Amylin potently activates AP neurons possibly via formation of the excitatory second messenger cGMP

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Amylin is secreted with insulin from the pancreas during and after food intake. One of the most potent actions of amylin in vivo is its anorectic effect, which is directly mediated by the area postrema (AP), a circumventricular organ lacking a functional blood-brain barrier. As we recently demonstrated, amylin also stimulates water intake most likely via its excitatory action on subfornical organ (SFO) neurons. Neurons investigated under equal conditions in an in vitro slice preparation of the rat AP were 15-fold more sensitive to amylin than SFO neurons. Amylin (10^{-11}–10^{-8} M) excited 48% of 94 AP neurons tested; the remaining cells were insensitive. The average threshold concentration of the excitatory response was 10^{-10} M and, thus, close to physiological plasma concentrations. Coapplication of the amylin receptor antagonist AC-187 reduced amylin’s excitatory effect. Amylin-mediated activation of AP neurons and antagonistic action of AC-187 were confirmed in vivo by c-fos studies. Peripherally applied amylin stimulated cGMP formation in AP and SFO neurons, as shown in immunohistochemical studies. This response was independent of nitric oxide (NO) formation in the AP, while coapplication of the NO synthase inhibitors N-monomethyl-L-arginine (100 mg/kg) and nitro-L-arginine methyl ester (50 mg/kg) blocked cGMP formation in the SFO. In contrast to the SFO, where NO-dependent cGMP formation seems to represent a general inhibitory transduction pathway, cGMP acts as an excitatory second messenger in the AP, since the membrane-permeable analog 8-bromo-cGMP stimulated 65% of all neurons tested (n = 17), including seven of nine amylin-sensitive neurons (77%). The results indicate that the anorectic effect of circulating amylin is based on its excitatory action on AP neurons, with cGMP acting as a second messenger.

Food intake; water intake; electrophysiology; nitric oxide; subfornical organ

AMYLIN IS A 37-AMINO ACID peptide that is secreted with insulin from pancreatic β-cells in response to nutrient stimuli (5, 36, 50). The most potent actions of circulating amylin affect the gastrointestinal system and ingestive behavior. As a partner hormone to insulin, amylin controls nutrient intake as well as nutrient influx to the blood by an inhibition of food intake, gastric emptying, and glucagon secretion (3, 13, 21, 26, 51, 52). At least the first two of these effects are mediated by the area postrema (AP) (9, 23), a hindbrain circumventricular organ (CVO) that lacks a functional blood-brain barrier (14) and contains a high density of amylin receptors (45).

We recently suggested that amylin might also be implicated in the stimulation of food-associated drinking, since subcutaneously applied amylin increased water intake in euhydrated rats to the same degree as ANG II in equimolar doses (34). It is conceivable that the subfornical organ (SFO) mediates the amylin-induced water intake, since amylin exerts an excitatory effect on ANG II-sensitive SFO neurons (34, 35), which is generally accepted to account for the ANG II-mediated induction of thirst.

To extend our investigations on the cellular mechanisms involved in the centrally mediated actions of amylin, we sought to examine the effect of amylin on the electrical activity of AP neurons in an in vitro slice preparation. With the use of AC-187 as a selective amylin receptor antagonist, the involved receptor type was characterized pharmacologically in electrophysiological experiments. These studies were supplemented by immunohistochemical experiments aiming at detecting c-Fos as a marker for neuronal activation subsequent to peripheral amylin administration.

Using immunohistochemical detection of cGMP formation after peripheral amylin application, we also addressed the possible role of intracellular cGMP signaling in amylin’s excitatory effect on AP neurons. Generation of cGMP as a second messenger signal may involve different signaling pathways. Membrane-bound guanylyl cyclase was shown to be coupled to the receptor for the atrial natriuretic factor. Soluble guanylyl cyclase serves as a target for nitric oxide (NO) formed after activation of the constitutive neuronal isoform of NO synthase (nNOS) (12, 28). In contrast to the SFO, where a large quantity of nNOS was detected immunohistochemically and by NADPH-diaphorase...
staining, the AP contains little nNOS (17, 38). In previous electrophysiological and immunohistochemical studies, NO-dependent cGMP formation in the SFO has been found to reduce neuronal activity (32).

