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Involvement of the extracellular signal-regulated kinase 1/2 signaling pathway in amylin’s eating inhibitory effect

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Submitted 7 July 2011; accepted in final form 28 November 2011

Potes CS, Boyle CN, Wookey PJ, Riediger T, Lutz TA. Involvement of the extracellular signal-regulated kinase 1/2 signaling pathway in amylin’s eating inhibitory effect. Am J Physiol Regul Integr Comp Physiol 302: R340–R351, 2012. First published November 30, 2011; doi:10.1152/ajpregu.00380.2011.—Peripheral amylin inhibits eating via the area postrema (AP). Because amylin activates the extracellular-signal-regulated kinase 1/2 (ERK) pathway in some tissues, and because ERK1/2 phosphorylation (pERK) leads to acute neuronal responses, we postulated that it may be involved in amylin’s eating inhibitory effect. Amylin-induced ERK phosphorylation (pERK) was investigated by immunohistochemistry in brain sections containing the AP. pERK-positive AP neurons were double-stained for the calciitonin 1α/β receptor, which is part of the functional amylin-receptor. AP sections were also phenotype using dopamine-β-hydroxylase (DBH) as a marker of noradrenergic neurons. The effect of fourth ventricular administration of the ERK cascade blocker U0126 on amylin’s eating inhibitory action was tested in feeding trials. The number of pERK-positive neurons in the AP was highest ~10–15 min after amylin treatment; the effect appeared to be dose-dependent (5–20 μg/kg amylin). A portion of pERK-positive neurons in the AP carried the amylin-receptor and 22% of the pERK-positive neurons were noradrenergic. Pretreatment of rats with U0126 decreased the number of pERK-positive neurons in the AP after amylin injection. U0126 also attenuated the ability of amylin to reduce eating, at least when the animals had been fasted 24 h prior to the feeding trial. Overall, our results suggest that amylin directly stimulates pERK in AP neurons in a time- and dose-dependent manner. Part of the AP neurons displaying pERK were noradrenergic. At least under fasting conditions, pERK was shown to be a necessary part in the signaling cascade mediating amylin’s anorectic effect.

ERK; MAPK; area postrema; amylin receptor; U0126

THE HOMEOSTATIC SYSTEM CONTROLLING food intake and body weight relies to a large extent on peripheral satiation signals. One of these signals is amylin, a peptide cosecreted with insulin by pancreatic β-cells in response to nutrient ingestion (12, 41). At near-physiological plasma concentrations, amylin effectively decreases food intake in rats, and is considered a physiological satiation signal (4, 27). The amylin analog pramlintide causes weight loss in obese humans that is accompanied by sustained reductions in 24-h food intake, portion sizes, fast food intake, and binge eating tendencies (5, 57).

Pharmacological and lesioning studies implicate the area postrema (AP) as the primary site of amylin’s anorectic action (29, 30, 57), and an excitatory action of amylin on AP neurons has been confirmed by electrophysiological studies (49). Immunohistochemical studies using the immediate early gene product c-Fos as a marker of neuronal activation showed that peripheral amylin activates the AP, and subsequently the nucleus of the solitary tract (NTS), the lateral parabrachial nucleus (LPB), the central amygdaloid nucleus (Ce) and the lateral subdivisions of the bed nucleus of the stria terminalis (BSTL) (50, 55). Amylin was also shown to increase the formation of the intracellular second messenger cGMP in the AP, which seems to mediate the excitatory action of amylin in AP neurons (49). Furthermore, local AP injection of a membrane permeable analog of cGMP decreased eating by a meal-size effect, similar to amylin (35). However, a necessary role of cGMP in mediating amylin’s anorectic effect via AP-neurons has not been determined.

It has been demonstrated that the extracellular-signal regulated kinase 1 and 2 (ERK1/2) signaling cascade is activated in the NTS in response to the administration of anorectic doses of cholecystokinin-8 (CCK) (59); moreover, the anorectic action of CCK depends on the ERK pathway, because pharmacological blockade of ERK phosphorylation attenuated CCK’s immediate anorectic response (59). The ERK1/2 cascade is one of the four members of the mitogen-activated protein kinase family (MAPK) and a highly conserved signaling pathway involved in various cellular processes (13, 64). Activation of this pathway leads to the rapid phosphorylation of ERK1/2 and subsequent activation of gene transcription. Importantly, ERK phosphorylation also leads to acute neuronal responses, such as activation or inhibition of ion channels that directly and quickly affect neuronal excitability (40, 70). Therefore, the ERK pathway may be functionally involved in fast eating-inhibitory effects elicited by satiation signals.

Because amylin stimulates ERK in osteoclasts, which carry specific amylin receptors (17, 38), we proposed that this cascade may also be triggered in AP neurons upon amylin binding. Hence, we hypothesized that amylin may inhibit eating by activating the ERK signaling pathway in AP neurons expressing amylin receptors. To study this issue we specifically investigated whether amylin induces ERK1/2 phosphorylation.
in the AP at a time when amylin exerts its satiating action and whether activation of the ERK pathway is involved in amylin’s anorectic action.

