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Amylin and Food Intake in Mice: Effects on Motivation to Eat and Mechanism of Action

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MORLEY, J. E., M. D. SUAREZ, M. MATTAMAL AND J. F. FLOOD. *Amylin and food intake in mice: Effects on motivation to eat and mechanism of action.* PHARMACOL BIOCHEM BEHAV **56**(1) 123–129, 1997.—Amylin is a hormone produced by the pancreatic islets of Langerhans. Amylin decreased food pellet consumption. Amylin also decreased lever pressing for milk solution whether or not the mice were prefed. Amylin did not produce a conditioned taste aversion in a two bottle test, whereas lithium chloride did. In addition, L-arginine, a precursor for nitric oxide synthesis, was demonstrated to inhibit the ability of amylin to decrease food intake. Amylin did not alter nitric oxide synthase activity in the fundus of the stomach. These studies demonstrated that amylin inhibits food intake at a higher range of doses than is typical of anorectic agents such as cholecystokinin. Amylin does not appear to decrease food intake by reducing the release of nitric oxide but may affect appetite by modulating serum glucose levels when co-released with insulin.

Amylin Appetite Food intake Nitric oxide L-arginine Nitric oxide synthase Lever press Anorexia Motivation

APPETITE regulation is a complex process involving a variety of central and peripheral mechanisms (20). The development of satiation appears to be due to the release of a variety of hormones that interact to terminate the meal (18,21). Amylin is a 37 amino acid peptide hormone that is co-released with insulin from the pancreatic islets of Langerhans in response to a meal (12). Amylin inhibited food intake after peripheral administration in mice (22) and in rats (1,25). This effect of amylin is independent of the vagus and does not involve the prototypic satiety agent, cholecystokinin (26,29). Further, intrahypothalamic amylin is anorectic in rats (1,8). Neuropeptide Y induced feeding in rats was also inhibited by intrahypothalamic administration of amylin (6). An inherent problem, in studies of the inhibiting of food intake, is whether drug administration results in altered hunger or induced "illness."

When a peptide decreases food intake, it is possible that the decrease is due to a nonspecific effect, such as decreased locomotion or due to illness (2,11). Illness, as such, is very difficult to measure or quantitate in rodents. Flood et al. (13) have utilized an experimental paradigm where mice have to work to get food (i.e., press a lever) coupled with and without prefeeding to allow the motivational aspects of peptide suppression of feeding to be more fully studied. They reasoned that if the animal is sufficiently ill that it significantly reduced its food consumption, then manipulating motivational levels will have little effect on food intake. For example, lithium chloride injected prior to testing suppressed food intake to the same degree in subjects that were feed or not feed prior to testing (13).

Nitric oxide synthase inhibition decreased food intake and produced weight loss in mice (4,23,24). This effect of nitric oxide appears to be due to a peripheral mechanism (unpublished observations) and may involve adaptive relaxation of the fundus of the stomach (10). L-arginine results in the production of nitric oxide, when it is converted to L-citrulline by nitric oxide synthase (19). As amylin has peripheral effects on food intake it appeared reasonable to investigate if its effects were produced through modulation of nitric oxide synthase.

The purpose of the studies reported here was to further our understanding of the effects of amylin on food intake by examining its effect on motivation to obtain food using the lever press and to determine if amylin might be sufficiently

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aversive to induce conditioned taste aversion. We determined if L-arginine attenuated the effect of amylin on food intake and if amylin decreased nitric oxide synthase activity in the fundus of the stomach.

METHODS

Male TAC(SW) mice 2–3 mo of age obtained from Taconic Farms Inc., Germantown, NY served as subjects. They were individually housed in plastic cages and maintained on a 12h light–dark schedule (lights off at 1800h) under controlled temperature (21–23°C). Water and food (Purina Rodent Laboratory Chow No 5001) were available ad lib except where noted. Amylin was obtained from Peninsula Laboratories Inc., Belmont, CA. L-Arginine was obtained from Sigma Chemicals, St. Louis, MO. Drugs were administered intraperitoneally.