Therefore, we also paid special attention to the NO dependency of amylin’s effect on cGMP formation in AP neurons. To discriminate NO-dependent from NO-independent effects, the NOS inhibitors N-monomethyl-l-arginine (l-NMMA) and nitro-l-arginine methyl ester (l-NAME) were used. Additionally, a double-labeling procedure was designed to detect cGMP-positive neurons immunohistochemically and NO-producing cells by NADPH-diaphorase staining. Finally, the membrane-permeating analog 8-bromo-cGMP (8-BrcGMP) was used in electrophysiological experiments to mimic the effect of intracellular cGMP formation on the activity of AP neurons.

**MATERIALS AND METHODS**

For all experiments, male adult Wistar rats (170–230 g) were used. They had ad libitum access to standard laboratory rat chow and water and were maintained on an artificial 12:12-h dark-light cycle (lights on at 7 AM).

**Electrophysiological Studies**

*Slice preparation and signal recording.* The extracellular recording method was described previously for the recording of SFO neurons (35). Rats were decapitated at 10 AM, and their brains were quickly removed and superfused with ice-cold artificial cerebrospinal fluid (aCSF) composed of (in mM) 124 NaCl, 5 KCl, 1.2 NaH2PO4, 1.3 MgSO4, 1.2 CaCl2, 26 NaHCO3, and 10 glucose, equilibrated with 95% O2-5% CO2 (pH 7.4, 290 mosmol/kg). A slice of the medulla oblongata was isolated by two coronal sections rostral and caudal to the AP. The slice was trimmed to contain only the AP and immediately adjacent parts of the nucleus of the solitary tract (NTS). The AP could easily be identified by its V-shaped appearance.

After 2 h of preincubation in aCSF at 35°C, the slice was transferred to the recording chamber and fixed to the bottom of the chamber with a small metal weight. The gold-plated brass recording chamber contained 0.7 ml of aCSF, which was constantly perfused at a rate of 1.6 ml/min after it was prewarmed to chamber temperature, which was maintained at 37.0°C by a feedback-controlled thermostatic element. Glass-coated platinum-iridium electrodes were used to make extracellular recordings from AP neurons. Action potential recordings were amplified and displayed on a storage oscilloscope (Gould), passed through a window discriminator (World Precision Instruments), and analyzed with custom software (spike2, Cambridge Electronic Design) on a personal computer.

*Drug application.* Rat amylin (Amylin Pharmaceuticals, San Diego, CA) and 8-BrcGMP (Sigma, Deisenhofen, Germany) were added to the aCSF shortly before application. Both drugs were stored in frozen aliquots (−24°C) and kept on ice until use during an experiment. As a standard stimulus, 10 ml of aCSF containing amylin (10−9–10−8 M) or 8-BrcGMP (10−3 M) were superfused per stimulus. After a stable recording from a single neuron had been established, its responsiveness was tested by switching to a perfusion solution containing the drug.

*Dose-response relationship and antagonist studies.* A dose-response relationship for the effect of amylin on AP neurons was established by superfusing 10−11–10−8 M amylin. To investigate whether the amylin-induced effect depends on amylin receptors, the amylin receptor antagonist AC-187 (10−7–10−6 M; gift from Amylin Pharmaceuticals) was applied with amylin.

**Data analysis.** The average discharge rate of each neuron was evaluated for 60 s before the stimulus from the continuously recorded ratemeter counts. This value (referred to as “control”) was used to normalize changes in firing rate, expressed as percent change from control. If the average change in discharge rate during the entire response time was larger than ±20%, the neuron was considered sensitive to the applied substance. Furthermore, to avoid possible false-positive responses, the effects of all agents had to be reversible to be included in this study. The effect parameters of the electrophysiological responses are expressed as means ± SE. Recording sites in the horizontal subregions of the AP were designated 1 (caudal), 2 (middle), and 3 (rostral). Differences between proportions of sensitive neurons in these subregions were evaluated using Fisher’s exact test. Differences were considered significant at P < 0.05.