The amylin receptor is a heterodimer of the type a or type b calcitonin receptor (CTR) as a core receptor and one of the known receptor activity-modifying proteins [RAMP; (15, 39)]. Therefore, we investigated whether the AP neurons displaying pERK specifically carry the amylin receptor by using double-fluorescence immunohistochemistry for CTR and pERK. Furthermore, because our previous studies indicated that a high percentage of amylin-activated neurons are noradrenergic (46), we phenotyped the pERK-positive AP-neurons using dopamine-β-hydroxylase (DBH) as a marker for noradrenergic neurons (2, 63). Finally, to substantiate whether activation of the ERK pathway in the AP is involved in amylin’s anorectic action, we assessed the effect of blocking the ERK cascade on amylin’s acute anorectic effect. We selectively blocked the mitogen-activated ERK kinase 1/2 (MEK) with U0126; MEK is responsible for ERK phosphorylation, and hence probably also its activation in AP-neurons. Specifically, we tested whether acute fourth ventricular pretreatment with U0126 blocks the amylin-induced ERK signaling in the AP and amylin’s acute inhibitory effect on eating.

**MATERIALS AND METHODS**

**Animals and Housing**

Male adult Sprague-Dawley rats (Elevage Janvier) were housed in a temperature-controlled room (21 ± 1°C) on an artificial 12:12-h dark-light cycle (lights on at 1:00 AM). Rats had ad libitum access to water and standard laboratory rat chow (cat. no. 3430, Provimi Klìba: Gossau, Switzerland), except during food deprivation as described below. All animal procedures were approved by the Veterinary Office of the Canton Zurich, Switzerland.

**Fourth Ventricle Cannulations**

Rats were anesthetized with isoflurane. The rats’ skull was fixed in a stereotaxic frame and a 22-gauge stainless steel guide cannula (model C313DC; Plastics One, Roanoke, VA) was implanted above the fourth brain ventricle (4V), using flat-skull coordinates of 11.6 mm caudal and 6.6 mm ventral to bregma (42). The guide cannula was fixed on the skull with three stainless-steel screws and dental cement. For cannula placement was verified by the 5-thio-glucose test. To assure correct intraventricular targeting.

**Perfusion and Brain Processing**

Rats were deeply anesthetized (pentobarbital sodium, 100 mg/kg ip, Kantonsapotheke Zürich, Switzerland) and transcardially perfused with ice-cold sodium phosphate buffer (PB 0.1 M, pH 7.2), followed by 4% paraformaldehyde in PB. Brains were postfixed for 2 h and cryoprotected in 20% sucrose/PB (48 h at 4°C). Three series of 20-μm coronal brain sections containing the AP, NTS, LPB, Ce, and the BSTL (according to Ref. 42), were cut in a cryostat (model CM3050S; Leica, Nussloch, Germany) and thaw mounted on adhesive glass slides (SuperFrost Plus; Menzel, Braunschweig, Germany).

**Immunohistochemistry for pERK1/2** (Phospho-p44/42 MAPK Thr202/Tyr204)

3,3'-Diaminobenzidine-tetrahydrochloride method. After the sections containing AP/NTS and LPB were air dried for 1 h at room temperature and rehydrated in phosphate-buffered saline (PBS; pH 7.4) containing 0.1% Triton X-100 (PBST), the sections were incubated for 15 min in PB containing 0.03 mol/l NaOH and 0.3% H2O2 to inhibit endogenous peroxidase activity. After being washed with PBS and blocked for 2 h in normal donkey serum (3% in 0.3% PBST; Jackson ImmunoResearch), sections were incubated in polyclonal rabbit anti-pERK1/2 antibody (1:1,000 in 0.3% PBST, cat. no. 9101, lot 26; Cell Signaling Technology) for 48 h at 4°C. The sections were then incubated in biotin-SP-conjugated donkey anti-rabbit IgG (H+L) (1:500 in 0.3% PBST, cat. no. 711–065-152, lot 76434; Jackson ImmunoResearch) for 2 h at room temperature. After incubation for 1 h in 1:100 avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector), pERK-labeled cells were stained by incubation for 4 min in 3,3’,5,5’-diaminobenzidine-tetrahydrochloride (DAB) chromogen solution (0.05% DAB, 0.018% H2O2, in PBS; Sigma-Aldrich). The slides were rinsed in PBS, dehydrated in graded alcohol, immersed in xylol, and coverslipped with Entellan (Merck, Germany).

**Fluorescence method (single-fluorescent staining)**. The sections containing the Ce and BSTL were stained by the fluorescence method because the use of H2O2 to eliminate nonspecific-DAB staining by endogenous peroxidase activity caused the formation of gas bubbles, which tended to damage the larger tissue sections of the forebrain. The same rabbit anti-p-ERK1/2 primary antibody (1:400 in 0.3% PBST) was used as described above in sections containing the Ce and BSTL. Detection was then achieved by 90-min incubation in Alexa Fluorophore 555-labeled donkey anti-rabbit IgG (H+L) (1:200 in 0.3% PBST, cat. no. A131572, lot 529473; Invitrogen). Sections were rinsed in PBS and mounted with Citifluor mounting medium (PBS/glycerol 1:1; Citifluor Products).

**Double-labeling of pERK and dopamine-β-hydroxylase.** Brain sections containing the AP were incubated in blocking solution followed by 0.3% PBST solution containing both mouse monoclonal anti-dopamine-β-hydroxylase (DBH) primary antibody (1:400, cat. no. MAB308, lot LV1390388; Chemicon) and the rabbit anti-p-ERK1/2 antibody (1:500) for 48 h at 4°C. The sections were then incubated for 90 min in 0.3% PBST containing Alexa Fluorophore 555-labeled donkey anti-rabbit IgG (1:200) and Alexa Fluorophore 488-labeled donkey anti-mouse IgG (1:250, cat. no. A21202, lot 536050; Invitrogen) and mounted with Citifluor.

