonistic property (Hezareh *et al*, 1996; Gonzalez *et al*, 1997). It is conceivable that this residual agonistic activity of PACAP6-38 can mask its inhibitory effect upon endogenous PACAP tone. This may also explain why PACAP6-38 did not completely suppress the effect of PACAP on food consumption. Second, the effect of PACAP6-38 was examined only during a 60-min period while blockage of the effects of endogenous PACAP by the antagonist usually requires several hours (Tohei *et al*, 2001; Borba *et al*, 2005). Third, it has been reported that the hypothalamic concentration of PACAP decreases after the onset of the light phase and falls to a nadir at 1200 h (Fukuhara *et al*, 1997). In addition, we observed that starvation significantly reduces PACAP mRNA content in the mice hypothalamus. These observations suggest that the levels of endogenous PACAP at the time our experiments were conducted (0900–1200 am) may not have been sufficient to induce a tonic stimulation of POMC neurons.

We have also investigated the possible effect of PACAP on the expression of several hypothalamic neuropeptides known to be involved in the control of feeding (Berthoud, 2002). The present data showed that acute i.c.v. administration of PACAP had no effect on AgRP, CART, NPY, OT and TRH mRNA levels. In contrast, i.c.v. injection of PACAP significantly reduced CRH, OX and MCH mRNA expressions 60 min after peptide administration. These results are in agreement with previous reports showing that central administration of PACAP reduces food intake in rats without altering the hypothalamic concentration of NPY (Chance *et al*, 1995) and exerts an inhibitory influence on CRH mRNA levels in rat (Mizuno *et al*, 1998). Clearly, the rapid (30 min) anorexigenic effect of PACAP cannot be accounted for by its inhibitory action on CRH mRNA level since (1) a response of CRH neurons was only observed

after 60 min, and (2) both peptides exert a negative influence on feeding (Hotta *et al*, 1991; Morley *et al*, 1992). Conversely, we found that mRNA levels of the orexigenic neuropeptides OX and MCH (Qu *et al*, 1996; Sakurai *et al*, 1998) were decreased only 60 min after PACAP administration. Further investigations are warranted to clarify the contribution OX and MCH in the long-lasting anorexigenic action of PACAP. Altogether, the present data indicate that the rapid anorexigenic action of PACAP is likely mediated through the melanocortinergic neuronal system, but does not involve other appetite-regulating neuropeptides including AgRP, CART, CRH, MCH, NPY, OT, OX and TRH.

The present study provides the first evidence that i.c.v. injection of PACAP produces hyperglycemia without affecting plasma corticosterone level. It is now firmly established that peripheral administration of PACAP also provokes a significant increase in plasma glucose level (Sekiguchi *et al*, 1994; Ozawa *et al*, 1999). Although PACAP can be transported out of the brain (Banks *et al*, 1993), two observations indicate that the effect of centrally-administered PACAP on glucose homeostasis cannot be accounted for by a peripheral action of the peptide. (i) The dose of PACAP injected i.c.v. in our study was much lower than the minimum effective doses of PACAP administered intravenously (Sekiguchi *et al*, 1994; Ozawa *et al*, 1999). In addition, it should be noticed that the half-life of PACAP38 in the systemic circulation is very short ($\approx$3.5 min; Birk *et al*, 2007; Bourgault *et al*, 2008) so that any PACAP diffusing from the brain to the periphery would be rapidly degraded. (ii) The hyperglycemic effect of PACAP occurs 15 min after intravenous injection (Ozawa *et al*, 1999) but only 30 min after i.c.v. administration of the peptide (this study). Taken together, these observations indicate that PACAP can act centrally to regulate glucose homeostasis. It has been previously reported that i.c.v. administration of the MC3-R/MC4-R antagonist MTII rapidly suppresses insulin release and elevates glycemia (Fan *et al*, 2000; Obici *et al*, 2001; Mizuno *et al*, 2003; Li *et al*, 2003, 2005). Collectively, these data support the notion that the central effect of PACAP on glucose homeostasis may be mediated through activation of the melanocortin system and does not involve the action of glucocorticoids.

In conclusion, the present study has demonstrated that PACAP, acting through the PACAP-preferring receptor PAC1-R, inhibits feeding in food-deprived mice. The reduction of POMC gene expression in PAC1-R KO mice strongly suggests that endogenous PACAP actually regulates the activity of POMC neurons. These data provide the first evidence that anorexia induced by central administration of PACAP is mediated, at least in part, through activation of the hypothalamic melanocortin system.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare that except for income received from their primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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