**Immunohistological Studies**

Detection of c-fos expression. All treatments were conducted during the first 4 h of the light phase. For the immunohistochemical detection of c-fos expression, amylin was injected at 5 μg/kg ip (n = 3). This dose of amylin has been adapted from previous studies demonstrating a potent amylin-mediated inhibition of feeding via AP neurons (20, 23). The amylin receptor antagonist AC-187 (500 μg/kg ip) was injected 10 min before amylin administration (n = 3); control animals received sterile saline (n = 3). At 90 min after amylin or control administration, animals were deeply anesthetized with pentobarbital sodium (100 mg/kg ip) and transcardially perfused with ice-cold sodium phosphate buffer (PB, 0.1 M, pH 7.4) and then with 4% paraformaldehyde in PB. Sections of the medulla oblongata containing the AP were postfixed and cryoprotected at 4°C in 4% paraformaldehyde (1 h) and 10% sucrose in PB (2 h), respectively.

Cyrossections (20 μm) were cut on a cryostat and thawed on poly-L-lysine-covered slides, which were air-dried at room temperature. After rehydration in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (PBS-T, pH 7.4), sections were incubated in 0.3% PBS-T for 48 h at 4°C using a 1:1,000 dilution of a rabbit polyclonal antiserum directed against c-Fos (Ab5, Calbiochem-Novabiochem, Bad Soden, Germany). After they were washed in 0.1% PBS-T, sections were incubated in 0.3% PBS-T for 48 h at 4°C using a 1:1,000 dilution of a rabbit polyclonal antiserum directed against c-Fos (Ab5, Calbiochem-Novabiochem, Bad Soden, Germany). After they were washed in 0.1% PBS-T, sections were incubated for 75 min at room temperature with Cy3-conjugated goat anti-rabbit immunoglobulin (Dianova, Hamburg, Germany). Sections were rinsed in PBS and mounted in Citifluor [1:1 (vol/vol) PBS-glycerol; Citifluor Products, Kent, UK]. Digitalized photographs were taken on a fluorescence microscope (Zeiss Axiioskop).

For quantification of the c-Fos response, c-Fos-immunoreactive (IR) cells were counted from 12–29 representative slices per animal. The cell counts from all animals of the same treatment group were pooled and are expressed as means ± SE. Statistical differences between the treatment groups were analyzed by Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn’s multiple comparison procedures. Differences were considered significant at P < 0.05.

Detection of cGMP formation. In the immunohistochemical studies for the detection of cGMP formation, animals were pretreated with 3-isobutyl-1-methylxanthine (IBMX, 10 mg/kg ip; Sigma) to prevent degradation of cGMP. At 15 min
after IBMX administration, amylin was subcutaneously injected at 784 μg/kg (1 ml/kg of 2 × 10^{-4} M, n = 3). The NOS inhibitors L-NMMA (100 mg/kg; Alexis, Grünberg, Germany) and L-NAME (50 mg/kg; Alexis) were injected with IBMX (n = 2) to analyze the NO dependency of cGMP formation. Control animals received IBMX or IBMX together with the respective NOS inhibitor (n = 2 per treatment group). Anesthesia and fixation were started 25 min after amylin administration; procedures were the same as those described for the c-fos studies.

In addition to the AP, the SFO was prepared by trimming the brain to a square block containing the entire hypothalamus, from which a coronal section was cut at the level of the anterior commissure. A slice of the body of the fornix containing the entire SFO was cut by hand. Tissue preparations were postfixed, cryoprotected, and sliced as described previously. The sections were incubated overnight at 4°C with sheep antiserum (8) directed against cGMP in paraformaldehyde-fixed tissue (1:6,000 in 0.5% PBS-T). After they were rinsed, the sections were incubated for 75 min at room temperature with FITC-conjugated donkey anti-sheep immunoglobulins (Dianova). Slides were washed in PBS and mounted with Citifluor. Photographs were taken using Kodak Ektachrome 400 film. The specificity of the NADPH-diaphorase staining for NOS had been confirmed by a previous study using the same protocol (32).

RESULTS

Electrophysiological Study

In total, 97 single-unit recordings were obtained from 64 preparations of the AP, including all studies on the effects of amylin and 8-BrcGMP. The spontaneous discharge rate of the recorded neurons ranged from 0.1 to 14 Hz. Stimulations were performed for ~6 min using 10^{-8} M amylin and 10^{-3} M 8-BrcGMP.

