Eating induced by perifornical cAMP is behaviorally selective and involves protein kinase activity

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ABUNDANT EVIDENCE IMPLICATES the perifornical hypothalamus (PFH), an area contiguous with the lateral hypothalamus, in the neural control of eating. Specifically, electrical stimulation of PFH neurons elicits eating in satiated animals (29), and neurons in this area are responsive to internal and external food-related stimuli (2, 6). In addition, several neurotransmitters (21, 22, 35) exert eating-stimulatory or eating-suppressive effects in the PFH that are important in the physiological control of eating (1, 16, 34).

METHODS

Subjects and surgery. Adult male Sprague-Dawley rats descended from breeder rats obtained from Charles River Laboratories were individually housed at 21°C in a vivarium with lights on at 1100 and off at 2300. Animals were maintained on Purina Rat Chow pellets and water ad libitum until 3 days postsurgery, when they were provided with a sweetened milk-mash diet (46% Purina Rat Chow powder, 37% sucrose, and 17% Carnation evaporated milk) to which they had free access for the duration of the experiment.

Animals weighing 350–450 g were anesthetized by Metofane inhalation and implanted stereotaxically with chronic bilateral 26-gauge stainless steel guide cannulas targeted at the medial PFH. With the incisor bar at –3.3 mm, the stereotaxic coordinates were 6.3 mm anterior to the interaural line (AP), 1.0 mm lateral to the midsagittal sinus (ML), and 8.0 mm ventral to the surface of the skull (DV). The cannulas were permanently anchored to the skull with dental acrylic and stainless steel screws, and a plastic guard was placed around the exposed portion of the cannulas. To maintain patency, a 33-gauge stainless steel obturator was inserted into the lumen of each cannula. Animals were allowed at least 1 wk of postoperative recovery, during which they were repeatedly handled and mock injected to accustom them to the testing procedure. Unless otherwise noted, experiments were conducted using naive animals.

General test procedure. Tests were conducted during the early portion of the light phase, with freshly prepared mash diet provided to each subject 1–2 h before testing to maximize satiety. All microinjections were administered in a volume of 0.3 µl through a 33-gauge injector terminating in the PFH 1.0 mm below the tip of each guide cannula. Unless otherwise noted, food intake was measured for each rat 1, 2, and 4 h after bilateral PFH injection or after the final bilateral injection set in a series. For each experiment, all treatments were administered in counterbalanced order, until each subject had received each treatment. Tests were separated by at least 72 h.

With unilateral injection, neither treatment was effective when given alone, and the feeding-stimulatory effect of the combined treatments was specific to the PFH, with injection into surrounding brain sites being ineffective (11). Here we report that MPB forskolin alone, when microinjected bilaterally into the PFH, is sufficient to elicit a behaviorally selective eating response that is reduced by prior treatment with the protein kinase inhibitor H-89. These results, portions of which have been reported in preliminary form (13), suggest that increased production of cAMP by adenylyl cyclase is sufficient to generate a complex patterned behavior that is virtually identical to naturally induced eating and that the eating response depends on activation of the cAMP-dependent protein kinase, protein kinase A (PKA).
Pharmacological agents. The water-soluble forskolin analog MPB forskolin and the membrane-permeant PKA inhibitor H-89 were obtained from Calbiochem, and the forskolin analog 1,9-dideoxyforskolin, which lacks adenylyl cyclase activating properties, was obtained from Sigma (St. Louis, MO). MPB forskolin was dissolved in artificial cerebrospinal fluid (aCSF, pH = 7.4) composed of (in mM) 147 Na\(^+\), 154 Cl\(^-\), 3 K\(^+\), 1.2 Ca\(^2+\), and 0.9 Mg\(^2+\). H-89 and 1,9-dideoxyforskolin were dissolved in DMSO.

Experiment 1: Patterns of eating and behavioral activity elicited by the adenylyl cyclase activator MPB forskolin. For determination of the patterns of eating and other behaviors elicited by increases in endogenous PFH cAMP production, satiated rats were given bilateral injections into the PFH of MPB forskolin (300 nmol) or its aCSF vehicle (n = 6). Rats were housed individually in clear Plexiglas cages from which their food bowls were accessible through the open bottom of a small clear Plexiglas box attached to the cage. Each food bowl rested on an electronic balance so that intake could be recorded without removing the food or disturbing the animals.

