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Neuropeptide Y Treatment and Food Deprivation Increase Cyclic AMP Response Element-Binding in Rat Hypothalamus

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SUMMARY

Intrahypothalamic (IHT) administration of neuropeptide Y (NPY) induces a robust feeding response in rats. We have shown previously that NPY-induced feeding is mediated by a pertussis-toxin-sensitive G protein in rats. NPY receptors are coupled to cAMP and Ca^{2+}. Because these second messengers are known to activate cAMP response element binding proteins, (CREB), cAMP response element modulators, or activating transcription factor 1, we investigated the involvement of these transcription factors in NPY-induced feeding in rats. Compared with control injections of cerebrospinal fluid (1 μl), IHT administration of NPY increased cAMP response element (CRE) binding to rat hypothalamic nuclear extracts in a time-dependent manner, as detected by an electrophoretic mobility shift assay. In contrast, IHT administration of the anorectic neuropeptide, pituitary adenylate cyclase activating polypeptide, strongly inhibited the CRE binding. Food deprivation for 48 hr also increased CRE binding, whereas 8 hr of refeeding normalized CRE activity. Preincubation of the hypothalamic nuclear extracts of NPY-treated and unfed rats with antibody specific to CREB blocked CRE binding, whereas preincubation with phosphoCREB antibody retarded the migration of CRE-protein complex, indicating that phosphoCREB is involved in this process. Consistently, immunohistochemical studies with food-deprived rats showed an intense phosphoCREB signal in the paraventricular nuclei and ventromedial hypothalamus in comparison to rats fed *ad libitum*. Hypothalamic calcium/calmodulin-dependent protein kinase II activity was also increased by IHT-NPY. These results suggest that calcium/calmodulin-dependent protein kinase II induced phosphorylation of CREB may be involved in regulating feeding behavior induced by NPY.

NPY, a 36-amino-acid peptide amide initially isolated from porcine brain, is present in high concentrations in mammalian central and peripheral nervous systems (1). NPY is currently considered the most powerful orexigenic agent isolated (2). The perifornical hypothalamus, a site known to regulate feeding, is richly innervated with neurons containing both NPY and catecholamines (3, 4). Moreover, although hypothalamic NPY levels and/or its mRNA have been found to be elevated in obese, hyperphagic, diabetic rats, NPY levels are reduced in anorectic tumor-bearing rats (5–7). Food-deprived rats showed a 5- to 10-fold increase in NPY concentration in the paraventricular nucleus and arcuate nucleus-median eminence of hypothalamus (8). Also, the central administration of NPY antibody dose-dependently inhibited feeding in hyperphagic rats with ventromedial hypothalamic lesions (9). These observations and the recent findings that leptin, the obese gene product, inhibits food intake and stimulates thermogenesis via inhibiting the synthesis and secretion of hypothalamic NPY (10) suggest that NPY may constitute a major central nervous system regulator of feeding behavior.

NPY receptors characterized to date, as well as those cloned, have been shown to be coupled to intracellular calcium and cAMP (11–14). We have demonstrated previously that hypothalamic receptors coupled to a pertussis toxin-sensitive G protein are involved in NPY-induced feeding in rats (15). However, the downstream elements involved in the signal-transduction process mediating NPY-induced feeding have not yet been characterized. It has been well documented that the elevation of intracellular calcium or cAMP leads to the activation of CRE binding transcription factors, including three families of proteins: CREB, CREM, and ATF-1 (16). The binding of these activated transcription factors to the CRE, TGACGTCA, which is present in the regulatory sequence of many eukaryotic genes, results in the stimulation

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ABBREVIATIONS: NPY, neuropeptide Y; AP-1, activator protein-1; ATF-1, activating transcription factor 1; CaM kinase II, Calcium/calmodulin-dependent protein kinase II; CRE, cAMP response element; CREB, cAMP response element binding protein; CREM, cAMP-response element modulator; CSF, cerebrospinal fluid; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IHT, intrahypothalamic or intrahypothalamically; PACAP, pituitary adenylate cyclase activating polypeptide; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PBS, phosphate-buffered saline; PKA, protein kinase A; PVN, paraventricular nucleus.

or repression of the target genes (17, 18). These observations led us to speculate that NPY-induced feeding may also be mediated by CRE binding transcription factors. Therefore, we investigated the effects of IHT administered NPY on CRE binding proteins in rat hypothalamic nuclear extracts. As a negative control, we also investigated the effects of IHT-PACAP, an anorectic neuropeptide (19). Results from these experiments show that hypothalamic CRE binding is increased in unfed and NPY-treated rats and decreased in refed and PACAP-treated rats.