Superfusion with amylin (10^{-9}–10^{-8} M) excited 48% of all neurons tested with this drug (n = 94). The remaining neurons (52%) were insensitive. All amylin-induced responses were reversible; inhibitory effects were not observed. Figure 1 shows a continuous rate-meter recording of a spontaneously active AP neuron that was dose dependently excited by amylin. The average threshold concentration for the excitatory responses was 10^{-10} M (Fig. 1, inset). In the top traces of Fig. 1, representative segments of the original spike recording taken at the end of each amylin application (traces 1 and 2) and during basal activity (trace 3) are illustrated. Compared with our previous studies on SFO neurons (34), AP neurons were 15-fold more sensitive to amylin applied under equal recording conditions, because quantitatively comparable responses were obtained at 15-fold lower amylin concentrations. The average effect parameters of all amylin-mediated responses induced by 10^{-8} M amylin on AP neurons are summarized in Table 1.

In 59 of the 94 recordings with amylin as a stimulus, the recording site was topographically identified. Amylin-sensitive neurons were not distributed evenly throughout the AP. As illustrated in Fig. 2, the per-

![Fig. 1. Continuous ratemeter recording of a spontaneously active neuron from rat area postrema (AP). Consecutive superfusions of amylin at different concentrations caused dose-dependent excitatory effects. Top traces: representative recordings of action potentials obtained during responses to amylin (traces 1 and 2) and after washout (trace 3). Inset: averaged mean excitatory responses of neurons, in which dose-response relationships could be obtained at 10^{-11}–10^{-8} M amylin.](http://ajpregu.physiology.org/DownloadedFrom)
neuronal activity in the AP, the membrane-permeating
tagandist, responsiveness to amylin almost completely
amylin-mediated excitation. After washout of the an-
moderate inhibitory response but strongly reduced the
shows a recording in which AC-187 alone exerted a
agonistic action of amylin was strongly reduced in all
rons without AC-187 (34, 35). To confirm that the amylin-induced excitation was
driven by specific amylin receptors, stimulations were
performed in the presence of the amylin receptor an-
agonist AC-187 (10⁻⁷–10⁻⁶ M), which effectively
blocks the action of amylin on SFO neurons (34, 35). When superfused alone, AC-187 caused inhibitory ef-
fects between -23% and -53% on four of six neurons
tested. Although amylin-induced excitations were not completely abolished by coapplication of AC-187, the
agonistic action of amylin was strongly reduced in all
cases compared with the stimulation of identical neu-
rons without AC-187 (50 ± 7%, n = 6). Figure 3
shows a recording in which AC-187 alone exerted a
moderate inhibitory response but strongly reduced the
amylin-mediated excitation. After washout of the
agonist, responsiveness to amylin almost completely
recovered (Fig. 3).

To disclose a potential role of cGMP formation for the
neuronal activity in the AP, the membrane-permeating
analog 8-BrcGMP (10⁻³ M) was superfused to mimic the
effect of intracellular cGMP formation in AP neu-
rons. 8-BrcGMP exerted reversible excitatory effects
(Fig. 4) on 65% of all neurons tested (n = 17). Data
quantifying the effect parameters are presented in
Table 1. Fourteen of 17 neurons tested with 8-BrcGMP
could additionally be tested with amylin. Amylin excited seven of nine neurons excited by 8-BrcGMP (Ta-
ble 2). Figure 5 displays a recording of an AP neuron
that was activated by 8-BrcGMP and amylin.

**Immunohistological Studies**

**Expression of c-fos.** The electrophysiological results,
demonstrating an excitatory effect of amylin on AP
neurons in vitro, could be confirmed in vivo by detec-
tion of c-fos expression in AP neurons after intraperi-
toneal injection of amylin. Although c-Fos-IR cells were
almost absent in the AP under control conditions, a
significant increase in c-Fos-IR neurons was detected
in the AP after amylin treatment (0.7 ± 0.1 and 44.7 ±
2.1 counts/section for control and amylin, respectively,
P < 0.001; Fig. 6). Analogous to the regional distribu-
tion of amylin-sensitive neurons, the density of posi-
tively labeled cells was higher in the caudal and middle
parts of the AP, whereas in the rostral part, c-Fos-IR
neurons were mainly restricted to the lateral regions
(not shown). Amylin also stimulated a strong c-fos
expression in the NTS (Fig. 6).