Food intake and behavior were monitored every minute, by an observer blind to the treatments, beginning 10 min before injection and continuing until 4 h postinjection. Meals were defined as at least 0.2 g of food intake separated from other bouts of eating by at least 10 min. Meal pattern analysis was performed to yield the average number of meals eaten, the average latency, duration, and size of each meal, and the average intermeal interval. For the behavioral analysis, the behavior of each rat during the experiment was scored as falling into 1 of 10 mutually exclusive, species-typical categories: eating, drinking, gnawing (on small blocks of wood provided to them or on any component of the cage), grooming, rearing (standing up on hind legs), sleeping (lying down with eyes closed), quiescence (lying down with eyes open), alert (weight supported on all 4 legs, without whole body movement), locomotion, or hyperactivity. At the completion of testing, the percentage of time spent engaging in each behavior was averaged across all rats in 10-min blocks.

Experiment 2: Is eating stimulation by MPB forskolin mediated by protein kinase activity? To determine whether MPB forskolin's feeding-stimulatory effect might depend on activation of a protein kinase such as PKA, the only known effector for cAMP in most cells, the membrane-permeant PKA inhibitor H-89 (100 nmol) or its DMSO vehicle was injected bilaterally into the PFH 30 min before MPB forskolin (300 nmol) or aCSF in a naive group of rats (n = 10). Food intake between the first and second sets of bilateral injections was measured as well as intake 1, 2, and 4 h after the final set of injections.

For determination of whether eating in response to MPB forskolin might be due to osmotic or chemically nonspecific effects independent of cAMP production, these animals subsequently received bilateral PFH injections of 1,9-dideoxyforskolin (300 nmol), which lacks the ability to stimulate adenylyl cyclase, or its DMSO vehicle (n = 9). Testing began 72 h after the last test day of the first phase of the experiment.

Experiment 3: Is suppression of MPB forskolin-induced eating behaviorally specific? To determine whether suppression of MPB forskolin-induced eating by H-89 might be due to malaise or other debilitating actions of the protein kinase inhibitor, we injected H-89 (100 nmol) or its DMSO vehicle bilaterally into the PFH 30 min before the onset of the dark cycle in a group including naive animals (n = 5) and animals from experiment 1 (n = 5). Food intake was monitored every 30 min until 3.5 h postinjection.

Histological verification of cannula placements and data analysis. Upon completion of testing, subjects were killed by CO\(_2\) inhalation and perfused transcardially with 10% Formalin. Brains were removed and postfixed for at least 24 h in Formalin before sectioning on a cryotome. Coronal sections (100 mm) were cut through the extent of the visible cannula track and stained with cresyl violet. The injection sites were then localized by tracing the image onto size-matched figures adapted from the atlas of Paxinos and Watson (31). Food intake data were averaged and analyzed for effects of treatment and time using ANOVA, with multiple comparisons (Duncan's multiple-range test) performed at an \(\alpha\) of 0.05.

RESULTS

Histology. Out of a total of 21 subjects, 20 injection sites were localized, and all of these were placed within the PFH. Experiment 1: Patterns of eating and behavioral activity elicited by the adenylyl cyclase activator MPB forskolin. As shown in Fig. 1, bilateral PFH injection of MPB forskolin (15) stimulated a robust eating response of up to 12.0 ± 3.1 g in 2 h. A significant effect of drug dose was found (\(F_{1,20} = 16.12, P < 0.003\)), with eating elicited by MPB forskolin significantly greater than vehicle scores at each postinjection time (\(P < 0.05\)).

The pattern of meal intake by MPB forskolin- and vehicle-injected rats is shown in Fig. 2A. As shown, after MPB forskolin injection, rats ate an average of two meals, a significant increase over the single meal eaten in the 4 h after vehicle injection (\(F_{1,20} = 5.87, P < 0.05\)). All of the rats began to eat soon after MPB forskolin injection (average latency 14 ± 8 min, ranging from 3 to 20 min), and the first meal was quite large, averaging 10.4 ± 3.1 g (ranging from 1.0 to 22.6 g) and lasting 17 ± 6 min. All of the MPB forskolin-injected rats also ate a second small meal within the 4-h test, averaging 2.0 ± 0.6 g and beginning an average of 73 ± 30 min after the end of the first meal. In contrast, in the
and meal duration were all statistically significant.