EXPERIMENT 1

Inhibition of Food Intake by Amylin

To determine how much food mice consumed, food pellets (Purina 5001) were weighed prior to beginning the experiment. After the mice were injected (0, 50, 100 or 200 ug/kg of amylin, IP) with 15 mice per group, we placed a food pellet in the hopper of the cage lid. Food intake was measured from 0 to 30 and from 31 to 60 min after amylin administration. Water was available during the test. After the first test period, consumption was determined by again weighing the pellet to determine the weight reduction and returning the food pellets to the hopper. The grams food intake was analyzed by a two-way ANOVA (dose of amylin by test period).

EXPERIMENT 2

Inhibition of Lever Pressing for Milk Reward by Amylin

In this experiment, we tested the effects of amylin on lever pressing for milk reinforcement in mice that were prefed or not prefed with milk. If amylin inhibits food intake by making the mice "ill", then it should suppress feeding to about the same extent in both paradigms. However, if amylin reduces hunger, then lever pressing should be reduced to a greater degree in prefed than non-prefed mice.

Rodents are reluctant to consume novel foods. Therefore prior to the study, mice were habituated to a milk solution consisting of 1 part evaporated milk (Carnation®; and two parts water in a 40 ml centrifuge tube with a dripless drinking stem. Habituation to the milk solution was accomplished by providing milk solution in place of food and water overnight (from 1400 h to 0700 hr) and replacing food and water in the morning. So that the mice would not lose more than 10% body weight, milk solution was provided for two consecutive nights followed by 1 night of standard rodent laboratory chow. After 1 week mice readily consumed most of the 40 ml provided per night.

After habituation to the milk solution, mice were trained in fully automated lever press boxes consisting of a small test cage (18.0 × 16.5 cm and 207 cm deep, Coulbourn Instruments Inc. Model E10-11) with one wall containing a lever (E22-01) situated 1.7 cm above the stainless steel grid floor. On the wall opposite the lever, a dipper module (E14-05) delivered 100 ul of milk solution when the mouse pressed the lever. Mice were initially trained to lever press for milk reinforcement after 18-hr food and water deprived. They were trained at 2- to 3-day intervals to press for milk reinforcement on a continuous reinforcement schedule. Each training session lasted 30 min. Mice were trained for 4 days; those reaching a criterion of at least 100 presses over the 30 min training session were used in the experiment.

After mice reached criterion for the lever press training, they were assigned to experimental groups so that the means and standard deviations for baseline lever pressing was about the same for each group. Six groups of 14-16 mice were used. Three groups were prefed with milk solution for 30 min, while the others received nothing. After a delay of another 30 min during which no food water or milk was available to either group, one set of mice from the prefed and non-prefed groups received an intraperitoneal injection of saline, 100 or 200 ug/kg of amylin. Immediately after the injection, the mice were placed in the lever press box. Their lever press performance was recorded during a 30 min test session with data automatically recorded after each 10 min period. The lever press data was analyzed in a three-way ANOVA (prefed status × drug dose × time data was collected). In addition, the present suppression in the means for lever pressing of the amylin treated groups relative to the saline control were calculated. The percent suppression of lever pressing during the 2nd and 3rd time periods was calculated using the means for the groups receiving 100 or 200 ug/kg of amylin relative to the mean of the group receiving 0 ug/kg.

EXPERIMENT 3

Effect of Amylin on Water Intake

Part of the effect of amylin on milk intake in the lever press apparatus might be attributed to an effect of amylin on water intake. The mice were water, but not food, deprived overnight. In the morning, separate groups of 15 mice were administered 0, 100 or 200 ug/kg of amylin IP. Immediately after drug administration, food and water were removed from the food hopper and water in a 50 ml centrifuge tube with rubber stopper and dripless spount was placed on the top of the cage. The amount of water consumed was determined by comparing the weight of the water fill centrifuge tube before and after the 30 min drinking test. The grams fluid consumed were analyzed by a one-way ANOVA.

