Transcript of protein kinase A knock-down modulates feeding behavior and neuropeptide Y gene expression in phenylpropanolamine-treated rats

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Submitted 14 May 2007; accepted in final form 31 July 2007

Phenylpropanolamine (PPA) is an over-the-counter appetite suppressant that is used in human dieters to reduce obesity or hyperphagia (7, 14, 50). Although PPA has been withdrawn from market due to the risk of stroke (38), there is still a reported reappraisal for the use of PPA in the US due to the proposed conclusions that chance, bias, and confounding are plausible alternative explanations for the observed findings of PPA (54). PPA is regarded as a sympathomimetic agent that is structurally and functionally related to amphetamine (AMPH)-like anorectic drugs. Pharmacological evidence reveal that the mechanism for the appetite-suppressing effect of PPA is linked to the central release of catecholamine that may act on α1-adrenoceptors and dopamine D1 receptors (12, 59), and then lead to an effect on hypothalamic NPY neurons to suppress appetite (21, 23).

It is unclear whether cAMP-dependent protein kinase (PKA) signaling is required for NPY gene expression during PPA treatment. Evidence reveal that, after ligand stimulation, adrenoceptors can activate G proteins, which elicit intracellular second messenger signals including cAMP, Ca2+, diacylglycerol, and inositol-trisphosphate in neurons (19). PKA signaling can be elicited by various physiological ligands in cells and is involved in the regulation of metabolism, cell proliferation, and apoptosis (18, 56). In the brain, PKA is critically involved in regulating behavioral responses to antidepressants, neuroleptics, and drugs of abuse (55) and is implicated in the regulation of dopamine agonist-induced feeding behavior (45). Moreover, PKA is involved in the regulation of NPY-induced feeding behavior in vivo (1, 20, 31, 53) or in vitro (36). Thus, one could hypothesize that PKA is involved in the regulation of NPY gene expression during PPA treatment.

In the current study, PKA antisense oligodeoxynucleotides (ODN) were employed to disrupt PKA gene expression in freely moving rats. Although the method of antisense knockdown had inherent difficulties, antisense were preferentially taken up by neurons in the rodent brain after intracerebral (42, 62) or intracerebroventricular (icv) administration (58). Moreover, NPY antisense ODN could be used to disrupt hypothalamic NPY gene expression in the investigation of feeding behavior and body weight reduction (26). Thus, it is rational to predict that icv administration of specific antisense ODN should be used to interrupt specific gene expression in the brain. In this study, we chose antisense, which had been previously used to specifically downregulate PKA gene expression (10, 27), to examine its effect on PPA anorexia following central ventricular administration.
MATERIALS AND METHODS

Animal treatments. Male Wistar rats (with a weight of 200–300 g, Animal Center of National Cheng Kung University Medical College) were housed individually in transparent plastic cages with stainless steel covering and hardwood bedding (Beta Chip, Northeastern Products). Food (LabDiet; PMI Nutrition International, Brentwood, MO) and tap water were provided ad libitum. Animals were maintained at 22 ± 2°C according to a 12-h light/12-h dark cycle (light on at 6:00 AM) and habituated to frequent handling. Drug administration, feeding, and body weight assessment were performed daily at the beginning of dark phase (6:00 PM). This study has been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee in Taiwan.

To examine the effect of PPA (Sigma-Aldrich, St. Louis, MO) on feeding behavior, rats were given the PPA (0, 70, or 110 mg/kg ip; n = 6–8 each group) daily for 5 days at the beginning of the dark phase (at 6:00 PM). Daily food intake and body weight change were examined at 24 h after daily drug treatment. The first injection of PPA was conducted at the end of day 0 (at 6:00 PM), and the intake data were calculated as the total amount of food during the previous day. The body weight data were calculated as daily body weight change when compared with the previous day.

To assess the effect of PPA on NPY, PKA, cAMP response element binding protein (CREB), and pro-opiomelanocortin (POMC) mRNA levels, rats (n = 5–6 each group) were given the PPA (0 or 70 mg/kg ip) daily for 1, 2, 3, or 4 days, and then were killed. Rats were anesthetized with pentobarbital (30 mg/kg ip) at 40 min after PPA treatment and then decapitated. The hypothalamus was removed from the brain immediately and subjected to determinations of mRNA levels or stored at −80°C until the day of use.