In line with the antagonistic action of AC-187 on
amylin-induced neuronal excitation, pretreatment
with AC-187 potently reduced the amylin-stimulated
c-fos expression (Fig. 6). Pretreatment with AC-187
significantly decreased the number of positively la-
beled neurons by 88% (44.7 ± 2.1 and 5.2 ± 0.5 counts/
section for amylin and AC-187 + amylin, respectively,
P < 0.001). Injection of AC-187 alone had no effect on
c-fos expression.

cGMP formation.** A peripheral subcutaneous injec-
tion of amylin (784 µg/kg) stimulated a strong cGMP
formation in cell bodies and fibers of AP neurons,
whereas no cGMP-labeled cells were detected under
control conditions (Fig. 7, top). The average number of
cGMP-IR neurons in the amylin-treated group was 40.8 ±
3.1 counts/section and was thus significantly higher
than in controls (P < 0.001). In contrast to the amylin-
stimulated c-fos expression, positively labeled cell bod-
ies were absent in the NTS region and were exclusively
restricted to the AP. However, cGMP-IR fibers appar-
ently projecting from the AP to the NTS were identi-
fied. The regional distribution of cGMP-IR neurons
throughout the AP matched the distributions of the
amylin-sensitive as well as the c-Fos-IR neurons, be-
cause cGMP-IR cells were preferably located in the
caudal, middle, and rostralateral parts of AP (not
shown).

Pretreatment with the NOS inhibitors L-NMMA (100
mg/kg ip) and L-NAME (50 mg/kg ip) did not affect the

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**Table 1. Effect parameters of excitatory responses on
AP neurons induced by amylin and 8-BrcGMP**

<table>
<thead>
<tr>
<th>Effect Parameter</th>
<th>Amylin (10⁻⁶ M)</th>
<th>8-BrcGMP (10⁻³ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean latency, s</td>
<td>95 ± 12</td>
<td>53 ± 7</td>
</tr>
<tr>
<td>Mean response, %</td>
<td>123 ± 19</td>
<td>42 ± 11</td>
</tr>
<tr>
<td>Mean response, Hz</td>
<td>4.5 ± 0.4</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>Mean peak response, Hz</td>
<td>7.6 ± 0.6</td>
<td>3.1 ± 0.7</td>
</tr>
<tr>
<td>Mean response duration, s</td>
<td>1,277 ± 99</td>
<td>502 ± 60</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 26 for amylin and 11 for 8-bromo-
cGMP (8-BrcGMP).
amylin-stimulated cGMP formation in AP neurons (40.8 ± 3.1, 45.4 ± 7.5, and 43.3 ± 1.4 counts/section for amylin, L-NMMA + amylin, and L-NAME + amylin, respectively), indicating that NO formation was not required in the AP to activate the intracellular production of cGMP. This contrasted with the previously reported essential role of locally released NO as the stimulus for cGMP formation in the SFO (41). In Fig. 7 (bottom), representative sections from animals treated with L-NMMA and L-NMMA + amylin demonstrate that L-NMMA did not stimulate cGMP formation when injected alone and also had no inhibitory effect on the amylin-dependent cGMP formation in the AP.

To confirm the effectiveness of the NOS inhibitors in the present study, their effect on cGMP formation in the SFO was analyzed in the same animals. Peripherally injected amylin significantly stimulated cGMP formation in SFO neurons located in the rostral and middle parts of the SFO (1.1 ± 0.3 and 13.1 ± 1.2 counts/section for control and amylin, respectively, P < 0.001). At the middle level, cGMP-IR cells were restricted to lateral and dorsal regions, forming a rim around the central part of the SFO (Fig. 8, top right). Unlike the NO-independent cGMP formation in the AP, pretreatment of the animals with L-NMMA and L-NAME significantly prevented the amylin-induced cGMP formation (13.1 ± 1.2, 1.7 ± 0.4, and 0.4 ± 0.2 counts/section for amylin, L-NMMA + amylin, and L-NAME + amylin, respectively). L-NMMA and L-NAME did not affect the number of positive cells when injected alone (Fig. 8, bottom). This indicates that the formation of cGMP in the SFO depends on the formation of NO induced by an excitatory stimulus.

To identify the spatial relationship between cGMP-positive neurons and NO-producing cells, double stainings were performed to identify amylin-stimulated cGMP formation and NOS activity (NADPH-diaphorase staining) in identical sections. Consistent with the finding that cGMP formation in the AP is NO independent, only a few diaphorase-positive cells were detected in this structure (Fig. 9). Moreover, there was no indication that cGMP immunoreactivity in the AP might be spatially associated with NADPH-diaphorase activity (Fig. 9, arrowheads). This is in contrast to the SFO, where NOS-positive cells were distributed along with cGMP-IR neurons (Fig. 10, arrowheads), although NOS activity and GMP immunoreactivity did not exist in the same cells.