The effects of drug treatment on the latency, meal size, and number of meals that occurred in each condition. Bar height indicates mean size (in g) of meal, and bar width indicates its average duration (in min). Abscissa indicates average time postinjection (min) at which meal occurred, and number of bars represents average number of meals that occurred in each condition. Bar height indicates mean size ± SE (in g) of meal, and bar width indicates its average duration (in min). *P < 0.05 greater than aCSF meal by ANOVA. B: behavioral selectivity of MPB forskolin. Percentage ± SE of time (averaged over 10-min blocks) spent engaging in eating, drinking, gnawing, grooming, and rearing behavior as a function of time (beginning 10 min before injection and ending 240 min postinjection) and MPB forskolin (300 nmol, •) or aCSF vehicle injection (○) (n = 6). On time scale, Pre is the 10-min block preceding injection, and numbers correspond to 10- or 20-min blocks postinjection ending on the respective number of minutes (e.g., 10 = 0–10 min postinjection). *P < 0.05 greater than vehicle scores at corresponding time.

Fig. 2: A: meal patterns after bilateral PFH microinjection of MPB forskolin (300 nmol) (solid bars) compared with those after microinjection of aCSF vehicle (open bar) (n = 6). Placement of each bar along abscissa indicates average time postinjection (min) at which meal occurred, and number of bars represents average number of meals that occurred in each condition. Bar height indicates mean size ± SE (in g) of meal, and bar width indicates its average duration (in min). *P < 0.05 greater than aCSF meal by ANOVA. B: behavioral selectivity of MPB forskolin. Percentage ± SE of time (averaged over 10-min blocks) spent engaging in eating, drinking, gnawing, grooming, and rearing behavior as a function of time (beginning 10 min before injection and ending 240 min postinjection) and MPB forskolin (300 nmol, •) or aCSF vehicle injection (○) (n = 6). On time scale, Pre is the 10-min block preceding injection, and numbers correspond to 10- or 20-min blocks postinjection ending on the respective number of minutes (e.g., 10 = 0–10 min postinjection). *P < 0.05 greater than vehicle scores at corresponding time.

4 h after vehicle injection, only three of the six rats ate, with their intake consisting of a single long-latency (117 ± 59 min) meal averaging 1.1 ± 0.5 g and lasting 3 ± 3 min. For the first meal, with the meal size of the three rats that did not eat a meal considered to be 0 g, the effects of drug treatment on the latency, meal size, and meal duration were all statistically significant (F1,7 = 6.54, P < 0.05; F1,10 = 9.83, P < 0.02; and F1,10 = 6.76, P < 0.03, respectively).

As shown in Fig. 2B, analysis of the percentage of time spent eating revealed a significant effect of drug treatment (F1,240 = 9.04, P < 0.02), time postinjection (F24,240 = 2.06, P < 0.005), and their interaction (F24,240 = 2.10, P < 0.005). During the first 10 min after MPB forskolin injection, animals spent 36.7 ± 13.1% of their time eating, significantly more time than was spent eating after aCSF injection (3.3 ± 3.3%) (P < 0.05). Animals spent at least 18% of their time eating during the 30 min immediately after MPB forskolin injection. In contrast, no other behaviors were affected during this time, and there were no significant effects on drinking, gnawing, rearing, locomotion, or hyperactivity at any time (Fig. 2B and Fig. 3). It is likely that the MPB forskolin-injected animals engaged in normal postprandial drinking; however, they were not observed to spend significantly more time drinking than when injected with vehicle (Fig. 2B), despite the fact that bouts of drinking were easily detected and easily distinguished from other behaviors. Measures of total water intake, which were not performed in these experiments, may be required to resolve the minimal prandial and postprandial drinking that occurs with the wet mash diet.