**Double-labeling of pERK and CTR.** Hindbrain sections of the AP were incubated for 15 min in PBS containing 0.03 mol/l NaOH and 0.3% H2O2 to inhibit endogenous peroxidase activity. After 2-h tissue blocking in 3% goat normal serum (Jackson ImmunoResearch) and 20% avidin (blocking kit, model SP-2001; Vector) the sections were incubated for 48 h at 4°C in 0.3% PBST solution containing 20% biotin (blocking kit; Vector) and the primary antibody recognizing CTR type 1a and 1b [1:35,000; polyclonal rabbit anti-CTR1(a+b) IgG, purified by protein A chromatography; Welcome Receptor Antibodies, Melbourne PA188/10, see also Refs. 9 and 62]. The sections were then incubated for 90 min at room temperature in 0.3% PBST containing biotinylated goat anti-rabbit (1:500; BA 1,000, Vector) and for 1 h in 1:400 avidin-biotin-peroxidase complex (Vector ABC kit; Vector). For detection of CTR-positive neurons, the tyramide-amplification procedure combined with a fluorophore was employed. The tyramide method was used due to the lack of available antibodies against pERK and CTR working for brain tissue that were...
raised in different species. This method was shown to be effective for detection of multiple targets with primary antibodies from the same host species without substantial crosstalk between the signals (25, 61, 66). Briefly, sections were incubated for 45 min in biotinylated tyramide solution (60 μl/10 ml PBS + 1:1,000 H2O2), followed by Alexa Fluor 488-conjugated Streptavidin (1:800; S-11223, Invitrogen). Sections were then washed and the immunohistochemical detection of pERK was performed as described above [in Fluorescence method (single-fluorescent staining)].

The specificity of the primary antibodies was demonstrated by the respective suppliers and by our own controls; namely, we confirmed the specificity of the anti-DBH antibody by detecting specific DBH-positive staining in the expected A1 (ventrolateral medulla), A2 (dorsomedial medulla including the AP and NTS) regions, and not in surrounding brain regions that are known not to contain noradrenergic neurons (gracile nucleus, hypoglossal nucleus) (2, 3). The secondary antibodies were also tested for the lack of unspecific tissue binding when omitting the primary antibody.

**Microscopy**

Brain sections were analyzed at 10- and 20-fold magnification using a microscope equipped with a digital camera (Axioskop; Carl Zeiss, Feldbach, Switzerland). The number of pERK1/2 immunoreactive neurons was quantified in four representative sections per rat, spanning the following brain levels for each area: AP and NTS (bilaterally), −13.80 to −14.16; unilateral LPBM −8.88 to −9.24; Ce −2.28 to −3.00; and BSTL, +0.12 to −0.12 (using bregma as reference according to Ref. 42). Three sections collected from the same region of the AP were quantified for the total number of pERK1/2, DBH, and DBH/pERK double-immunoreactive neurons. Counting of positive cells in each region was conducted manually by an investigator blinded to the treatment. Group means were calculated from the averaged cell counts per slice for each animal and presented as group means ± SE.

**Study 1: Time Course and Dose Dependence of Amylin-Induced ERK1/2 Phosphorylation**

Sixty-four rats weighing 240–280 g at the time of perfusion were used in this study. Animals were handled and habituated to the investigator and to the housing conditions for at least 10 days before perfusion. Twenty-four four-fasted rats were injected subcutaneously (1 ml/kg body wt) at dark onset with saline (control) or amylin (20 μg/kg; Bachem, Dübendorf, Switzerland). The number of pERK1/2 immunoreactive neurons was quantified for the total number of pERK1/2, DBH, and DBH/pERK double-immunoreactive neurons. Counting of positive cells in each region was conducted manually by an investigator blinded to the treatment. Group means were calculated from the averaged cell counts per slice for each animal and presented as group means ± SE.

**Study 2: Effect of MEK Inhibition on Amylin-Induced Anorexia**

The method was adapted from studies using similar methodology (58, 59). Rats were habituated to both subcutaneous and 4V injection with sterile saline before the trials. The Doses of U0126 and the vehicle were chosen based on previous reports using this inhibitor (18, 47, 58). Rats were tested under four different conditions (A, B, C, and D). For conditions A and B, 25 rats weighing 250–310 g at the time of stereotaxic surgery and 400–560 g at the time of perfusion were handled and habituated to the investigator and to the housing conditions for at least 1 wk before surgery. Nineteen rats with correct 4V cannula placement were tested in a within-subjects design (3 days between trials) for the influence of the MEK1/2 inhibitor U0126 (V112A; Promega) on amylina’s eating-inhibitory action. Body weight and 24-h basal food intake were assessed before each trial and food was removed 2 h before dark onset (ad libitum condition). In rats tested under condition A, U0126 was injected into the 4V of rats pharmacologically inhibit ERK1/2 signaling in the AP. Rats received a 4V injection of U0126 (5 μg in 3 μl of 50% DMSO/saline) or vehicle 1 h before dark onset; subcutaneous injections of saline (control) or amylin (20 μg/kg sc) were administered shortly before dark onset. Food was then returned and cumulative intake (± 0.1 g) was measured during the first 2 h of the dark phase and corrected for spillage. The same rats were tested under condition B in which a higher dose of U0126 was administered; rats were injected (4V) with 10 μg of U0126 dissolved in 4 μl of vehicle and then injected subcutaneously with saline (n = 9) or 20 μg/kg amylin (n = 9). This higher U0126 dose was only tested in a single trial.