To determine the effect of PPA antisense on the anorectic response of PPA, rats (n = 6–8 for each group) were treated with antisense (20 µg in a 10-µl vehicle icv) at 1 h before PPA (110 mg/kg ip) daily for 5 days. Daily food intake was calculated as the total amount of food during the previous day.

Another control experiment was designed to determine the effects of PKA antisense pretreatment on PPA and NPY mRNA levels in PPA-treated rats. Rats (n = 6–8 each group) were injected daily with antisense or missense (20 µg in a 10-µl vehicle icv) at 1 h before daily PPA (110 mg/kg ip) for 5 days. Rats receiving PPA at 40 min before death were anesthetized with pentobarbital (30 mg/kg ip) and then decapitated. The hypothalamus was removed from the brain immediately and subjected to determinations of mRNA levels, or stored at −80°C until the day of use.

To determine the effect of PPA on CREB DNA binding activity, rats were given the PPA (70 mg/kg ip, n = 4–6 each group) daily for 4 days at the beginning of the dark phase (at 6:00 PM). At 40 min after daily PPA treatment, the hypothalamus was removed to determine CREB DNA binding activity by the electromobility shift assay (EMSA) technique daily for 4 days.

To determine the effect of PPA on NPY protein content, rats were treated with PPA (0 or 70 mg/kg ip, n = 6–8 each group) daily for 5 days at the beginning of the dark phase (at 6:00 PM). Daily food intake was examined at 24 h after daily drug treatment. To examine whether the change of NPY content is due to the effect of PPA treatment or is secondary to reduced feeding, rats (including pair-fed) were saline-treated daily for 5 days. The amount of food in pair-fed animals was restricted and was daily equal to that of normal-fed rats (including pair-fed) daily for 5 days at the beginning of the dark phase (at 6:00 PM).

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RNA extraction. Hypothalamic NPY mRNA levels in a block of mediobasal hypothalamic tissue were measured as described previously (40). In brief, total RNA was isolated from tissue block using the modified guanidinium thiocyanate-phenol-chloroform method (13). Each hypothalamic block was homogenized in 1 ml of TRIZOL reagent (Life Technologies, Grand Island, NY) using an Ultrasonic Processor (Vibra Cell model CV17; Sonics & Materials, Danbury, CT). After an incubation at 22°C for 5 min, 0.2 ml of chloroform was added to each sample, shaken vigorously for 15 s, incubated at 22°C for 5 min, and then centrifuged at 12,000 g for 15 min at 4°C. After removal of aqueous phase and precipitation with 0.5 ml isopropanol, samples were incubated at 22°C for 10 min and centrifuged at 12,000 g for 15 min at 4°C. The gel-like RNA pellets were washed with 75% ethanol by vortexing and centrifugation at 7,500 g for 5 min at 4°C. Thereafter, RNA pellets were dried briefly, dissolved in RNase-free water, and stored at −80°C. The content of RNA was determined spectrophotometrically at 260 nm (Hitachi U-3210, Japan).

Reverse transcription-polymerase chain reaction. Using the 1st Strand cDNA Synthesis Kit (Boehringer Mannheim, Mannheim, Germany), RNA was reversely transcribed into single-stranded cDNA.
For each sample, 8 μl of sterile diethyl pyrocarbonate (DEPC) water containing 2 μg of RNA were added to oligo-p(dT)15 primer (0.8 μg/μl) followed by a heating at 65°C for 15 min, a cooling at 25°C for 10 min, and then added to a reaction mixture consisting of 10× reaction buffer (100 mM Tris, 500 mM KCl; pH 8.3), deoxynucleotide mix (10 mM each), MgCl₂ (25 mM), RNase inhibitor (40 unit/μl), and avian myeloblastosis virus reverse transcriptase (25 unit/μl). Reaction mixtures were incubated at 42°C for 2 h and then brought to 95°C for 5 min to terminate the reaction followed by soaking at 16°C. We subsequently carried out PCR by mixing 3 μl of cDNA product with mastermix solution consisting of DEPC water, 10× reaction buffer, MgCl₂ (25 mM), deoxynucleotide mix (10 mM each), P1 and P2 primers (1 μg/μl each), and Taq polymerase (5 unit/μl). GAPDH was used as the internal standard calibrator. PCR reactions for NPY were carried out on a PCR thermocycler (Perkin-Elmer GeneAmp 2400) for 28 cycles with the following steps: 91°C for 1 min (denaturing), 60°C for 1 min (annealing), and 72°C for 30 s (extension), followed by a final elongation step at 72°C for 7 min, and finally the PCR products were soaked at 16°C. PCR reactions for the other molecules analyzed were carried out in steps similar to those described above except the changes of two steps (annealing and cycles) that were described as follows: PKA (60°C, 28 cycles); CREB (60°C, 35 cycles); POMC (55°C, 25 cycles); GAPDH (52°C, 25 cycles). These PCR products were measured during the exponential phase of the DNA amplification in the present study. The sequences of primers used in RT-PCR were shown in Table 1.