EXPERIMENT 4

A Two-Bottle Test of Conditioned Taste Aversion

The study was conducted in two parts. In the first part, we tested whether the experimental design was sensitive enough to yield conditioned taste aversion using a known aversant, lithium chloride (150 mg/kg, IP) following the design of Chance et al. (6). Mice were habitutated for 48 hours to drinking tap water from 50 ml centrifuge tubes fitted with rubber stoppers and a drippless drinking spouts. Mice were water but not food deprived for 18 h prior to the study. Mice were divided into two groups, half of which received tap water and the others received water with 0.1% saccharin solution to drink; food was available. There were 15 mice in each group. After 30 min, half the mice in the tap water group received an IP injection of saline or lithium chloride and the mice drinking saccharin were similarly treated. Forty-eight hours later, all mice were tested with one tube of tap water and one tube of saccharin solution on their cage after 18 hours water deprivation. Fluid consumption from each tube was determined after 30 min. Since the consumption of water and saccharin are not indepent events on the 48 h aversion test and are two depedent variables, we took difference between water

and saccharin consumption and analyzed it in a two-way ANOVA (fluid by drug). A positive mean meant that the group consumed more water than sacchrin.

As the preliminary study was successful, we proceeded to determine if amylin would induced conditioned taste aversion. Naive mice were used in this study. There were 14–15 mice in each group. The experimental design was the same except that 0, 100, or 200 ug/kg of amylin was injected in place of lithium chloride. The rationale of the test is that if amylin induced "illness", then the mice will reduce consumption of the novel saccharin solution (e.g., conditioned taste aversion) but it will not affect consumption of the familiar tap water. The data was analyzed in the same way in which the data for the lithium chloride study was analyzed.

EXPERIMENT 5

Effect of L-Arginine on Suppression of Food Intake by Amylin

The purpose of this experiment was to determine if the release of nitric oxide is involved in amylin-induced anorexia by testing if L-arginine reduces the suppression of food pellet consumption by amylin. Administration of L-arginine would by pass nitric oxide inhibition of the NMDA receptor. In this experiment, mice were food and water deprived for 18 hr. Immediately prior to testing, mice received two injections as follows: saline-saline, L-arginine-saline, saline-amylin or L-arginine-amylin. Both injections were given intraperitoneally and the second injection was given immediately after the first injection. Amylin was administered intraperitoneally at 200 ug/kg. In a preliminary study, we determined that 500 mg/kg of L-arginine suppressed food intake relative to a saline treated group ($t = 3.01$, d.f. = 28, $p < 0.01$). A dose of 250 mg/kg of L-arginine was used in this study, so that we could determine if a dose of L-arginine that did not inhibit food intake would reduce the anorectic effect of amylin. There were 11–12 mice per group. Following injections, weighed food pellets were placed in the cage and food intake measured after 30 min by weighing the pellet and determining the weight change. The grams food consumed was analyzed by a one-way ANOVA.