For conditions C and D, 26 rats weighing 260–310 g at the time of stereotaxic surgery and 390–480 g at the time of perfusion were used. Twenty-one rats with correct 4V cannula placement were tested in a within-subjects design (3 days between trials). For condition C, body wt and 24-h basal food intake were assessed before each trial and food was removed 2 h before dark onset (ad libitum). Seven micrograms of U0126 or vehicle was injected into the 4V of rats 30 min before dark onset; subcutaneous injections of saline (control) or amylin (5 μg/kg sc) were administered shortly before dark onset. Food was then returned and cumulative intake (± 0.1 g) was measured as before. The same rats were tested under condition D, in which the same doses of U0126 and amylin were injected as in condition C, but rats were fasted for 24 h before amylin or saline injection at dark onset (fasted). The time between trials under condition D was of 3–4 days.

**Study 3: Effect of MEK Inhibition on Amylin-Induced pERK Expression in AP Neurons**

To test whether the MEK inhibitor prevents the amylin-induced ERK phosphorylation in AP neurons, pERK immunohistochemistry was performed in rats that were treated with U0126 or vehicle (4V) before amylin or saline (subcutaneous). The 19 rats tested under conditions A and B in study 2 were divided into four groups 5 days after the last feeding trial. Rats were fasted for 24 h, injected with U0126 (5 μg in 3 μl DMSO/saline; 4V) or vehicle 1 h before dark onset and injected with saline or 20 μg/kg amylin sc at dark onset. Fifteen minutes later, the animals were deeply anesthetized and transcardially perfused.

Twenty-one rats tested under conditions C and D in study 2 were divided into four groups. Rats were fasted for 24 h, injected in the 4V with U0126 (7 μg in 3 μl DMSO/saline) or vehicle 30 min before dark onset and injected with saline or 5 μg/kg amylin sc at dark onset. Fifteen minutes later, the animals were perfused. Three series of 20-μm coronal brain sections containing the AP were cut in a cryomicrotome; one series was stained for pERK by the DAB method.

**Statistical Analyses**

In study 1, two-way-ANOVA was used to test the effect of amylin and time of perfusion in the pERK time course evaluation. Bonferroni post hoc tests were used to detect differences between time points. The effect of the different amylin doses in study 1 was analyzed by one-way ANOVA with Bonferroni multiple comparison tests; the Kruskal-Wallis with Dunn’s post hoc tests was used.
for nonparametric comparisons. The unpaired Student t-test was used to compare saline- and amylin-injected animals in the double-labeling analysis. In study 2, data from the feeding trials employing 5 and 7 μg U0126 (conditions A, C, and D) were analyzed by repeated-measures two-way ANOVA; the data from the feeding trials with 10 μg U0126 (condition B) were analyzed by two-way ANOVA, because this dose was tested in a single trial. Bonferroni post hoc tests evaluated individual differences between treatments. The difference in food intake of each animal under saline and under amylin treatment was calculated, and the comparison between vehicle and U0126 administration was performed by paired t-test. In study 3, analysis of amylin-induced ERK phosphorylation in the AP after 4V injection of U0126 or vehicle was performed by one-way ANOVA followed by the Student-Newman-Keuls post hoc tests. Calculation of the percentage of pERK cells in the AP took the respective saline groups as baseline. For all statistical tests a P value < 0.05 was considered significant.

RESULTS

Study 1: Time Course and Dose Dependence of Amylin-Induced ERK1/2 Phosphorylation

Two-way ANOVA indicated that the effects of amylin, time of perfusion, and interaction between amylin treatment and time in the AP were all significant (P < 0.001); in other words, amylin significantly increased the number of pERK-positive cells in the AP compared with saline at all time points investigated (Fig. 1D). Amylin-induced ERK1/2 phosphorylation peaked ~10 to 15 min after injection; the number of pERK neurons decreased thereafter. The pERK signal was observed both in the nuclear and cytoplasmic cell compartments (Fig. 1, B and C). In the NTS, the number of pERK cells in amylin-treated rats increased significantly above baseline until the 20 min time point (Fig. 2A). In the LPB, amylin treatment increased significantly the number of pERK neurons at 15 min (Fig. 2B).

Double-labeling analysis revealed pERK-positive neurons in the AP that expressed the CTR (Fig. 3). Because CTR was not only found in the membranes of neuronal cell bodies, but was also prominent in their cellular processes, and because it was difficult to clearly distinguish between these two entities in the CTR staining, an exact cell count of double-labeled cells was not possible.

The number of pERK-positive neurons and the number of pERK/DBH positive cells in the AP was significantly higher in amylin-injected rats than in saline-treated animals (Fig. 4). Double-fluorescence analysis revealed that 22% of the neurons displaying amylin-induced pERK within the AP were also DBH-positive (Fig. 4A). However, only 7.5% of the total number of DBH-positive neurons showed ERK1/2 phosphorylation after amylin. There was no difference in the total number of DBH-positive neurons in the AP between amylin and saline-treated rats.