**Gel electrophoresis.** At the completion of reverse transcription-polymerase chain reaction (RT-PCR), 8 μl of each PCR product was subsequently separated by flat-bed gel electrophoresis on a 3% agarose gel. Gels stained by ethidium bromide (0.5 μg/ml, Sigma-Aldrich) were visualized under UV light, photographed, and then scanned densitometrically. Ratios of NPY and GAPDH mRNA were calculated to determine relative NPY mRNA levels. Contents of NPY mRNA in PPA-treated group were indicated as the percentage of the control group. The ratio of NPY/GAPDH mRNA was measured by radioimmunoassay specific for CREB consensus sequence 5′-AGAGATTGCCTGACGT-3′, which was labeled on the 3-end with biotin.

The PKA antisense was targeted to the α-catalytic subunit of PKA (5′-GCGATCGGGACAT-CATGTTG-3′) complementary to nucleotides 6–23, which could interrupt PKA gene expression. The missense sequence was 5′-CTGCGT-GAGACATTGC-3′, which did not show significant matches in the database. We used ODNs that were phosphorothioate-modified (S-ODNs) only on the three terminal bases of both the 5′- and 3′-ends (Proligo Pty, Singapore). The sequence of PKA antisense used in this study can specifically reduce with the expression of either PKA mRNA level or protein content. Before PPA treatment, rats were icv injected with similar dose of antisense daily for about 3 days (from day 2 to day 0) until the response of normal feeding behavior was slightly reduced. This is due to the fact that either continuous or repeated icv injections of antisense may be necessary to maximize behavioral effect and especially to block the synthesis of constitutively active gene product (43, 63). Both antisense and missense S-ODNs were dissolved in artificial corticospinal fluid containing 140 mM NaCl, 3.35 mM KCl, 1.15 mM MgCl₂, 1.26 mM CaCl₂, 1.2 mM Na₂HPO₄, and 0.3 mM NaH₂PO₄; pH 7.4.

**CREB binding assay.** Binding of CREB in nuclear extracts was assessed by EMSA with double-stranded deoxyoligonucleotides specific for CREB consensus sequence 5′-AGAGATTGCCTGACGT-3′, which was labeled on the 3-end with biotin. EMSA was carried out using the Lightshift kit (Promega Life Science). Briefly, 1 μg of nuclear protein was preincubated with 10 mM Tris, 50 mM KCl, 1 mM DTT, 5 mM MgCl₂, 2 μg poly(dI·dC), and 2 pmol of oligonucleotide probe for 20 min at room temperature. Specific binding was confirmed by using a 200-fold excess of unlabeled probes as specific competitor. Protein-DNA complexes were separated by a 6% nondenaturing acrylamide gel electrophoresis. Complexes were transferred to positively charged nylon membranes and UV-crosslinked in a streptavidin-horseradish peroxidase followed by hemiluminescent detection (11).

**Radioimmunoassay.** PPA-treated and vehicle-treated (including pair-fed and normal-fed) rats were decapitated under anesthesia with pentobarbital (30 mg/kg ip). The target of cannulation was close to the junction between the right lateral ventricle and the third ventricle (coordinates: 0.8 mm posterior to Bregma, 1.5 mm from the midline, and 3.5–4.0 mm below the dura) (44). A 23-gauge stainless steel guide cannula was implanted and secured to the skull with stainless steel screws and dental cement. We confirmed the accuracy of placement by observing a transient and rapid inflow of vehicle via polyethylene tubing connected with a 28-gauge injector cannula. The cannula was then occluded with a 28-gauge stylet. For icv infusion of PKA antisense, the stylet was replaced with a 28-gauge injector cannula extending 0.5 mm below the tip of the guide cannula. Behavioral testing began at 1 wk after the surgery. During this period, rats were daily treated with the antibiotic tetracycline to prevent the infection. For all experiments, verification of cannula placement was done by the administration of angiotensin II (100 ng/rat, Sigma-Aldrich). Angiotensin II reliably induced water drinking in nondeprived rats when administered into the ventricles (47). Only data from rats drinking more than 10 ml within 30 min were included in this study.