As shown in Fig. 2B, after the increase in eating behavior, grooming behavior was significantly increased (F1,240 = 11.94, P < 0.01), with peaks 60 min (i.e., 50–60 min) and 130 min (i.e., 120–130 min) postinjection (P < 0.05). Both of these peaks in grooming occurred within 20 min after the termination of an MPB forskolin-elicted meal, as shown in Fig. 2. Although there was no significant correlation between the amount of food eaten and the time spent grooming by individual MPB forskolin-treated rats, the two subjects that consumed the most food spent the most time engaged in grooming. Compared with vehicle scores, sleeping (Fig. 3) was decreased after MPB forskolin injection (F1,240 = 13.81, P < 0.005), with a significant effect of time (F24,240 = 5.87, P < 0.001) and a significant interaction between treatment and time (F24,240 = 4.97, P < 0.001). As shown in Fig. 3, this decrease was significant from 50 min (i.e., 40–50 min) to 160 min postinjection (P < 0.05), a period of time spanning the two MPB forskolin-elicited meals and elevated grooming behavior. Quiescent (F1,240 = 5.72, P < 0.05) and alert behavior (F1,240 = 8.82, P < 0.02) were also increased in MPB forskolin-injected animals, with the significant increases occurring during the period of decreased sleeping (P < 0.05) (Fig. 3).

Experiment 2: Eating stimulation by MPB forskolin is reduced by a protein kinase inhibitor. As shown in Fig. 4, bilateral PFH injection of MPB forskolin elicited a dramatic eating response of 15.7 ± 2.3 g in 2 h. This response was reduced by as much as 50% by prior injection of the PKA inhibitor H-89. Significant effects of drug treatment (F3,99 = 11.7, P < 0.001) and postinjection time (F3,99 = 36.32, P < 0.001) were found. Multiple comparisons showed that MPB forskolin-induced eating was significantly greater than vehicle scores 1, 2,
and 4 h postinjection and that H-89 significantly reduced this elicited response 1 and 2 h postinjection (P < 0.05). H-89 alone was without effect on food intake between sets of injections and, when given alone, had no effect on food intake at any time (mean food intake 2 h postinjection was 2.7 ± 0.3 g, data not shown).

When food intake 1, 2, and 4 h after injection of 1,9-dideoxyforskolin or its DMSO vehicle was compared with the eating elicited by bilateral MPB forskolin or vehicle in the first phase of the experiment, significant effects of treatment and time were found (F_{3,66} = 21.34, P < 0.001 and F_{2,66} = 16.79, P < 0.001). As shown Fig. 4, despite its structural similarity to MPB forskolin, 1,9-dideoxyforskolin failed to elicit significant eating in the same group of animals, and eating scores after 1,9-dideoxyforskolin injection were significantly lower than eating elicited by MPB forskolin at all time points (P < 0.05), suggesting that the effects of MPB forskolin were pharmacologically specific.

Experiment 3: Suppression of MPB forskolin-elicited eating is behaviorally specific. To control for the possibility that H-89 injection might suppress MPB forskolin-elicited eating by rendering the animals nauseous or unable to eat, we tested the ability of H-89 to inhibit nocturnal eating. As shown in Fig. 5, the 100 nmol dose of H-89 that suppressed MPB forskolin-induced eating failed to reduce nocturnal eating at any time measured.

DISCUSSION

We show here for the first time that bilateral PFH administration of MPB forskolin, an activator of the cAMP-synthesizing enzyme adenylyl cyclase, is sufficient to elicit an intense eating response (Fig. 1) at a dose that is ineffective when injected unilaterally (13). In contrast, the same dose of the forskolin analog 1,9-dideoxyforskolin, which does not stimulate adenylyl cyclase (3) but has the same non-cAMP-dependent effects as forskolin (19), failed to stimulate eating.
MPB forskolin in the present study also raises the stimulatory effects. The magnitude of the effects of not act on the same neurons to produce their eating-activation of all currently known NPY receptors inhib-
circuit stimulating natural eating. However, because cAMP and NPY may act on a common neural
previous findings that the PFH is most sensitive to the deprivation or after injection of neuropeptide Y (NPY)
large first meal in comparison with subsequent meals, on meal parameters, as well as the stimulation of a
possible for meal termination (23). This pattern of effects suggest a concomitant inhibition of mechanisms respon-
cAMP may stimulate mechanisms of meal initiation, whereas the increases in meal size and meal duration
forskolin occurs in discrete meals and is characterized by a large, long-lasting meal beginning an average of 14
stimulating cAMP production in the PFH.