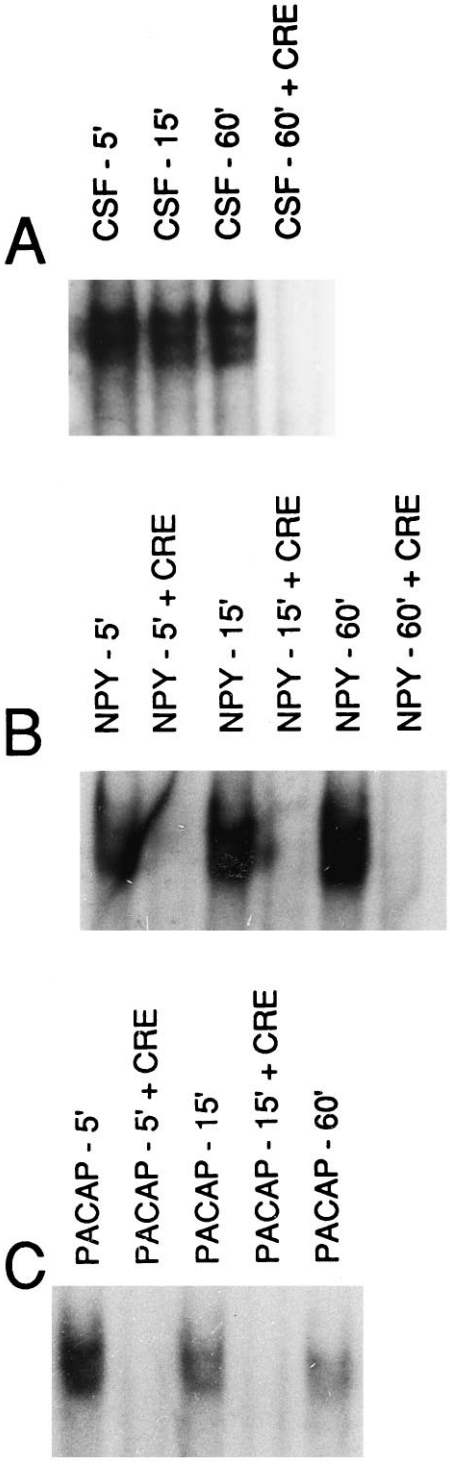

Fig. 1. Time course of CRE binding in rat hypothalami after IHT administration of (A) 1 μl CSF, (B) 1 μg NPY, and (C) 2 μg PACAP. After treatment, rats were killed at the indicated time without giving food or water. Hypothalamic nuclear proteins (10 μg) were analyzed for CRE binding activity. Unlabeled CRE (125-fold excess) was used to determine the nonspecific binding.

Experimental Procedures

Materials. Male Sprague-Dawley rats (350–500 g at time of death) were obtained from Zivic Miller (Zelienople, PA) and housed individually in a temperature- and humidity-controlled environment under a 12-hr light/dark cycle (lights on at 7:30 a.m.), with *ad libitum* access to rat chow and water. After anesthetization (ketamine/xylazine), stainless steel cannulae (24 gauge) were surgically implanted into the perifornical hypothalamic area of the rats according to our previously published procedures (19). Experiments were conducted after at least 2 weeks of recovery. The hypothalami were taken from the treated rats as described previously (20). Briefly, the hypothalamus, taken to a depth of approximately 2.5 mm from just anterior to the optic chiasm and extending to the posterior mammillary area and bounded laterally by the choroid fissure, was removed from the ventral surface of the brain and frozen in liquid nitrogen. NPY was synthesized in our laboratory according to our previous report (21). PACAP-38 and 3,3′-diaminobenzidine reagent (Sigma Chemical, St. Louis, MO), CREB, CREM, and ATF-1 antibodies (Santa Cruz Biochemicals, Santa Cruz, CA) and the phosphoCREB antibody (Upstate Biotechnology, Lake Placid, NY) were obtained commercially and used without further purifications. Biotinylated anti-rabbit IgG and Avidin: Biotinylated enzyme Complex kit (PK-6101) were obtained from Vector Laboratories (Burlingame, CA). All buffer reagents, polynucleotide kinase, and double-stranded 39-mer oligonucleotide 5′-GATC*TGACGTCA*TGAC*TGACGTCA*TGAC*TGACGTCA*TCA-3′, containing a tandem repeat of the consensus sequence for the CRE DNA binding site, -TGACGTCA (13379–011), AP-1 double stranded 40-mer oligonucleotide 5′-GATCCTTCG*TGACTCA*GCGG-GATCCTTCG*TGACTCA*GCGG-3′, containing tandem repeat oligonucleotide for the AP-1 binding site, -TGACTCA (13381–017), CaM kinase II substrate (syntide-2) were obtained from Life Technologies (Grand Island, NY). Nuclear factor κB double-stranded 22-mer oligonucleotide 5′-AGTTGA*GGGGACTTTCCC*AGGC-3′ (E3291) was obtained from Promega (Madison, WI). The sensitivity of the DNA binding to the proteins has been reported to be significantly enhanced by using DNA probes containing multiple binding sites that are spaced (22).