**ICV administration of PKA antisense.** The PKA antisense was injected with 2 μg in 4 μl of sterile saline into the lateral ventricle. The antisense was co-injected with an equal volume of 10 μM of a misense sequence of PKA gene. This combination was targeted to the catalytic subunit of PKA (5′-GAGACATTGC-3′), which did not show significant matches in the database. We used S-ODNs that were phosphorothioate-modified (S-ODNs) on the three terminal bases of both the 5′- and 3′-ends (Proligo Pty, Singapore). The sequence of PKA antisense used in this study can specifically reduce with the expression of either PKA mRNA level or protein content. Before PPA treatment, rats were icv injected with similar dose of antisense daily for about 3 days (from day 2 to day 0) until the response of normal feeding behavior was slightly reduced. This is due to the fact that either continuous or repeated icv injections of antisense may be necessary to maximize behavioral effect and especially to block the synthesis of constitutively active gene product (43, 63). Both antisense and missense S-ODNs were dissolved in artificial corticospinal fluid containing 140 mM NaCl, 3.35 mM KCl, 1.15 mM MgCl₂, 1.26 mM CaCl₂, 1.2 mM Na₂HPO₄, and 0.3 mM NaH₂PO₄; pH 7.4.

**Table 1. Sequences of primers used in the experiment of RT-PCR**

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<thead>
<tr>
<th>Primer</th>
<th>Sequence 5′→3′ Products (base pairs)</th>
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<tbody>
<tr>
<td>NPY</td>
<td>GGGCTGTGGGCTGAATGGCC 264</td>
</tr>
<tr>
<td>PKA</td>
<td>GGAAGGCTTCTCAGGCTCT 383</td>
</tr>
<tr>
<td>CREB</td>
<td>GAGACGGATGGAGGAGCTTGTA 616</td>
</tr>
<tr>
<td>POMC</td>
<td>GGGCTACAGGCTTGGTGAGGATGCA 742</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AGATGACCAACAGGATACATT 309</td>
</tr>
</tbody>
</table>

NPY, neuropeptide Y; PKA, protein kinase A; CREB, CAMP response element binding protein; POMC, pro-opiomelanocortin.

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for each NPY as described previously (3). Authentic antibody against NPY and radioactive tracer $^{125}$-labeled NPY were purchased from Peninsula Labs Hypothalamic concentration of NPY (pg/ml) is calculated by the mass of NPY per unit weight of protein (pg/μg) that is measured by the mass of protein per unit volume of hypothalamus (μg/ml).

Western blotting. Protein samples extracted from hypothalamic tissue were separated in a 12.5% polyacrylamide gel, transferred onto a nitrocellulose membrane, and then incubated separately with specific PKA antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or CREB antibodies (Cell Signaling Technology, Beverly, MA) and α-tubulin antibodies (Sigma-Aldrich). After incubation with horseradish peroxidase goat anti-rabbit IgG, the color signal was developed by 4-chloro-1-naphthol/3,3′-diaminobenzidine, 0.9% (wt/vol) NaCl in Tris-HCl (Sigma). Relative photographic density was quantified by scanning the photographic negative film on a Gel Documentation and Analysis System (Alphalmager 2000; Alpha Innotech, San Leandro, CA).

Statistical analysis. Data were presented as means ± SE. A t-test or one- or two-way ANOVA followed by Dunnett’s test was used to detect significances of difference among groups. P < 0.05 was considered to be statistically significant.

RESULTS

Effect of PPA on feeding behavior. Changes of daily food intake in rats receiving PPA are shown in Fig. 1, top. Statistical analysis (two-way ANOVA) revealed significant dose-dependent [F(2,21) = 10.21, P < 0.05] and time-dependent effects [F(5,42) = 5.15, P < 0.05]; however, the interaction effect failed to achieve significance. It revealed that a treatment with 70 mg/kg PPA reduced the food intake day 1 to day 3, and a treatment with 110 mg/kg PPA reduced daily body weight continuously from day 1 to day 5 compared with controls. Moreover, a treatment with 70 mg/kg PPA on day 5 restored the body weight to normal level compared with that on day 2.