Fig. 1. Representative example of amylin-induced ERK phosphorylation (pERK) in the area postrema (AP). Immunohistochemical stainings of pERK by the 3,3′-diaminobenzidine-tetrahydrochloride (DAB) method in AP sections from a rat injected with saline (A) or 20 μg/kg of amylin (B). Cells that are positive for pERK immunoreactivity are labeled in brown (arrows). The high-power magnification picture (C; ×40) displays neurons where pERK is found in the nucleus (small arrow), the cytoplasm (large arrow), and in both cellular compartments (arrowhead). The time course of amylin-induced ERK1/2 phosphorylation in the AP is shown in (D); number of pERK-positive neurons in saline (white bars) or amylin (20 μg/kg; black bars)-treated rats. Rats were perfused 10, 15, 20, or 30 min after injection. Data are expressed as group means ± SE; ***P < 0.001 saline vs. amylin; #P < 0.01 comparison of amylin-induced pERK between different time points. The dose dependence of amylin-induced ERK1/2 phosphorylation in the AP is shown in (E). Shown is the number of neurons showing pERK in rats injected with saline (white bar), 5 μg/kg (checkered bar), or 20 μg/kg of amylin (black bar) and perfused 15 min later. Data are expressed as group means ± SE. Bars with different letters are significantly different (P < 0.05).
Based on the outcome of the time course analysis, the 15-min time point was chosen to evaluate the effect of a lower amylin dose on pERK stimulation. At 5\mu g/kg, amylin induced a significant increase in the number of pERK cells in the AP, and there was a clear dose-dependent effect of amylin when compared with the effect of the 20\mu g/kg dose (Fig. 1E). The low dose of amylin did not induce pERK in the NTS and LPB (Fig. 2, C and D). The Ce and BSTL displayed significantly higher numbers of pERK cells after administration of either dose of amylin compared with saline (Fig. 5), and the number of pERK cells was comparable after both doses. In addition to the more typically observed cytoplasmic pERK staining, the pERK signal was also detected in both the nuclear and cytoplasmic cell compartments in some cells of the Ce and BSTL (not shown).

Study 2: Effect of MEK Inhibition on Amylin-Induced Anorexia

Test conditions A and B. Two-way-ANOVA revealed a significant amylin (20\mu g/kg) effect at all time points and under both test condition A (Fig. 6A; 5 \mu g U0126, repeated-measures
analysis 30 and 60 min $P < 0.001$) and condition B (Fig. 6B; 10 $\mu$g U0126, 30 min $P < 0.001$, 60 min $P < 0.05$). Post hoc analysis showed that amylin significantly reduced 30- and 60-min cumulative food intake in 4V-vehicle-treated rats. Two-way ANOVA did not reveal an effect of U0126 or an interaction with amylin treatment; U0126 did not affect food intake by itself. Post hoc analysis further demonstrated that 5 $\mu$g U0126 given 1 h before amylin did not attenuate the eating-inhibitory effect of amylin at any time point (Fig. 6A). Ten micrograms of U0126 did not affect the eating-inhibitory effect of amylin 30 min after injection (Fig. 6B). There was no significant anorectic effect of amylin in animals treated with 10 $\mu$g U0126 at the 60-min time point; however, food intake of these animals did not differ from amylin-injected rats treated...
with vehicle, indicating that U0126 was not able to block amylin’s eating inhibitory action (Fig. 6).

**Test conditions C and D.** Two-way ANOVA revealed a significant amylin (5 μg/kg) effect at all time points and under both condition C (Fig. 7A: 7 μg U0126 ad libitum condition, 30 and 60 min P < 0.001) and condition D treatment conditions (Fig. 7B: 7 μg U0126 fasting condition, 30 min P < 0.01, 60 min P < 0.001). Post hoc analysis showed that amylin significantly reduced 30- and 60-min cumulative food intake in 4V vehicle-treated rats under both conditions. Seven micrograms of U0126 did not attenuate the eating-inhibitory effect of amylin at any time point in rats tested under the ad libitum condition (Fig. 7A). Two-way ANOVA did not detect an effect of U0126 given 30 min before amylin, but there was a significant interaction between U0126 and amylin treatment at 60 min in fasted rats (P < 0.05; Fig. 7B). Furthermore, post hoc analysis demonstrated that 7 μg U0126 significantly attenuated the eating-inhibitory effect of amylin at the 60 time point in fasted rats (vehicle/amylin vs. U0126/amylin P < 0.05; Fig. 7B). Analysis of the individual differences in food intake clearly demonstrated the effect of U0126 to attenuate the reduction in food intake induced by amylin; this attenuation was significant at the 60 min time point (P < 0.05; Fig. 7C). U0126 given 30 min before the feeding trial did not affect food intake by itself under either feeding condition (Fig. 7, A and B).

**Study 3: Effect of MEK Inhibition on Amylin-Induced pERK Expression in AP Neurons**

Under all conditions, amylin induced a significant increase in the number of pERK-positive AP neurons in U0126 and in vehicle treated rats, relative to their respective controls (Fig. 6C and 7D). In study 3a, the number of pERK-positive neurons in the AP was significantly reduced by 30% in amylin-injected rats that were pretreated with 5 μg U0126 1 h before amylin injection (Fig. 6C). In study 3b, the number of pERK-positive neurons in the AP of amylin-injected rats that were pretreated with 7 μg U0126 30 min before amylin was reduced by 27% compared with amylin-injected rats pretreated with vehicle; however, the effect of U0126 did not reach statistical significance in the post hoc analysis (Fig. 7D).