Methods. The oligonucleotide was labeled with [^{32}P]-ATP using polynucleotide kinase and purified on a sephadex G-50 column ac-

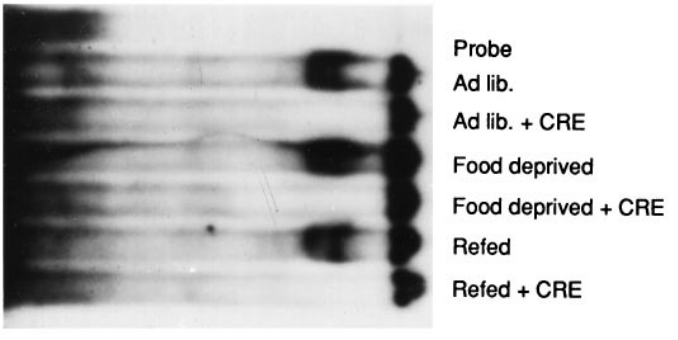

Fig. 2. Effect of nutritional status on CRE binding in rat hypothalamus. Hypothalamic nuclear extracts from three different groups: *ad libitum*, rats had free access to food and water; food deprived, deprived of food but not water for 48 hr; and refed/food deprived as above but allowed to eat for 8 hr before sacrifice. All animals from the three different groups were killed at the same time. Hypothalamic nuclear proteins (10 μg) were analyzed for CRE binding activity.

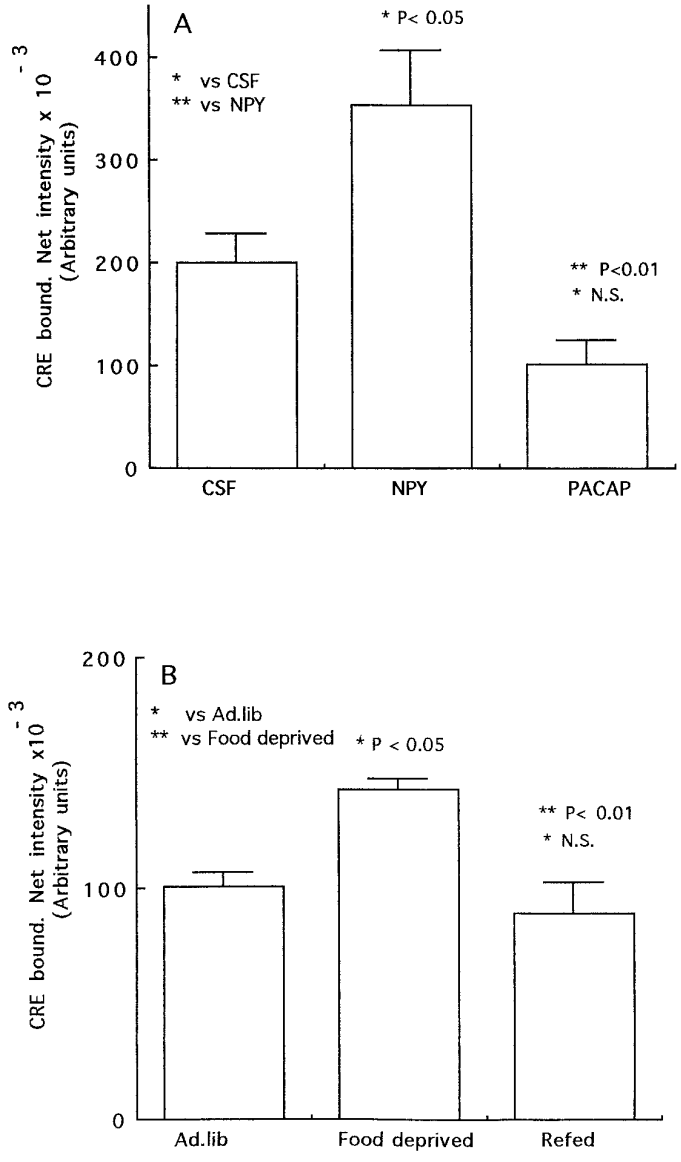

Fig. 3. Effect of peptide treatment (A) or alteration in nutritional status (B) on CRE binding activity. Eight rats from each group were used for the CRE binding assay. A, CSF (1 μl), NPY (1 μg), and PACAP (2 μg) were administered IHT and rats were killed 60 min later. No food was given after the peptide treatment. B, Eight rats from each group were used for this study. Specific binding of [^{32}P]CRE was quantitated by densitometry. Statistical significance was determined by ANOVA, followed by Tukey's protected *t* test.

cording to the protocols supplied by the manufacturer (Pharmacia, Piscataway, NJ).

CaM kinase II activity. Hypothalami were homogenized and extracted for CaM kinase II activity as described by Erondu and Kennedy (23). Calmodulin-dependent CaM kinase II activity was assayed using syntide-2 as substrate as described in Life Technologies protocol.