Effect of PKA antisense on PPA anorexia. As shown in Fig. 3, PKA antisense could partially block the anorectic response of PPA (110 mg/kg), indicating the involvement of PKA gene in PPA-treated rats. Statistical analysis with two-way ANOVA revealed a significant treatment effect [F(3,28) = 5.6, P < 0.05] and time effect [F(5,42) = 3.9, P < 0.05]. Comparing the intake between antisense/CSF-treated and PPA-treated rats every day revealed significant effects from day 1 to day 5 (P < 0.05). These results indicate that PKA knock-down could reduce the feeding response of PPA.

No statistical significance was obtained in 110 mg/kg PPA-treated rats receiving missense/CSF (vehicle) injection (shown in Fig. 2). The effect of daily treatment of PPA (70 mg/kg) on hypothalamic NPY, PKA, CREB, POMC, and GAPDH mRNA levels over a 4-day period. Top: the RT-PCR results of hypothalamic NPY, PKA, CREB, POMC and GAPDH mRNA levels. Bottom: relative densitometric values for RT-PCR products of hypothalamic NPY, PKA, CREB, POMC mRNA when compared with the controls. Content of each mRNA in PPA-treated group was indicated as the percentage of control. Bars are means ± SE; n = 5–6 per group. *P < 0.05 vs. control. NPY, neuropeptide Y; PKA, cAMP-dependent protein kinase; CREB, cAMP response element binding protein; POMC, pro-opiomelanocortin.
in Fig. 3) or not (shown in Fig. 1) (t-test), indicating the noninterference of missense treatment and vehicle on PPA’s action.

Effect of pretreatment with PKA antisense on NPY mRNA levels. Results shown in Fig. 4 reveal that the effect of pretreatment with PKA antisense can (1) reverse partially the decreased level of NPY mRNA and (2) attenuate the increased level of PKA mRNA in PPA-treated rats. Using GAPDH as the internal standard, we calculated and compared the ratio of NPY/GAPDH mRNA or PKA/GAPDH mRNA in each group. A one-way ANOVA revealed that NPY mRNA content was decreased in both PPA and PKA antisense/PPA groups [F(7,34) = 3.82, P < 0.05] compared with the control (missense) group. Moreover, significant effects were also observed in PKA antisense/PPA group when compared with the PPA group. This result suggests that PKA gene is involved in the regulation of NPY gene expression in PPA-treated rats.

Similarly, a one-way ANOVA revealed that PKA mRNA content was increased in PPA (missense)-treated group but decreased in PKA antisense group [F(7,34) = 2.75, P < 0.05] compared with the control (missense) group. Moreover, significant effects were also observed in PKA antisense/PPA group when compared with the PPA group. This result suggests that PKA antisense is sequence specific and is effective in the interruption of PKA-mediated action in PPA-treated rats.

Effect of PPA on CREB DNA binding activity. Results shown in Fig. 5 revealed that PPA could increase CREB DNA binding activity in the hypothalamus. Analysis with one-way ANOVA revealed an increase of CREB from day 1 to day 2 [F(4,25) = 2.48, P < 0.05] compared with the control. This result revealed that CREB DNA binding activity was increased during the first 2 days of PPA treatment.

Effect of PPA on NPY contents. Results shown in Table 2 indicate the effect of PPA on the change of hypothalamic NPY content. Results revealed that PPA treatment could decrease NPY contents [F(5,36) = 2.68, P < 0.05, one-way ANOVA] and that changes of NPY contents paralleled that of NPY mRNA levels during a 5-day period of PPA treatment. Moreover, NPY contents in paired-fed animals were not changed in groups from day 1 to day 5 if compared with that of the normal-fed control (day 0).

Effects of PPA on PKA and CREB protein contents. Results shown in Fig. 6 reveal that daily PPA increase PKA and CREB protein contents during a 4-day period. Using α-tubulin as the internal standard, we calculated and compared the ratio of PKA (or CREB) over α-tubulin in each group. Analysis with one-way ANOVA revealed an increase of PKA contents [F(4,25) = 3.5, P < 0.05] from day 1 to day 3 and an increase of CREB contents [F(4,25) = 2.1, P < 0.05] on day 1 and day 2.
may involve the inhibition of hypothalamic NPY gene expression.