**DISCUSSION**

In the present study, we found several important pieces of evidence suggesting that the ERK1/2 pathway is activated by amylin in AP neurons and that it is involved in the amylin-induced inhibition of food intake. First, we found that amylin activates the ERK1/2 pathway in the AP because amylin increased the number of cells showing the phosphorylated, active form of ERK. Second, the pERK signal in the AP showed a time and dose dependence that corresponds to the typical timing and dose dependency of amylin’s anorectic action. Third, pERK expression colocalized to some extent
with DBH- and CTR-containing neurons that appear to be the primary target cells for amylin in the AP. Fourth, brain structures shown to be synaptically activated by amylin (45, 50), namely the NTS, LPB, Ce, and BSTL, also showed increased ERK1/2 phosphorylation after amylin treatment. Fifth, a blockade of the ERK cascade in the AP by injection of the MEK1/2 inhibitor U0126 into the 4V reduced the number of neurons displaying amylin-induced pERK in the AP by about 30%. Furthermore, U0126 attenuated amylin’s ability to reduce food intake in rats at least under certain experimental conditions, namely when the rats had been fasted for 24 h prior to amylin administration. Interestingly, the time course of pERK formation in the AP parallels the onset of amylin-induced anorexia (28). ERK phosphorylation peaked within the first 15 min after peripheral amylin, which is similar to the dynamics of CCK-induced pERK in the NTS (59), a satiating hormone that also acts within minutes after injection (22, 33). The pERK levels started to decrease 20 min after injection; however, the pERK signal remained significantly higher than in controls for up to 30 min after the injection of amylin. We did not investigate amylin-induced ERK phosphorylation at later time points. The pERK signal was mainly observed in the cytoplasm. It is known that pERK may have either membrane bound, cytosolic, or nuclear targets (23). It seems plausible that cytoplasmic or membrane-bound targets are involved in amylin’s fast effect to reduce eating, potentially by influencing ion channel activity, and hence neuronal activity directly.

Given that the AP seems to be the primary brain target for amylin action (44), we propose that the ERK cascade may be triggered by direct activation of the amylin-receptor in the AP, which consists of a heterodimer of CTR1(a) (7, 24) as a core receptor and a RAMP (15, 39), probably RAMP3 (7). This notion is supported by our observation that some AP neurons in which amylin-induced pERK also showed CTR expression. In contrast to our previous studies (9), we detected generally fewer CTR-positive cell bodies in the AP, while CTR staining was clearly prominent in cellular processes. This was most likely due to the experimental settings used in the tyramide amplification approach, in which the concentration of the primary antibody used is greatly reduced. For this reason, the number of CTR-positive cell bodies in the AP may have been underestimated; a formal quantification was therefore not performed, because it would most likely yield erroneous results. Furthermore, it was difficult to clearly distinguish between the CTR signal in cell body membranes from dendritic/axonal CTR
signal in the present staining in some cases, making it impos-
sible to perform an exact cell count of the double-labeled
neurons.

Amylin is known to bind directly to the CTR subunit, which is a
seven-transmembrane or G protein-coupled receptor type
(GPCR) (26). GPCR activation can activate the ERK cascade
in neurons via both Rap-1 or Ras/Raf dependent pathways (23,
43, 60, 65), but how amylin receptor activation is linked to
ERK cascade activation in the AP still needs clarification.
PcERK signaling may also result from an indirect effect via
synaptic interactions, which might explain that not all pERK-
positive cells showed detectable CTR expression after amylin
treatment.

In the present study, we found that 22% of the neurons
displaying amylin-induced pERK within the AP were norad-
renergic. Assuming that pERK signaling is functionally re-
levant for amylin’s eating inhibitory effect, this finding is in line
with our previous report of an important role of noradrenergic
neurons in amylin’s effect (46). The relatively low number of
noradrenergic AP neurons that also showed amylin-induced
ERK phosphorylation contrasts with a higher percentage of
c-Fos expressing noradrenergic neurons in the AP after amylin,
which amounted to ~50% (46). The reason for the lower
percentage of colocalization with pERK is unknown, and one
should be cautious with direct quantitative comparisons across
different methods. Interestingly, c-Fos expression may be
brought about by pERK via Elk-1 or RSK2 mechanisms (65,
67). Since c-Fos expression reflects neuronal activation, ERK
phosphorylation may be part of the intracellular processes that
lead to c-Fos expression and hence neuronal activation in the
AP. If this were the case, one would expect a blockade of
amylin-induced c-Fos formation by the MEK inhibitor U0126;
however, this has not yet been investigated. Such result would
imply that neurons that show amylin induced pERK are ident-
tical with cells that show an amylin-induced c-Fos response.
Such colocalization studies for amylin-induced c-Fos and
pERK are technically problematic because of the different time
courses for the induction of the two signals. We therefore
chose an indirect approach that demonstrates an overlap be-
tween the biochemical phenotype of amylin-responsive neu-
rons in the c-Fos (46) and the pERK studies. This finding is
complemented by our observation that CTR-expressing AP
neurons show a c-Fos response after amylin treatment (71); the
finding is also in line with our present results showing that
some pERK-expressing AP neurons are CTR-positive.

Similar questions may be asked in respect to the role of
cGMP signaling in the AP in amylin action. Amylin induces
cGMP formation in the AP, and cGMP may mediate the exci-
tatory effects of amylin in AP neurons (49). Furthermore,
reduction of meal size by local administration of the membrane
permeable analog 8-Br-cGMP into the AP suggests a func-
tional role of cGMP signaling in amylin’s eating inhibitory
effect (35). Interestingly, cGMP accumulation is able to ac-
ivate the ERK cascade in neurons in vitro (14, 19, 32) but the
potential link between cGMP accumulation in the AP and ERK
cascade activation, and the necessity for such a link for amy-
lin’s eating inhibitory effect, remains to be elucidated.