Preparation of hypothalamic nuclear extracts. The hypothalami from unfed and CSF- (1 μl), NPY- (1 μg) or PACAP- (2 μg) treated rats were dissected out and immediately frozen in liquid nitrogen. Nuclear protein extract from hypothalami was prepared by slight modification of the methods described by Morooka *et al.* (24). Briefly, two hypothalami from each treatment were homogenized at 4° in 20 mM HEPES buffer, pH 7.4, containing 50 mM β-glycerophosphate, 2 mM EGTA, 1 mM vanadate, 1 mM PMSF, and 2 μg/ml aprotinin. The homogenate was held in ice for 15 min and then centrifuged at 3,300 $\times$ *g* for 15 min. The supernatant was discarded, and 0.5 volume of buffer A (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 1 mM PMSF, 1 mM vanadate, 1 mM DTT, and 2 mg/ml aprotinin) was added to 1 volume of the pellet and rehomogenized on ice. To this homogenate, 0.5 volume of buffer B (same as A but containing 0.8 M KCl) was added and mixed continuously in a rotary mixer at 4° for 1 hr. The crude nuclear suspension (~300 μl) was dialyzed in a slide-A-Lyzer dialysis cassette with M_r 10,000 cut-off (Pierce Chemical Company, Rockford, IL) in 500 volumes of dialysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM vanadate, 2 mg/ml aprotinin) for 8 hr. The suspension was centrifuged at 13,000 $\times$ *g* for 30 min at 4° and the supernatant was collected. After estimating the protein content using BioRad protein assay reagent (BioRad, Hercules, CA), the supernatant was frozen in liquid nitrogen and stored at −80°.

Electrophoretic mobility shift assay. Binding reactions were performed at room temperature in 10 ml of 10 mM Tris buffer, pH 7.5, containing 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 20% glycerol, 0.4 mg/ml salmon sperm DNA, 10 mg protein, and approximately 0.1 ng of ^{32}P-labeled CRE probe (5–6 $\times$ 10^4 cpm). After 20 min of incubation, the reaction mixture was subjected to electrophoresis on a 3% glycerol and 1 $\times$ Tris/borate/EDTA buffer containing 6% non-denaturing polyacrylamide gel at 180 V in 0.25 $\times$ Tris/borate/EDTA buffer (.25 $\times$ = 25 mM Tris, 22.5 mM boric acid, 0.25 mM EDTA, pH 8.0) at 4°. Gels were dried, autoradiographed, and the bands were quantified using Eletrophoresis Documentation and Analysis System (Kodak Digital Science, Rochester, NY). In competition experiments, the reaction mixture was incubated with 125-fold molar excess of unlabeled double stranded oligonucleotide for 15 min at 4° before the addition of the labeled probe. Antibody supershift assay was performed by incubating the reaction mixture with 2 μg of CREB, CREM, or ATF-1 antibody for 25 min at room temperature or with 2 μg of phosphoCREB antibody for 3 hr at 4° before loading.

PhosphoCREB immunohistochemistry. Immunohistochemistry was performed as described by Ginty *et al.* (25). In brief, rats were deprived of food for 48 hr or were fed *ad libitum*. These rats were anesthetized with pentobarbital (100 mg/kg intraperitoneally) and perfused transcardially with 200 ml of 4% paraformaldehyde in PBS (pH 7.5, 4°). The brains were removed and postfixed in 4% paraformaldehyde in 10% sucrose/PBS for 2 hr. Subsequently, the brains were transferred to 30% sucrose in PBS overnight at 4°. The brains were cut in the frontal plane on a freezing microtome at 50 μm, and every second section was processed for phosphoCREB detection using rabbit polyclonal phosphoCREB antibody as primary antibody (1:1000 dilution), biotinylated anti-rabbit IgG as secondary antibody (1:250 dilution), and 3,3′-diaminobenzidine as substrate.

All procedures were reviewed and approved by the University of Cincinnati animal care and use committee.

Results

Artificial CSF (1 μl), NPY (1 μg), or PACAP (2 μg) were administered IHT to groups of six rats, and two rats from each treatment were killed 5, 15, and 60 min later. The hypothalamic nuclear extract preparations of these rats were analyzed for their ability to bind to the [^{32}P]CRE probe, and the binding was quantitated by densitometry. As shown in Fig. 1A, CRE binding remained nearly unaltered for 5 (100%), 15 (85%), and 60 (105%) min after CSF treatment. Compared with controls, CRE binding did not increase 5 min after NPY treatment. However, NPY treatment increased the CRE binding by 142% and 380% in 15 and 60 min,