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Fig. 3. Recording from a neuron of rat AP shows that amylin-induced excitation was strongly reduced in the presence of the receptor antagonist AC-187. After washout, responsiveness to amylin was almost completely recovered. AC-187 moderately decreased the discharge rate when superfused alone.

Fig. 4. Recording from a neuron of rat AP superfused with the membrane-permeating analog 8-bromo-cGMP (8-Br-cGMP), which induced an excitatory and reversible effect.
DISCUSSION

The present electrophysiological studies provide the first evidence that amylin potently activates neurons in the AP, which is considered an essential mediator of the anorectic effect as well as the slowing of gastric emptying induced by this pancreatic hormone. The excitatory effect of amylin was dose dependent and reversible and occurred at a threshold concentration of 10^{-2} M, which is close to physiological plasma concentrations of amylin, which are between 5 and 100 pM (5). The sensitivity of AP neurons to amylin was 15-fold higher than that for neurons located in the SFO (34), another CVO that is equally accessible to blood-borne peptides. These different sensitivities as well as different couplings to intracellular second messenger systems (see below) suggest the involvement of different receptor subtypes in the SFO and the AP. However, if different receptor subtypes were involved, the amylin receptor antagonist AC-187 would bind to both receptor subtypes, since it blocked the amylin-induced excitatory effects in both structures (34, 35).

On the basis of these results, we propose that circulating amylin reduces food intake via an excitatory action on AP neurons located outside the blood-brain barrier. This hypothesis is strongly substantiated by in vivo studies demonstrating that the anorectic effect of peripherally administered amylin is blunted in AP-lesioned rats (23). The role of the AP as a physiological target for circulating amylin is furthermore supported by the topographical correspondence of neuronal amylin responsiveness determined electrophysiologically in vitro and by demonstration of c-fos activation after amylin application in vivo. The distribution of excitatory neurons across the AP found in vitro was similar to that of c-Fos-IR cell nuclei determined immunohistochemically after systemic application of amylin at 5 μg/kg ip. The doses of amylin and its receptor antagonist applied in vivo in the present study to demonstrate their opposing actions on c-fos expression were the same as those used previously for the pharmacological characterization of amylin as an anorectic hormone (24) and, consequently, were not chosen with the intention to estimate the threshold of the amylin action in vivo. The injection of amylin at 5 μg/kg ip represents an ~28-fold higher dose than the ED_{50} for the anorectic effect of amylin reported recently (33) in a study in which amylin was infused intravenously (2.9 pmol·kg^{-1}·min^{-1} for 15 min). The threshold dose of continuously infused amylin was estimated to be 1–3 pmol·kg^{-1}·min^{-1}, which increased the plasma levels of amylin by 9–24 pM (2). These plasma levels of amylin were only slightly above the increase in plasma amylin after a large meal given to rats after 18 h of food deprivation (8 pM).

It was a further objective of the present work to analyze the involvement of intracellular cGMP formation in the effect of amylin on neuronal activity. Special attention was paid to a possible mediation by the messenger molecule NO, which is known to be released after activation of the enzyme NOS (12, 28). As an activator of the soluble guanylyl cyclase, NO may induce cGMP formation and thus alter neuronal activity. In the SFO, different excitatory stimuli (glutamate and ANG II) triggered the release of NO from NOS-containing neurons with the subsequent formation of NO-dependent cGMP, and NO and cGMP exclusively showed inhibitory effects (32). These observations resulted in the hypothesis that excitatory agents in general might exert this action as a protective negative-feedback mechanism against excessive neuronal activation (41). In line with this hypothesis, it was found that calcitonin, a dipsogenic peptide exerting exclusively excita-

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**Table 2. Numbers of AP neurons responsive to amylin and 8-BrcGMP**

<table>
<thead>
<tr>
<th></th>
<th>Amylin</th>
<th></th>
<th></th>
<th>8-BrcGMP</th>
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<tbody>
<tr>
<td></td>
<td>Excited</td>
<td>No effect</td>
<td>Total</td>
<td>Excited</td>
<td>No effect</td>
</tr>
<tr>
<td>8-BrcGMP</td>
<td>7 (50)</td>
<td>2 (14)</td>
<td>9 (64)</td>
<td>7 (50)</td>
<td>2 (14)</td>
</tr>
<tr>
<td>No effect</td>
<td>4 (29)</td>
<td>1 (7)</td>
<td>5 (36)</td>
<td>4 (29)</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Total amylin</td>
<td>11 (79)</td>
<td>3 (21)</td>
<td>14 (100)</td>
<td>11 (79)</td>
<td>3 (21)</td>
</tr>
</tbody>
</table>

Values in parentheses represent percentage of total number of cells tested with both substances.