Previous studies, most of them relying on feeding data and
on c-Fos immunocytochemistry, suggested that the excitatory
effect of amylin is transmitted from the AP via the NTS to the
LPB, and possibly to the Ce and BSTL. Lesions of the AP
blocked the eating-inhibitory effect of peripheral amylin and
the amylin-induced c-Fos expression in these downstream
projection sites (29, 30, 50, 56). It appears plausible that the
amylin-induced pERK signal in the NTS, LPB, Ce, and BSTL
might be a consequence of synaptic inputs originating in the
AP rather than a direct effect of amylin in these areas, but this
needs to be tested. Rats with electrolytic LPB lesions have a
significantly reduced eating-inhibitory effect of amylin and a
marked reduction in the number of c-Fos-positive neurons in
more rostral brain areas, namely in the Ce, though the level of
amylin-induced c-Fos-expression in the AP and NTS remained
unaltered (8). This indicates that a LPB lesion specifically
blocks the amylin signal transmission from the hindbrain to the
forebrain, and that an intact LPB is necessary for amylin’s
effect on eating. Assuming that the induction of ERK phos-
phorylation in the NTS and other brain areas is synaptically
mediated after a primary effect in the AP, it was somewhat
surprising that the pERK signal in some of these projection
sites, and in particular in the NTS, did not follow the same
kinetics as in the AP. The reason for this apparent discrepancy
is unknown. It is, however, important to note that the pERK
signal in the LPB appeared to follow similar time lines as in the
AP, i.e., there was a higher degree of stimulation at the 15-min
time point. Our recent tracing studies showed that there is a
prominent projection from the AP to the LPB (45). These
studies had suggested that the amylin signal may at least in part
be transmitted directly from the AP to the LPB, and that the
indirect transmission via the NTS may be less important. Our
present study showing similar pERK kinetics in the AP and the
LPB, but not the NTS, is in principle in line with this idea.

When targeting the 4V, U0126 may not only influence the
AP but also partly the NTS. Therefore, we also analyzed pERK
expression in the NTS under U0126 pretreatment in both
experimental conditions used in study 3. In study 3a, 5 μg
U0126 pretreatment did not significantly reduce the number of
NTS neurons expressing pERK after 20 μg/kg amylin (data not
shown). Furthermore, the relative pERK induction with the 20
μg/kg amylin dose in the NTS was in fact small compared with
the AP (see Figs. 1 and 2). At the peak 15-min time point, there
was a 95% increase in pERK expression in the AP but only a
42% increase in the number of pERK cells in the NTS. The
relative effect of U0126 to block the ERK cascade in the NTS
also seemed to be less pronounced than in the AP. The specific
experimental condition where we observed an effect of a
blockade of ERK phosphorylation in attenuating amylin’s
anorectic action was using the 5 μg/kg amylin dose in combina-
tion with 7 μg U0126 pretreatment after 24-h fasting. This
amylin dose was not able to induce pERK levels above base-
line in the NTS, as shown in Fig. 2. Consistently, we also
observed in study 3b that the 5-μg/kg amylin dose did not
induce pERK in the NTS above baseline, and 7 μg U0126 did
not influence pERK expression in the NTS (data not shown).
Therefore the MEK inhibitor U0126 did not seem to exert any
measurable influence on the NTS neurons to block the ERK
cascade. Overall, we cannot exclude the possibility that some
amylin effects may be NTS-mediated, especially because amy-
lin did induce pERK expression in the NTS; however, for the
reasons explained, the contribution of NTS pERK expression
in amylin’s anorectic action, if existent, is most likely minor.

In general, amylin-induced AP pERK expression in study 3
was higher than in study 1. The exact reason for this is
unknown, but it may be due to the fact that rats of study 3 had previously been tested with the various drugs in several feeding trials (study 2) prior to death, which could have also somehow influenced their sensitivity to amylin. Additionally, differences in stain intensity may be related to other interassay variables, such as the antibody batch (even if using the same lot number), ambient temperature during incubation (even if the same incubation conditions and timings are used), or due to simple biological variation.

We found in study 3b that animals treated with 7 µg MEK inhibitor U0126 30 min before 5 µg/kg amylin showed 27% less amylin-induced pERK-positive neurons in the AP, even if this reduction did not reach statistical significance. At a functional level, the blockade of the ERK cascade in the AP was able to almost completely abolish the ability of peripheral amylin to reduce 30 and 60 min food intake in rats that were fasted for 24 h before the feeding trial (condition D), and the potency of this blockade was comparable to studies by Sutton and colleagues (58, 59) that suggested a role of the ERK cascade in CCK and melanotan II anorectic actions. This finding suggests the involvement and a necessary role of the ERK cascade in amylin’s anorectic action via the AP. However, blockade of the ERK cascade did not abolish amylin’s anorectic action under ad libitum conditions even when using a relatively high dose of U0126 (10 µg, condition B); similar observations were made using different amylin dosages and timing schedules as explained below.