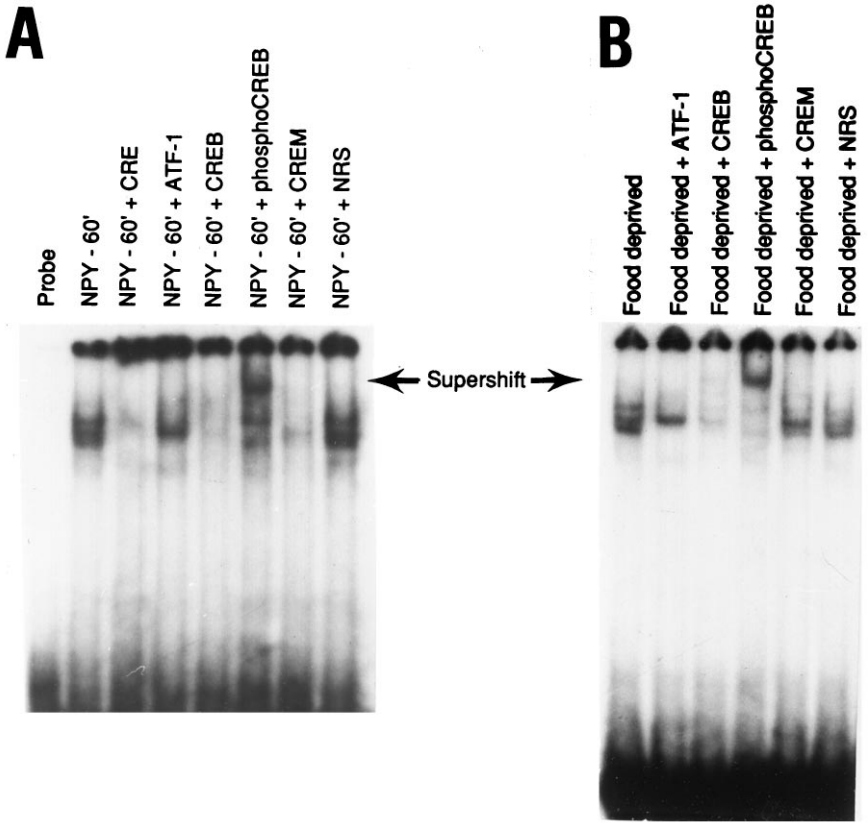

Fig. 4. Analysis of NPY-induced (A) or food-deprivation-induced (B) CRE binding proteins in rat hypothalamus. Nuclear proteins (10 μg) from NPY-treated (60 min) or food-deprived (48 hr) rats were preincubated with (2 μg) antibodies specific to CRE binding proteins and analyzed on gel shift assay. As a negative control, normal rabbit serum (*NRS*; 2 μl) was tested to show no nonspecific binding to the [^{32}P]CRE probe.

respectively (Fig. 1B). On the other hand, PACAP injections decreased the CRE binding by 0%, 59%, and 66% in 5, 15, and 60 min, respectively (Fig. 1C). Preincubation of the hypothalamic nuclear extracts of CSF-, NPY-, or PACAP-treated rats with unlabeled CRE inhibited [^{32}P]CRE binding (Fig. 1).

The effects of altering endogenous NPY on CRE binding proteins in rat hypothalamus were investigated in a group of six rats by altering the nutritional status of the animals. Hypothalamic nuclear extract of rats who were not fed for 48 hr exhibited 151% increased CRE binding compared with that of rats that were fed *ad libitum*, which exhibited 100% (Fig. 2). However, refeeding of the unfed rats for 8 hr reduced the CRE binding to 52% compared with controls. Again, preincubation of the nuclear extracts with unlabeled CRE blocked [^{32}P]CRE binding.

To confirm the reproducibility of the results, the above experiments were repeated three more times. As shown in Fig. 3A, 60 min after IHT-NPY treatment, CRE binding was significantly increased as compared with CSF rats. On the other hand, IHT-PACAP reduced the CRE binding to hypothalamic nuclear proteins compared with controls by 51 $\pm$ 9%. However, this reduction was not statistically different from that of control rats because of variability within this treatment group (Fig. 3A). Food deprivation for 48 hr significantly increased the CRE binding compared with that of controls, which was normalized after refeeding (Fig. 3B).

To identify the transcription factors responsible for increased CRE binding in NPY-treated and unfed rats, we performed supershift assays using antibodies to CREB, CREM, ATF-1, and phosphoCREB. Two groups of four rats were used in this study. One group was killed 60 min after the injections of NPY (1 μg), and the other group was killed after 48 hr of fasting. Preincubation with CREB antibody completely blocked the CRE binding to the nuclear extracts of both NPY-treated and unfed rats (Fig. 4). On the other hand, phosphoCREB antibody retarded the migration of the CRE binding bands of unfed and NPY rats. Although normal rabbit serum, used as a control, had no effect, pretreatment with ATF-1 antibody partially inhibited the CRE binding in both NPY-treated and unfed rats. Although the CREM antibody completely blocked the CRE binding in NPY-treated rats (Fig. 4A), only partial inhibition was observed in unfed rats (Fig. 4B).

The association between the nutritional status of the animals and the phosphorylation of CREB in hypothalamus was evaluated in rats that were fed *ad libitum*, rats that were deprived of food for 48 hr, and rats that were deprived of food for 48 hr and then refed for 8 hr. The presence of phosphoCREB was observed in all three groups (Fig. 5). Compared with rats that were fed *ad libitum* (100%), food deprivation increased phosphoCREB signal to 239%, whereas refeeding reduced it to 63% (Fig. 5). Unlabeled CRE oligonucleotide inhibited [^{32}P]CRE binding in a concentration-dependent manner. At 31.25-fold excess, inhibition was 66%, whereas 74% and 100% inhibition was observed at 62.5- and 125-fold excesses, respectively. Whereas related oligonucleotide AP-1 (125-fold excess) inhibited 29% of CRE binding, an unrelated oligonucleotide (nuclear factor κB) exhibited no effect (Fig. 4). Consensus AP-1 sequence, TGACTCA, has been reported to bind CREB with 5- to 10-fold lower affinity than CRE (26).