This finding was supported by previous reports showing that cAMP agonist or PKA activator administered into hypothalamus could decrease NPY-induced feeding behavior (53). However, some in vitro and in vivo studies had shown that NPY gene was activated by phorbol ester or cAMP analog (1, 36) and that PKA inhibitors could attenuate the stimulatory effect of feeding induced by ghrelin or orexin in NPY neurons (31). Mechanisms underlying this contradictory effect of PKA on NPY gene expression are unknown. To clarify this contradiction, icv injection of PKA antisense was administered to PPA-treated rat, and results showed that PKA knock-down in the brain could interrupt the anorectic response of PPA with a restoration of NPY gene expression. This result supported our hypothesis that activation of hypothalamic PKA signaling was involved in the inhibition of hypothalamic NPY gene expression in PPA-treated rats.

Although it has been reported previously that PKA signaling could activate NPY gene expression (31, 32, 39), there are some differences between those reports and the present data. First, the previous data are from studies in a single NPY-containing neuron isolated from arcuate hypothalamus (31, 32) or in PC 12 cells in vitro (39), while the present data are from in vivo hypothalamus. Second, the effect of PKA signaling was examined through the activation of ghrelin (or leptin) receptors (32) or adenosine receptors (39), while the present data were examined through the activation of catecholamine receptors.

On the basis of the present findings, together with the previous results, the apparent discrepancy of PKA on NPY neurons described above can be explained by the fact that downregulation of NPY neurons linked to inhibition of food intake takes place in arcuate nucleus, while action sites of PPA/AMPH are not the arcuate but nucleus of dopaminergic and noradrenergic neurons (12, 21, 45); PKA may be implicated in the regulation of these neurons that secondarily innervate and regulate the arcuate NPY neurons. In contrast, cAMP-PKA pathway appears to be the direct upregulator of arcuate

Table 2. Effects of PPA treatment on the protein level of hypothalamic NPY

<table>
<thead>
<tr>
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<tr>
<td>Saline-treated</td>
<td></td>
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<tr>
<td>Day 0 (normal-fed control)</td>
<td>66.67 ± 3.28</td>
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<td>Day 1 (pair-fed control)</td>
<td>67.97 ± 2.55</td>
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<tr>
<td>Day 2 (pair-fed control)</td>
<td>65.36 ± 2.91</td>
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<tr>
<td>Day 3 (pair-fed control)</td>
<td>67.75 ± 1.68</td>
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<td>Day 4 (pair-fed control)</td>
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<td>Day 1 (70 mg/kg)</td>
<td>51.12 ± 2.35*</td>
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The protein level of NPY was measured by radioimmunoassay at 24 h after intraperitoneal injection of drug. The amount of food in pair-fed animals was equal to that of the saline-treated control (day 0) or phenylpropanolamine (PPA)-treated animals of each day (day 1–day 5) as described in materials and methods. *P < 0.05 vs. saline-treated controls (Dunnett’s test).

**DISCUSSION**

Although the anorectic action of PPA has been attributed to its suppressive effect on hypothalamic NPY, the exact molecular mechanisms underlying this action are still unknown. In this study, we found that PKA mRNA levels were elevated during daily PPA treatment and the alteration in PKA mRNA content was closely related to changes of feeding behavior and NPY mRNA level, suggesting that PKA gene was involved in the regulation of NPY gene expression. However, instead of being inhibited as observed in NPY gene expression, PKA gene was activated following daily PPA treatment. This result suggests that the participation of hypothalamic PKA signaling

From the figure, it is clear that CREB DNA binding activity is significantly increased on the first and second days of PPA treatment compared to the control. The bar graph shows that PKA knock-down in the brain could interrupt the anorectic response of PPA with a restoration of NPY gene expression. This result supported our hypothesis that activation of hypothalamic PKA signaling was involved in the inhibition of hypothalamic NPY gene expression in PPA-treated rats. However, instead of being inhibited, CREB DNA binding activity is increased, suggesting that PKA gene was involved in the regulation of NPY gene expression. The exact molec-