EXPERIMENT 6

Effect of Amylin on Nitric Oxide Synthase Levels in the Fundus of the Stomach

Mice were food deprived for 18 hr. Mice were then injected with saline or 100 ug/kg of amylin. After 30 min, the mice were sacrificed under methoxyflurane anesthesia and the stomach fundus removed and placed on dry ice. Tissue (20–50 ng) from the fundus was homogenized in 10 volumes of (wt/vol) 0.32 M sucrose/10mM Hepes/1mMDTT (pH7.4), using 20 up and down strokes of a teflon-glass homogenizer (800 rpm). The nuclear material was removed by centrifugation at 500 × g for 10 min. The supernatant S_1 was removed and centrifuged again at 1000 rpm to obtain a crude pellet. The pellet was resuspended in 2 –3 ml of the homogenizing buffer using 10 up and down strokes of a teflon homogenizer. The suspension was then centrifuged at 100,000 × *g* for 30 min to obtain the soluble fraction. Protein in the soluble fraction was determined using the method of Bradford (3). A 100 ul aliquot of the supernatant was then added to a mixture of 1.0 uM[^{3}H] L-arginine (43.5 Ci/mmol, Amersham), 1.0 mM NADPH, 0.1 mM DTT, 100 uM H_4biopterin, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$ and calmodulin (10 ug/ml) in 400 ul of TRIS-HCL (0.1 M buffer, pH 7.4) and incubated for 30 min at 37°C. The reaction was terminated by adding 40 ul of 20% HClO4. The mixture was cooled on ice and centrifuged at 4,000 rpm. A 100 ul aliquot of the supernatant was injected into a HPLC, equipped with a Zorbax 300 SCX silica column (4.6 mm × 25 cm). The eluting solvent was: 0.1M KH_2PO_4 (Arginine, R_t 10 min, (Citrulline, R_t 4.5 min). The flow rate was 1.0 ml/min. Fractions of 1.0 ml were collected (ISCO, Retriver-2) and radioactivity in the fractions was determined using liquid scintillation spectrometry. Blanks consisted of 100 ul of buffer incubated 30 min.

STATISTICS

All results are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined by analysis of variance (ANOVA). Significant differences among group means was determined by Dunnett's *t*-test following a one-way ANOVA or Tukey's *t*-test for design using multifactorial design (16, 31).

RESULTS

Experiment 1

Inhibition of food intake by amylin. A two-way ANOVA detected a significant main effects for the dose of amylin injected, $F(3,112) = 4.13$, $p < 0.01$ and for the time data was collected, $F(1,112) = 91.28$, $p < 0.001$ and for the interaction of the main effects, $F(3,112) = 4.26$, $p < 0.01$. The significant interaction was due to differential effects of amylin during 0–30 and 31–60 min feeding periods (Fig. 1). Amylin produced a dose-dependent decrease in feeding during the first, but not during the second, feeding periods. During the first feeding period, the means of the groups receiving 100 ug/kg ($p < 0.05$) and 200 ug/kg ($p < 0.01$) were significantly smaller than the mean of the group given saline (0 ug/kg) by Tukey's *t*-test (Fig. 1).

Experiment 2

Inhibition of lever pressing for milk reward by amylin. In this experiment, we tested whether amylin had a differential effect on lever pressing for milk reinforcement in mice that were prefed or not prefed with milk. The ANOVA indicated that the main effects of prefeeding, $F(1,258) = 94.83$, $p < 0.001$, and drug dose, $F(2,258) = 12.83$, $p < 0.001$ were significant as was the interaction of drug dose and time, $F(4, 258) = 5.88$, $p < 0.001$. The main effect of the time that data was collected and the other interactions were not significant. In Fig. 2, the distribution of mean lever presses is about the same for each group over time except that the mice which were not prefed with milk have higher means. The significance of the main effect of prefeeding was that mice which were not prefed whether they were treated with saline or amylin made 2–3 times as many lever presses as prefed mice (Fig. 2). The main effect of drug dose was significant because amylin-treated mice pressed overall about half as much as those given saline. The lack of a significant main effect of time was due to a strong interaction between drug and time in which mice receiving saline pressed more often during the 11–20 and 21–30 min test periods than at 1–10 min (frequently referred to as warm up effect) while the amylin treated groups tended to show the opposite trend. The mice treated with 100 ug/kg of amylin pressed significant less than the control group during