To identify the nuclei responsible for the food-deprived induced increase in phosphoCREB levels in hypothalamus, the phosphoCREB immunohistochemistry study was performed. Intense phosphoCREB signal was noticed in the

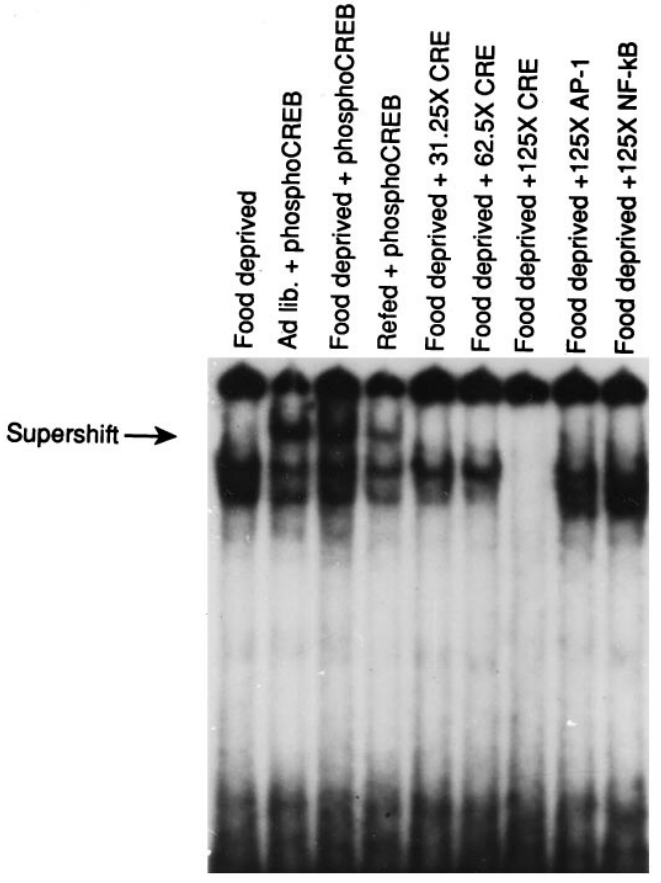

Fig. 5. Effect of nutritional status on the phosphorylation of hypothalamic CREB. Hypothalamic nuclear proteins (10 μg) from *ad libitum*, food-deprived (48 hr), and refed rats (8 hr) were preincubated with 2 μl phosphoCREB antibody and analyzed on gel shift assay. Also shown is the dose-dependent displacement effect of unlabeled CRE and 125-fold excess of related, unrelated oligos on food-deprivation-induced [^{32}P]CRE binding.

PVN and ventromedial hypothalamus of the rats that were deprived of food for 48 hr (Fig. 6, B and D). In comparison, only faint and diffused phosphoCREB signal was observed in the PVN, lateral, and ventromedial hypothalamus of the rats that were fed *ad libitum* (Fig. 6, A and C).

The effects of IHT-NPY (1 μg) (nine experiments) on rat hypothalamic CaM kinase II activity were also investigated. Three rats were killed 5, 15, and 60 min, respectively, after administering NPY, and the CaM kinase II activity was estimated using published procedures (23). The CaM kinase II activity was increased by 63% within 5 min of NPY administration (3.55 $\pm$ 0.43 nmol/min/mg protein) compared with untreated groups (2.17 $\pm$ 0.32 nmol/min/mg protein). CaM kinase II activity returned toward the basal level in 15 min (2.75 $\pm$ 0.59 nmol/min/mg protein) and remain unchanged at 60 min (2.83 $\pm$ 0.58 nmol/min/mg protein). There was no stimulatory effect of CSF treatment on CaM kinase II activity at 5 min (three experiments; 2.55 $\pm$ 0.47 nmol/min/mg protein).

Discussion

The results presented herein show that IHT administration of NPY increases CRE binding to rat hypothalamic nuclear extracts. This binding is specific to the CRE oligonucleotide because preincubation with unlabeled CRE dose-dependently inhibited the [^{32}P]CRE binding, whereas unrelated oligos had no effect. Food deprivation, which has been well established to increase the hypothalamic NPY levels (8), also increased CRE binding. On the other hand, refeeding, which decreases NPY levels in arcuate and median eminence areas of the hypothalamus (8), or IHT administration of PACAP, which antagonized NPY-induced feeding (19), attenuated CRE binding. These observations are consistent with the possible role for CRE binding proteins in modulating the target gene(s) responsible for NPY-induced feeding. Furthermore, our preliminary results suggest that CRE binding is elevated in obese Zucker rats, which also exhibit increased NPY levels in the hypothalamus.