The vigorous eating elicited by bilateral PFH MPB forskolin occurs in discrete meals and is characterized by a large, long-lasting meal beginning an average of 14 min postinjection and a second meal of normal size occurring 73 min later on average (Fig. 2A). The relatively short latency suggests that the increase in cAMP production by MPB forskolin is rapid and that cAMP can also rapidly engage all of the subsequent mechanisms required to affect neural activity, ultimately eliciting eating behavior. The MPB forskolin had a relatively long duration of action (≈110 min), consistent with the longevity of second messenger effects in general. The increase in meal frequency induced by MPB forskolin suggests that increased PFH cAMP may stimulate mechanisms of meal initiation, whereas the increases in meal size and meal duration suggest a concomitant inhibition of mechanisms responsible for meal termination (23). This pattern of effects on meal parameters, as well as the stimulation of a large first meal in comparison with subsequent meals, is very similar to that observed after >6 h of food deprivation or after injection of neuropeptide Y (NPY) into the PFH (27). These similarities, combined with previous findings that the PFH is most sensitive to the eating-stimulatory effects of both NPY (35) and combined IBMX/MPB forskolin treatment (11), suggest that cAMP and NPY may act on a common neural circuit stimulating natural eating. However, because activation of all currently known NPY receptors inhibits cAMP production (10), NPY and MPB forskolin may not act on the same neurons to produce their eating-stimulatory effects. The magnitude of the effects of MPB forskolin in the present study also raises the possibility that derangements of the cAMP signaling system in neural circuits controlling eating might contribute to some disorders of eating and body weight control.

In contrast to the intense stimulatory effect on eating, MPB forskolin treatment had no effect on drinking, gnawing of wood blocks, rearing, or locomotion at any time, nor was there any evidence of hyperactivity. Except for the initial increase in time spent eating (Fig. 2B), there was no effect on any behavior until 50 min (i.e., 40–50 min) postinjection, which began a period of decreased sleeping and an apparently compensatory increase in alert behavior and, later, quiescent (resting) behavior (Fig. 3). Similarly, increases in grooming behavior occurred 50–60 min and 120–130 min postinjection (Fig. 2B). These findings may suggest that eating stimulation was the primary effect of MPB forskolin and that the other delayed effects occurred consequent to the stimulation of eating. Consistent with this, the two significant bouts of grooming, a typical postprandial behavior, occurred soon after the two meals elicited by MPB forskolin, and the animals that consumed the most food exhibited the largest subsequent increases in grooming behavior. Similarly, the delayed decrease in sleeping and increase in alert and quiescent behavior may have been secondary to the intense, long-lasting activation of feeding control systems. In any case, stimulation of feeding is clearly not merely a consequence of general, nonspecific arousal. The behavioral selectivity of PFH injection of MPB forskolin is much greater than that of the membrane-permeant analog 8-BrcAMP; we have shown that although stimulation of eating by 8-BrcAMP is not accompanied by any other oral behavior, locomotion can increase (14), and others have reported convulsions and death after central injection of large doses of other cAMP analogs in the rat (4). The greater selectivity of MPB forskolin may be due to its dependence on the intracellular cAMP-synthesizing machinery of individual cells, in contrast to 8-BrcAMP, which functionally increases cAMP levels extracellularly as well as intracellularly across different cell types. That MPB forskolin elicits eating of discrete meals with minimal effects on other behaviors suggests that increases in endogenous intracellular cAMP in some PFH cells elicit a complex, patterned behavior that more closely mimics naturally induced eating, underscoring a role for the intracellular actions of PFH cAMP in the control of eating.