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<tr>
<td>Day 2 (pair-fed control)</td>
<td>65.36 ± 2.91</td>
</tr>
<tr>
<td>Day 3 (pair-fed control)</td>
<td>67.75 ± 1.68</td>
</tr>
<tr>
<td>Day 4 (pair-fed control)</td>
<td>65.36 ± 1.87</td>
</tr>
<tr>
<td>Day 5 (pair-fed control)</td>
<td>66.56 ± 1.86</td>
</tr>
<tr>
<td>PPA-treated</td>
<td></td>
</tr>
<tr>
<td>Day 1 (70 mg/kg)</td>
<td>51.12 ± 2.35*</td>
</tr>
<tr>
<td>Day 2 (70 mg/kg)</td>
<td>48.75 ± 2.38*</td>
</tr>
<tr>
<td>Day 3 (70 mg/kg)</td>
<td>55.10 ± 2.20*</td>
</tr>
<tr>
<td>Day 4 (70 mg/kg)</td>
<td>61.49 ± 2.62</td>
</tr>
<tr>
<td>Day 5 (70 mg/kg)</td>
<td>66.14 ± 2.72</td>
</tr>
</tbody>
</table>

The protein level of NPY was measured by radioimmunoassay at 24 h after intraperitoneal injection of drug. The amount of food in pair-fed animals was equal to that of the saline-treated control (day 0) or phenylpropanolamine (PPA)-treated animals of each day (day 1–day 5) as described in materials and methods. *P < 0.05 vs. saline-treated controls (Dunnett’s test).

**DISCUSSION**

Although the anorectic action of PPA has been attributed to its suppressive effect on hypothalamic NPY, the exact molecular mechanisms underlying this action are still unknown. In this study, we found that PKA mRNA levels were elevated during daily PPA treatment and the alteration in PKA mRNA content was closely related to changes of feeding behavior and NPY mRNA level, suggesting that PKA gene was involved in the regulation of NPY gene expression. However, instead of being inhibited as observed in NPY gene expression, PKA gene was activated following daily PPA treatment. This result suggests that the participation of hypothalamic PKA signaling
NPY, PKA, AND PHENYLPROPANOLAMINE ANOREXIA

Western blotting analyzing the contents of PKA and CREB.

increasing cAMP serves as a positive regulator. NPY mRNA and feeding, while at the arcuate NPY neuron, the cAMP-PKA pathway in the brain is linked to suppression of the concept that PPA- and AMPH-related drugs activated PKA and CREB protein contents over a 4-day period. Top: the results of Western blotting analyzing the contents of PKA and CREB. Bottom: relative densitometric values for Western blotting of PKA and CREB in saline- and PPA-treated groups. Contents of PKA and CREB in PPA-treated groups are indicated as the percentage of the control group. Bars are means ± SE, n = 4–6 each group. *P < 0.05 vs. control.

NPY neuron, which has been well demonstrated by the literatures including a recent study (32). The present data support the concept that PPA- and AMPH-related drugs-activated cAMP-PKA pathway in the brain is linked to suppression of NPY mRNA and feeding, while at the arcuate NPY neuron level cAMP serves as a positive regulator.

Restoration of the NPY mRNA level on day 4 during PPA (70 mg/kg) treatment was accompanied by a gradual decrease in the PKA mRNA level, implying a disinhibitory effect of PKA on NPY gene expression. Possibly, this disinhibitory effect of PKA might be relevant to a gradual decrease in catecholamine released from presynaptic nerve terminals during a repeated treatment of PPA as described in our recent report (23). The physiological state during the first 2 days in PPA-treated rats was similar to that of fasting, which is in a state of negative energy balance, resulting in the induction of NPY gene expression on subsequent days.

CREB is a downstream nuclear transcription factor of PKA. Increasing evidence reveal that CREB is required for dopamine-dependent gene expression (4, 15) and that CRE-mediated gene induction may be involved in NPY gene expression in dopamine agonist-treated rats (24). Thus, it is possible that the CREB gene may be activated during PPA treatment. The present result revealed that the CREB mRNA level was increased following PPA treatment and that the CREB DNA binding activity was elevated during PPA treatment, which paralleled the change of PKA gene expression. This result suggests that the hypothalamic PKA-CREB signaling may participate in the central regulation of NPY gene and PPA-mediated appetite suppression.