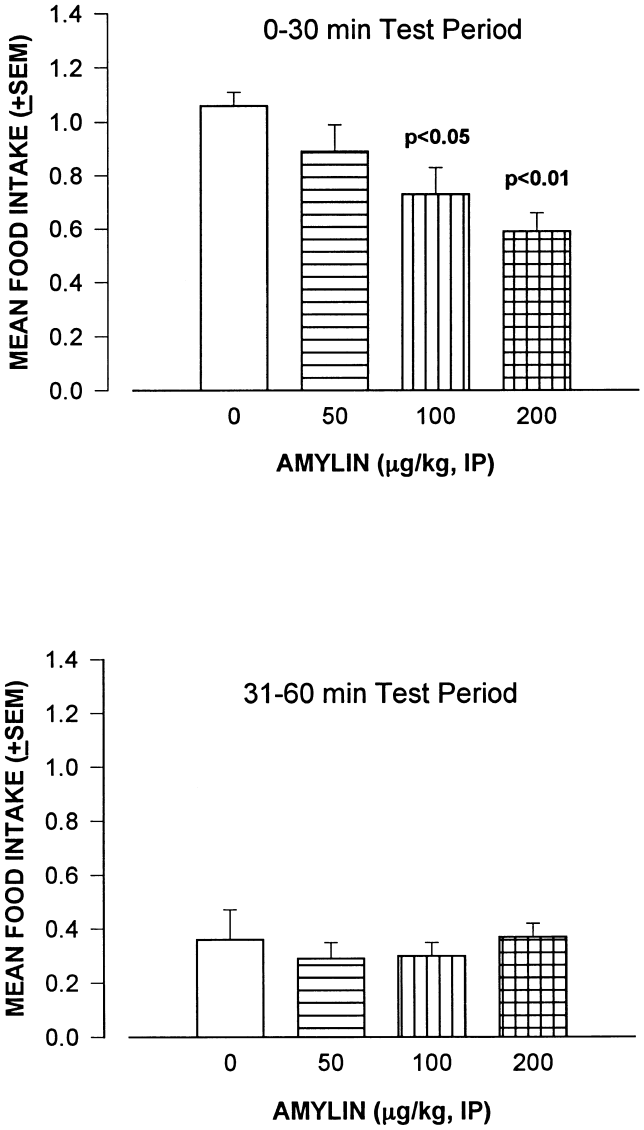

FIG. 1. Amylin suppressed food pellet intake in the home cage. The error bars represent the standard error of the means.