How might intracellular cAMP act in the PFH? PKA is the only known effector for cAMP in most cells, suggesting that the eating-stimulatory effects we observed may depend on activation of this protein kinase, which can phosphorylate a wide variety of targets, including neurotransmitter receptors (18) and both voltage-gated and ligand-gated ion channels (24, 25). Consistent with this, we show that the vigorous eating in response to PFH MPB forskolin is reduced up to 50% by the selective protein kinase inhibitor H–89 during the first 2 h postinjection (Fig. 4). The Ki value of H–89 is an order of magnitude lower for PKA than for the cGMP-dependent protein kinase (PKG) and at least three orders of magnitude less than the Ki for other

![Fig. 5. Behavioral specificity of H–89. Cumulative food intake beginning at onset of nocturnal phase as a function of time postinjection (0.5, 1, 2, and 3.5 h) and PFH microinjection of vehicle or H–89 (100 nmol) (n = 10).](attachment:image)
protein kinases, such as protein kinase C (17). Although the dose of H-89 used in the present study might have been sufficient to affect PKG activity, it was found that a membrane-permeant analog of cGMP fails to elicit eating (12) when injected into the PFH, arguing that such inhibition is unlikely to account for the reduction in MPB forskolin-induced eating. In addition, that the dose of H-89 that inhibited MPB forskolin-induced eating did not reduce natural eating at the onset of the nocturnal phase (Fig. 5) argues that the eating suppressive effects of H-89 are unlikely to be mediated by malaise or debilitation. It is possible that the combination of H-89 and MPB forskolin might have caused aversion, although the robust eating observed in the control condition on multiple test days may suggest that this is unlikely. Although these results may also suggest that nocturnal eating is not dependent on cAMP and PKA, this issue clearly warrants further attention; further tests will also be needed to determine whether MPB forskolin-induced eating can be completely blocked by this inhibitor. The present results provide the first evidence suggesting that activation of PKA mediates the eating elicited by agents that increase endogenous cAMP. This is particularly important given that excessively high concentrations of cAMP and its analogs applied to whole cells can inhibit, rather than stimulate, PKA activity (8).

Current evidence suggests that the cAMP/PKA system is well suited to play an integrative role in the nervous system. The subcellular distribution of PKA parallels the distribution of adenylyl cyclase (28), with a high concentration at synaptic specializations (7). The major PKA regulatory subunit in the brain, RIα, is concentrated, in neurons, in the soma and proximal dendrites and processes (26). What is the advantage of the synaptic localization of these components of the cAMP signaling pathway? The ability of the cAMP synthesizing enzyme adenylyl cyclase, of which at least eight types are known, to serve as a coincidence detector for the activation of a variety of neurotransmitter receptors (5), in combination with its presence at synaptic sites, suggests that the cAMP/PKA system may be optimally positioned to integrate the effects of a wide variety of signals impinging on neurons. Given the abundant evidence implicating PFH neurons in the control of eating, it is likely that the eating-stimulatory effects of cAMP analogs, and agents that increase endogenous cAMP, are a result of actions on neurons in this area. The present results suggest the possibility that the cAMP/PKA system may integrate some of the multiple feeding-related signals to which PFH neurons respond (30, 32) and that this second messenger system may integrate the effects of some eating-stimulatory (35) and eating-inhibitory (20–22) neurotransmitters in this area to ensure that eating occurs in conditions of physiological need.

Perspectives

The present results suggest that an increase in cAMP production in a single brain area is sufficient to generate a very specific and goal-oriented behavior, eating, by activating cAMP-dependent protein kinase. The regulation of cAMP levels, both directly and indirectly, by the activation of a wide variety of neurotransmitter receptors, combined with the relatively short latency of the eating response to agents that functionally increase cAMP levels, suggests the possibility that the cAMP/PKA system may provide a remarkably swift molecular trigger for the generation of eating behavior in circumstances in which there is both physiological need and an availability of food resources. The cAMP/PKA system in this area may also play a role in the neuronal plasticity that accompanies food-related learning (4), as well as in the long-term neural control of eating and body weight.

Although it is not currently known which neuropeptides may elicit eating via increases in PFH cAMP, the recent discovery of several neuropeptides, such as hypocretin (orexin; Refs. 9, 33), produced by lateral hypothalamic and perifornical neurons suggests that there are, potentially, many other unidentified neuropeptides involved in the hypothalamic control of eating.

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