It has been well established that three families of CRE binding transcription factors, CREB, CREM, and ATF-1, mediate the final events in the signaling cascades induced by cellular cAMP or calcium (16). These second messengers also have been shown to be coupled to the NPY receptors characterized to date (11–14). We also have shown that hypothalamic NPY receptors coupled to pertussis-toxin-sensitive G proteins mediate NPY-induced feeding in rats (15). It therefore seems logical to believe that NPY-induced increases in CRE binding may be due to the phosphorylation of at least one of the above transcription factors. Supershift assays performed to delineate the transcription factor(s) revealed that the normal rabbit serum had no effects on the CRE binding induced by NPY or fasting (Fig. 4). However, preincubation with CREB antibody, which has no cross-reactivity with ATF-1 or CREM, completely blocked the NPY- and fasting-induced CRE binding. Consistent with this observation, the antibody that recognizes the phosphorylated CREB supershifted the CRE-protein complex (Fig. 4). These findings suggest that phosphorylation of CREB may be responsible, at least in part, for the increased CRE binding associated with NPY and fasting. Although the observed inhibition of CRE binding by CREM may be attributed to the known cross-reactivity of CREM antibody with CREB and ATF-1, presently we do not know the reason for the partial inhibition by ATF-1 antibody. It may, however, be speculated that ATF-1 may also be involved in this signal transduction cascade via the formation of heterodimers with CREB (27). These possibilities have not yet been investigated.

Our suggestion that increased CRE binding may be due to the phosphorylation of CREB proteins is in agreement with the findings by Nichols *et al.* (28). This report demonstrated that DNA binding to highly purified rat brain CREB is increased by phosphorylation and decreased by dephosphorylation, induced by PKA and phosphatase 2A, respectively. However, there are other reports that did not find any effect of PKA- or protein kinase C-induced phosphorylation on DNA binding by CREB proteins (26, 29). Alternatively, NPY or PACAP treatment may modulate the formation of heterodimers containing CREB, resulting in an increase or decrease in CRE binding in hypothalamus. In contrast to the prevailing hypothesis that CREB is a constitutive DNA binding protein, there is evidence suggesting that central CREB expression changes during postnatal development (30) and drug abuse (31). We observed changes in CRE binding by NPY and PACAP as early as 15 min after the administration of the peptides. These alterations in binding are most likely

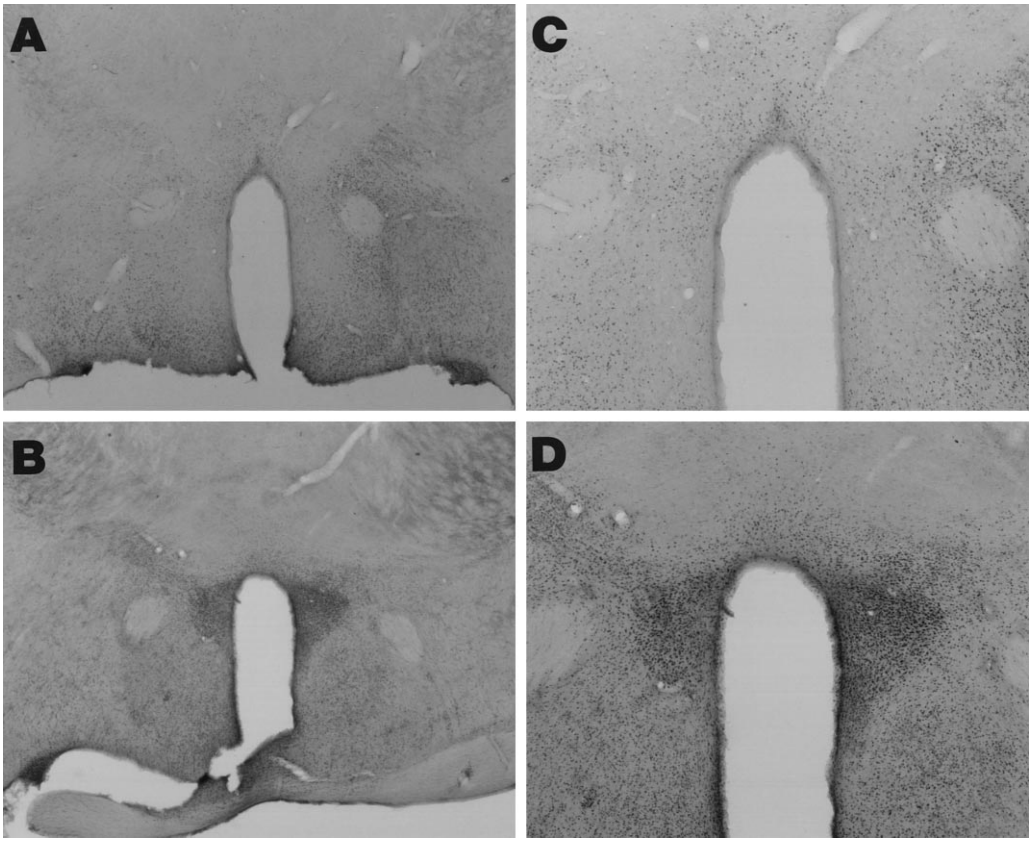

Fig. 6. Food-deprivation-induced phosphorylation of CREB in rat hypothalamus. Photomicrograph at lower magnification (10.9×) showing intense nuclear phosphoCREB signal in the PVN and ventromedial hypothalamus of food-deprived rats (B). In comparison, weak and diffused phosphoCREB labeling was observed in the lateral and ventromedial areas of hypothalamus in rats that were fed *ad libitum* (A). At higher magnification (21.8×), the PVN nuclei of food-deprived rats shows the abundant phosphoCREB signal (D) in comparison to rats that were fed *ad libitum* (C).