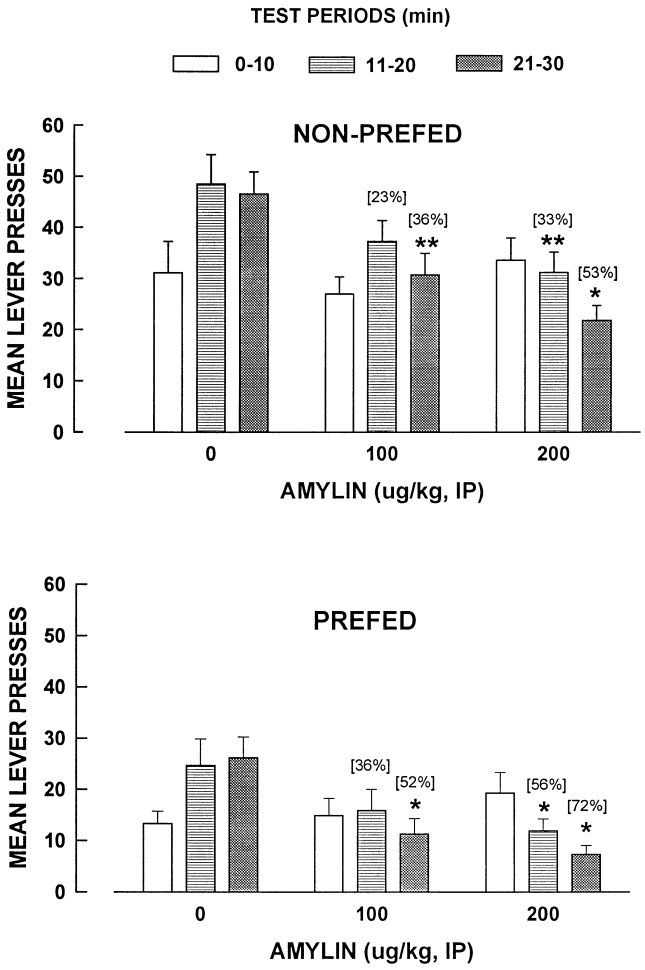

FIG. 2. Amylin suppresses lever pressing for milk reinforcement in non-prefed mice (top) and prefed mice (bottom) on a thirty min test data collected after each of 3 ten minute test periods. The numbers in brackets indicates the percent suppression of lever pressing relative to the vehicle control (0 ug/kg) for the same time period. Prefed mice showed greater suppression even when a relative measure is used. The * indicates treatment means differing from the mean of the control for the same time period at $p < 0.01$ or ** at $p < 0.05$ using Tukey's *t*-test following an ANOVA. The error bar represent the standard error of the mean.

the third test period whether or not the groups received milk prior to test (Fig. 2). The mice treated with 200 ug/kg of amylin pressed significantly less than the control group during both the second and third test sessions. The percent suppression in lever pressing induced by amylin was from 23% to 53% in mice not prefed and was from 36% to 72% in prefed mice.

Experiment 3

Effect of amylin on water intake. The results of the one-way ANOVA indicated that there was no significant effect of amylin on water consumption, $F(2,42) = 0.83$. The means and SEM were as follows: saline 1.61 + 0.11; 100 ug/kg of amylin 1.55+0.11; 200 ug/kg of amylin 1.39 ± 0.06.

Experiment 4

A two-bottle test of conditioned taste aversion. In the test of lithium chloride conditoned taste aversion for saccharin, the ANOVA indicated that the main effects of fluid consumed during the conditioning phase, $F(1, 56) = 52.15$, and the main effect of the drug injected after fluid consumption, $F(1, 56) = 11.56$, had significant effects at $p < 0.001$. The interaction of these factors was significant at $p < 0.05$, $F(1,56) = 4.35$. The group given water to drink and then an injection of saline, showed no preference for either solution (Fig. 3a). The group given saccharin to drink and then injected with saline clearly preferred saccharin on the test. Of the groups treated with lithium chloride, the one given water to drink showed some preference for the saccharin solution on the test, while the group given saccharin to drink clearly avoided the saccharin solution. Thus, pairing the consumption of saccharin and lithium chloride during the conditioning phase, was the only treatment that resulted in avoiding the saccharin solution on the test of conditioned aversion.