The activation of PKA signaling may be a critical pathway for AMPH-like anorectic drugs to induce an appetite-suppressing effect. Except for the current finding that PKA is activated in PPA treatment, much evidence reveals that 1) the intracellular concentration of cAMP is increased following PPA treatment in minces of rat heart (25); 2) PKA signaling is activated following AMPH treatment (24); 3) intracellular cAMP is accumulated following ephedrine treatment in rat leukemia cells (49), Chinese hamster ovary cells (57), or human adipose tissue (16); and 4) daily oral administration of phentermine to rats can change the activity of the adenylate cyclase-cAMP system in renal and hepatic tissues (28). PPA, ephedrine, and phentermine are AMPH-like anorectic drugs and are classified as sympathomimetic agents that may exert their effect via the activation of monoaminergic system in the brain (2, 12, 41). Except for sympathomimetic agents, it has been suggested that psychostimulant and opiate drugs, such as AMPH and cocaine, may target similar CREB genes to induce behavioral responses (9, 24). It was then rational to speculate that induction of PKA signaling by AMPH-like anorectic drugs should be viewed as a group of concerted events that occurred against a complex background of intra- and intercellular signal pathway.

The present data reveal that while PKA antisense almost completely counteracts the effect of PPA on PKA, it only partially, ~60%, counteracts the effect of PPA on NPY message, suggesting a component of PKA-independent pathway for PPA suppression of NPY. It has been documented in our previous reports that the changes in PKA mRNA level following AMPH treatment were in a manner similar to that of some types of protein kinase C isozymes (PKC-α, PKC-δ, and PKC-λ) during a 4-day period of drug treatment, suggesting that the coactivation of PKA and PKC signaling in hypothalamus might play a synergistic role in modulating NPY gene expression in AMPH-treated rats (22, 24).

Some evidence revealed that the induction of PKA signaling might serve to activate NPY gene transcription (52); thus, it was possible that PPA might activate PKA signaling in a distinct population of hypothalamic neurons, such as anorexigenic POMC neurons, and in turn inhibit NPY neurons. It was reported that both NPY- and α-melanocyte-stimulating hormone (MSH)-immunoreactive neurons were embedded in dense, intermingling networks of NPY- and α-MSH axons and their neuronal wiring in hypothalamus possessed a different role in feeding behavior (30, 37). Moreover, activation of cAMP/PKA signaling is involved in the regulation of POMC gene expression (8, 34). The present data revealed that POMC
mRNA levels were elevated following PPA treatment and were expressed in a manner parallel to the alteration of PKA mRNA levels. However, they were opposite to the changes of NPY mRNA levels. Therefore, it is possible that the increased PKA following PPA treatment might preliminarily activate POMC-containing cells, which then went on to reduce downstream NPY gene expression. Conversely, PKA signaling might at first activate NPY-containing neurons, which in turn reduced the inhibitory action on downstream POMC-containing cells since the activity of two populations of neurons (NPY/AgRP- and POMC-containing neurons) in hypothalamus is reciprocally regulated by a number of peripheral and central neuro-modulatory systems that influence energy balance (48, 51). The possible role of POMC-containing cells during PPA treatment needs to be investigated further.

Although evidence revealed that substantial food deprivation might lead to increased NPY gene expression (46), we ruled out the possibility that the changes in NPY level were simply secondary to reduced feeding, rather than the rapid action of PPA on hypothalamic NPY, because pair-fed (nondrug-treated) animals from day 0 to day 5 showed no change in NPY level compared with that in normal-fed animals.

In this study, application of PKA antisense S-ODN into the cerebral ventricle attenuated the response of PPA anorexia in conscious rats, revealing an effective control of feeding behavior by PKA manipulation at the molecular level. Compared with the unmodified ODNs, S-ODNs are known to exert a greater effect at much lower concentrations due to their higher intracellular stability (17). For in vivo application, S-ODNs were more stable than ODNs after both icv and intracerebral administrations (60). An intracerebral infusion study found that signals of S-ODNs in cell bodies could be observed for much longer periods of time (up to 6–16 h) than unmodified ODNs (up to 2–4 h) (43). It is suggested that the antisense S-ODN method may be used as a tool to study causal relationships between molecular processes in the brain and behavior.

In conclusion, the present data provide a message that is relevant for understanding the PKA regulation of the NPY neurons in hypothalamus. The current findings may provide a molecular basis for the anorectic effect of PPA and imply that manipulations at the molecular level of PKA or cAMP at a specific site in the brain may allow the development of therapeutically effective agents to improve the undesirable properties of PPA, such as hypertension and stroke, or other AMPH-like anorectic drugs.

**GRANTS**

This study was supported by grants partly from the National Science Council (NSC-96-2320-B-040-018-MY2) and partly from the Department of Health (DOH95-NNB-1018).

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