due to post-translational changes in CREB (phosphorylation) rather than transcriptional activation. However, we can not rule out the changes in CREB expression during the later stages after peptide treatment (1 hr) or during the alterations in nutritional status of the animals. These possibilities have not yet been investigated.

Activation of protein kinases is required to phosphorylate the transcription factors (16). Because IHT-NPY increased the hypothalamic CaM kinase II activity by 63% and NPY-receptor interaction results in the elevation of intracellular calcium, it would seem that CaM kinases regulate NPY-induced phosphorylation of CREB. However, hypothalamic NPY receptors are also negatively coupled to cAMP (15), and it is also known that both calcium- and cAMP-dependent pathways engage in "cross-talks" at various levels in the signal transduction cascade (16). Therefore, one can not rule out the involvement of cAMP/PKA in the activation or deactivation of CREB. On the other hand, the involvement of Ca^{2+}-activated protein kinase C in this signal transduction cascade can be ruled out because the [^{32}P]AP-1 probe showed no binding to NPY-treated hypothalamic nuclear proteins (not shown).

The data presented herein suggested an involvement of phosphoCREB as a possible regulator of transcription of the gene(s) controlling NPY-induced feeding. On the other hand, the reduction of CRE binding on refeeding (and PACAP treatment) suggests that no CRE binding transcription factors are activated in response to a satiety signal. Moreover, there is a decrease in phosphorylated CREB upon refeeding. These observations suggest that dephosphorylated CREB may negatively modulate the gene(s) responsible for the initiation of feeding and may constitute the signal for satiety. As has been shown by Lamph *et al.* (32) with protooncogene *jun* promoter, it seems possible that phosphorylation and dephosphorylation of hypothalamic CREB may act as an on/off switch mediating the feeding and satiety induced by increased (food deprivation) and decreased (refeeding) hypothalamic NPY levels. Presently, however, the events leading to dephosphorylation of CREB on satiety are not clear. The degree of phosphorylated CREB under physiological conditions is kept in check by the opposite effects of protein kinases and phosphatases (16). In this study, we have found that CaM kinase II activity reaches the basal levels within 15 min after an initial 63% increase. It is possible, therefore, that after initial activation of transcription factors by CaM kinases, increased phosphatase activities may down-regulate the transcriptional activities of phosphoCREB via dephosphorylation.

Xu *et al.* (33) recently reported that intracerebroventricular administration of NPY stimulates c-*fos* expression in the paraventricular nucleus of the hypothalamus and other nuclei in the forebrain. It should be noted that the major responsive element to Ca^{2+}/cAMP in the c-*fos* promoter is TGACGTTT, which, although different from palindromic CRE site, binds CREB. These studies are therefore consistent with our findings that CREB may mediate NPY-induced feeding.

Phosphorylation of hypothalamic CREB has been shown to mediate a number of functions, including the hypothalamic homeostatic mechanisms (34), circadian rhythms (25), and memory (35). These reports are of interest because NPY also has been shown to play a role in controlling memory (36) and circadian rhythms (37). Therefore, NPY effects on feeding,

memory, and circadian rhythms may seem to be mediated by a common pathway. However, the effects of NPY on circadian rhythms and memory are mainly localized to hypothalamic suprachiasmatic nucleus (37) and hippocampus (36), respectively. These sites are distant from the perifornical hypothalamus, where NPY was administered in this study (19). Moreover, the immunohistochemical studies show that fasting-induced phosphorylation of CREB is localized mainly to paraventricular and ventromedial hypothalamus of rats. In addition, it has been suggested that NPY effects on feeding are mediated by the recently cloned Y-5 subtype of NPY receptors, which are abundantly distributed in PVN, lateral hypothalamus, and arcuate nucleus (38). On the other hand, most of the central effects of NPY, including those on memory and circadian rhythm, are mediated by either Y-1 or Y-2 receptor subtypes (39). These observations therefore suggest that the effects of NPY on feeding, memory, and circadian rhythms may be mediated by independent mechanisms or by similar intracellular mechanisms with the behavioral effect depending on neurological locations.

In summary, CRE binding transcription factors that may be involved in NPY-induced feeding have been investigated for the first time. These studies suggest that CaM kinase II-induced phosphorylation of CREB may regulate the NPY action. Similar results obtained on fasting and refeeding may also suggest a possible involvement of CREB in partially mediating the signals of hunger and satiety. These suggestions are consistent with the general consensus that NPY may constitute a major regulator of feeding behavior.

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