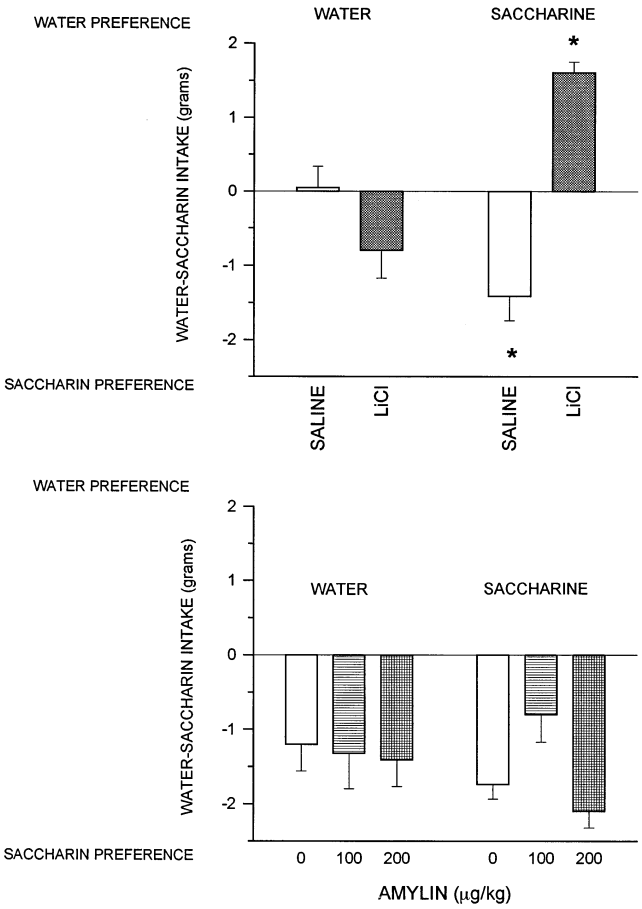

FIG. 3. Lithium chloride (top) but not amylin (bottom) induced conditioned taste aversion of 0.1% saccharin solution. The error bars represent the standard error of the mean.

Amylin failed to induce significant taste aversion to saccharin at either 100 or 200 ug/kg as the ANOVA indicated that neither the main effects of fluid consumed during conditioning, $F < 1$, nor the dose of amylin injected after consuming the fluid, $F(2,78) = 1.77, p > 0.10$, were significant. The interaction of the main effects was not significant, $F(2,78) = 1.55$, $p > 0.10$. (Fig. 3b).

Experiment 5

Effect of L*-arginine on suppression of food intake by amylin.* An ANOVA run on grams food consumed indicated a significant treatment effect when mice were treated with 250 mg/kg of L-arginine and 200 ug/kg of amylin, $F(3,44) = 4.36$, $p < 0.001$ (Fig. 4). Tukey's *t*-test indicated that mice treated with 200 ug/kg of amylin had a significantly lower mean than the means of either the saline control group ($p < 0.01$), or the mean for mice treated with 250 mg/kg of L-arginine ($p < 0.01$) or the mean of those treated with L-arginine plus amylin ($p < 0.05$). Therefore, the anorextic effect of amylin was reduced by co-administration of L-arginine an dose which itself did not affect food intake.

Experiment 6

Effect of amylin on nitric oxide synthase levels in the fundus of the stomach. There was no difference in the nitric oxide

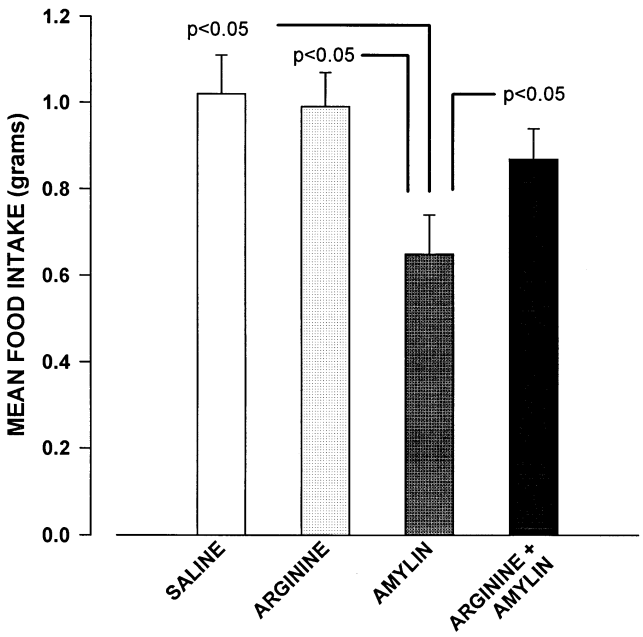

FIG. 4. L-arginine suppress the anorectic effect of amylin on food pellet consumption. The error bars represent the standard error of the mean.

synthase levels in those mice given amylin (17 ± 1 nmoles/mg/min) compared to those mice given saline (17 + 1 nmoles/mg/min).

DISCUSSION

The pancreatic islet cell peptide, amylin, suppressed feeding and lever pressing at 100 and 200 ug/kg. Amylin suppressed lever pressing to a greater extent in prefed mice than in nonprefed mice. Amylin did not produce a conditioned taste aversion for the novel tasting saccharin solution at either 100 or 200 ug/kg. L-arginine inhibited the ability of amylin to decrease food intake. Amylin did not decrease nitric oxide synthase in the fundus of the stomach.