The lowest dose of U0126 that we tested (5 µg), which was given 1 h prior to amylin and which was ineffective under ad libitum conditions in blocking amylin’s anorectic action, effectively blocked the eating-inhibitory effect of CCK and of melanotan II in rats fasted for 16 h prior to the trial (58, 59). To assess the possibility that the lack of effect could be related to an insufficient duration of U0126 action rather than the dose of U0126 or the nutritional status of the animal, we tested animals injected with U0126 30 min before feeding (conditions C and D). The kinetics of CCK’s anorectic action are similar to that of amylin (51, 59); however, because the target site in the present study was the AP and not the NTS, as in the studies by Sutton and colleagues (58, 59), the uptake of U0126 and duration of inhibitor activity may differ between the two brain areas. Furthermore, cell culture studies have shown that U0126 effectively blocked ERK1/2 phosphorylation as early as after 30 min of incubation with U0126 in colon cancer cells (48). In parallel, the effect of amylin dosage was also assessed. In trials of conditions C and D we used a lower dose of amylin (5 µg/kg) than in conditions A and B (20 µg/kg). This change was based on the premise that the initial dose of amylin was too high for a single injection of U0126 to prevent possible ERK-mediated effects induced by amylin. However, even under these new test conditions, U0126 was still not able to reduce the effect of amylin on eating in ad libitum fed rats (condition C).

Such an effect of U0126 was only seen in rats that received U0126 after an extended fasting period of 24 h (condition D). The reason why blockade of ERK phosphorylation attenuated amylin’s ability to reduce eating under some but not all conditions is unclear at present, but may be related to differences in the energy status that affect amylin action (34). Similar to our recent studies using c-Fos immunocytochemistry (34), preliminary data from our group suggest that the amylin-induced pERK-expression is lower in rats kept ad libitum prior to amylin-injection and perfusion compared with rats fasted for 24 h (unpublished observation). Consistent with this view, recent studies demonstrate that MAPK/ERK pathway activation is indeed modulated by the nutritional status (20, 21). Accordingly, in the study by Sutton and et al. (59) showing that the anorectic action of CCK depends on the ERK pathway, animals that were fasted for 16 h before the pharmacological blockade of ERK phosphorylation with U0126 were used and not ad libitum rats.

Furthermore, the activation of many other intracellular pathways is dependent on the nutritional state of the organism or the cell. For instance, the AMP protein activated kinase signaling pathway, which is triggered in the arcuate hypothalamic nucleus by several hormones involved in the control of eating, and the mammalian target of rapamycin (mTOR) pathway are equally modulated by the nutritional status of the organism (16, 69).

Alternatively, the relative inefficacy of U0126 to totally block ERK phosphorylation in the AP and to affect amylin’s anorectic effect under some experimental conditions may be related to some difficulty to penetrate the AP. The incomplete blockade of ERK phosphorylation may also be due to the action of other kinases (other than MEK) that may partly phosphorylate ERK when MEK is inactivated. In a recent study by Aksamitiene et al. (1), the epidermal growth factor (EGF) was able to induce a crosstalk between PI3K/Akt and Ras/MAPK signaling pathways in breast cancer cells. Their data suggested that initial ERK1/2 activation at early time points was exclusively mediated by MEK, but at later times MEK’s contribution became less significant in this model and U0126-resistant ERK activation was shown to be dependent on kinases located downstream of PI3K/Akt.

Taken together, future studies may shed more light in defining the role of pERK signaling and of other intracellular signaling cascades for amylin action in a broader range of experimental conditions.

**Perspectives and Significance**

In conclusion, the present study showed that amylin activates the ERK cascade in the AP in amylin receptor-carrying neurons and in noradrenergic neurons. Furthermore, the ERK cascade acts as a mediator of amylin’s anorectic signal in the AP, at least under some experimental conditions, in particular when the rats had been fasted prior to injection. Because we were not able to completely block ERK phosphorylation, we may in fact underestimate the role of this cascade in amylin’s anorexia under other conditions, such as ad libitum feeding. Amylin induced the ERK cascade not only in the AP, but also in brain areas of the NTS-LPB-Ce-BSTL axis. Using c-Fos immunodetection techniques, this pathway had previously been shown to be activated by amylin (50, 55) and by other gastrointestinal peptides that inhibit eating, namely CCK (37, 52), glucagon-like peptide 1 (6), and peptide YY 3–36 (11). Interestingly, amylin is known to modulate CCK’s anorectic effect, and the combination of these two hormones produces augmented reductions in food intake (10, 36). Because both
CCK and amylin were shown to induce pERK in the NTS and AP, respectively (Ref. 59 and present study), it will also be interesting to test whether pERK signaling in the AP or NTS may be involved in this hormonal interaction.

The pERK signal was observed not only in the cytoplasm but also in the nuclear compartment of neurons, especially in the AP. Nuclear pERK may induce the expression of target genes involved in the mediation of long-term neuronal adaptive responses, or integration with other hormones. Since most reports suggest that amylin’s effect to increase energy expenditure requires chronic amylin infusion or the effect of a longer acting amylin-receptor agonist like salmon calcitonin, it may be possible that these effects in part require transcriptional events (31, 54, 68). It will therefore also be interesting to investigate whether the ERK cascade is involved in some long-term responses of amylin.

ACKNOWLEDGMENTS

The technical help of K. Forster, K. Spleithoff, and D. Zueger is gratefully acknowledged. The authors thank Prof. Hans-Rudolf Berthoud from the Pennington Biomedical Research Center in Baton Rouge, LA, for his valuable input during the development of the present study.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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