Milk reinforcement was used in the lever press apparatus because mice will readily press for it, while they are much less likely to press for Noyes food pellets and will not press for water. The use of milk reinforcement in the lever press might be thought of as testing the effect of amylin on food and fluid intake at the same time. Previous findings indicate that mice perceive milk as primarily food. NPY increased the intake of food and milk solution but decreased the intake of water in mice (14). Mice normally consume 3–4 ml of water per day but will consume 35 to 40 ml of milk solution. In mice that were water deprived overnight, amylin did not significantly suppress water intake when amylin was injected prior to testing water intake (Exp. 3). Thus, the effect of amylin on lever pressing for milk solution appears to alter hunger and not thirst.

A number of methods have been utilized to determine whether food inhibition by drugs alters appetite or produces gustatory aversion (i.e., induces illness). The classical method to determine this had been the utilization of the conditioned taste aversion paradigm (5,9,25,28). We have previously argued that inducing conditioned taste aversion requires that either the food substance or the environment in which it is eaten be novel; therefore, this paradigm does not necessarily

detect "illness" (13). In the food pellet and in the lever press studies, the mice consumed the food in the test chamber on several occaisions prior to amylin administration. Thus, neither the food nor the test situation has any novelty upon which inducing conditioned taste aversion depends. Chance et al. (7) reported that 1 ug of amylin injected intrahypothalamically did not produce conditioned taste aversion. Billington et al. (2) developed the differential satiety paradigm which manipulated the duration of food deprivation. It was found that true satiety substances decreased food intake to a greater extent in animals who were less hungry (shorter periods of food deprivation) than in those who were more hungry. The lever press paradigm, with or without prefeeding, represents the reverse of this paradigm. Flood et al. (13) found that the aversive agent, lithium chloride, decreased lever pressing equally in prefed and non-prefed mice. Previously, they reported that the time spent prefeeding enhanced suppression of food intake by gastric releasing peptide and cholecystokinin-octapeptide. Both of these peptides are putative gastrointestinal hormones that play a role in the peripheral satiety cascade system (15,21,27). The data presented in this manuscript are compatible with the concept that amylin inhibited feeding by decreasing appetite. The failure to produce a conditioned taste aversion in the two-bottle test suggested that peripherally administered amylin was not aversive. A similar finding has been reported for centrally administered calcitonin gene-related peptide (17).

Both nitric oxide and amylin alter food intake by peripheral mechanisms. It has been postulated that nitric oxide enhances food intake by producing adaptive relaxation of the fundus of the stomach (10). L-arginine is converted to citrulline by nitric oxide synthase with the elaboration of nitric oxide (19). Thus, the administration of L-arginine should increase nitric oxide. We found that L-arginine could reverse the inhibitory effect of amylin on food intake, possibly by increasing the availability of nitric oxide. Thus, amylin could decrease food intake by decreasing the release of nitric oxide. Based on unpublished studies, we found that the nitric oxide synthase inhibitor, *N*-^{G}nitro-arginine methyl ester, did not decrease food intake in mice when administered intracerebroventricularly, or directly into the ventromedial hypothalamus. However, Squadrito et al. (30) have reported that both peripheral and intracerebroventricular injection of N-^{G}nitro-arginine suppressed food intake in rats. Further study is needed to determine if amylin produces its effect on feeding by altering nitric oxide release at central or peripheral sites. In an attempt to confirm this, we measured nitric oxide synthase activity in the fundus of the stomach, as nitric oxide may promote increased feeding though adaptive relaxation of the stomach (10). The lack of a difference in nitric oxide synthase levels in the fundus of mice treated with saline or amylin neither confirms nor disproves the possibility that amylin produces its effects on feeding by modulating nitric oxide in other tissues.

In conclusion, these studies provide evidence that amylin is a pancreatic hormone that reduces food intake. Amylin may produce its effect on feeding by preventing the release of nitric oxide.

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