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**Fig. 5.** Recording from a neuron of rat AP consecutively superfused with the membrane-permeating analog 8-BrcGMP and amylin at effective concentrations. Both substances induced excitatory and reversible effects.

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tory actions on SFO neurons (43), induced an NO-depen-
dent cGMP formation in part of these neurons (40).

The present study further confirms the compatibility
of this hypothesis with the excitatory action of amylin
on SFO neurons by showing that it induced cGMP
production in SFO neurons that could be blocked by
pretreatment with NOS inhibitors. Thus the notion
may be generalized that NO-dependent cGMP forma-
tion is stimulated in the SFO by excitatory peptide
hormones. Consequently, in the SFO, cGMP can be
excluded as the direct second messenger of amylin,
as well as of ANG II and calcitonin, since the consistently
inhibitory actions of NO and cGMP, respectively, con-
trast with the primary excitatory actions of each of
these peptides.

The exclusively excitatory action of amylin on SFO
neurons is most likely mediated by cAMP, which has
been shown to account for various other effects of
amylin (6, 30, 31, 47). Stimulation of cGMP in the SFO
by amylin in an NO-dependent fashion would fit with
the idea of a secondary protective role of cGMP as a
mediator limiting excessive neuronal excitation in this
particular CVO.

Interestingly, in many brain tissues including the
SFO, it appears that two different subsets of neurons,
which are tightly codistributed, form part of the NO-
cGMP signaling cascade. One subset represents nNOS-
containing and NO-producing neurons; the other cells
contain soluble guanylyl cyclase and are thus able to
produce cGMP in response to NO release (7). The
present results obtained by double staining of amylin-
induced cGMP and NADPH-diaphorase activity under-
line the physiological relevance of this spatial relation-
ship. In the SFO, NOS and cGMP were never
colocalized in the same neuron, but NO-producing neu-
rons were located close to those cells that produced
cGMP in response to amylin, suggesting a coupling of
nNOS activation and the subsequent release of NO
with the formation of cGMP in the surrounding cells.
This observation is consistent with the codistribution
of NO-producing and NO-reactive SFO neurons de-
tected by in vitro studies using the NO donor sodium
nitroprusside to induce cGMP formation (32). In these
and other studies (17), the identity of cells positively
labeled for NADPH-diaphorase activity with nNOS-
containing neurons was confirmed immunohistochemi-
cally to validate the use of NADPH-diaphorase activity
as a marker of nNOS.

The present study shows that amylin-induced cGMP
in the AP differs fundamentally from that in the SFO,
both with regard to its mechanism of formation and its
action. In fact, the immunohistological data of this
study show that the amylin-induced cGMP formation
in the AP is completely independent of the NOS sys-
tem, since treatment with the NOS inhibitors L-NMMA
and L-NAME did not affect the formation of this second
messenger. This observation is in line with the low
abundance of nNOS in the AP (1, 17).

A blockade of NO release by NOS inhibitors, as well
as a reversal of this effect by the substrate L-arginine,
has been used in electrophysiological approaches to
characterize a potential influence of NO and subse-
quent cGMP formation on neuronal activity. In line
with our histological observations, the discharge rate
of AP neurons remained unaltered in response to inhibi-
tion of NOS (48), while neuronal activity could be
influenced in the SFO (32) and in other sites where the
NOS system has been proposed to modulate neuronal
function such as the NTS (48) and the spinal cord (42).

Our histochemical evidence of a very low incidence of
nNOS in the AP is consistent with other studies report-
ing a low abundance of NOS in this structure (17). The
distribution of the few NADPH-diaphorase-positive
neurons in the AP was completely unrelated to that of

![Fig. 6. Immunohistochemical staining of 20-μm-thick coronal sec-
tions of rat AP processed for c-Fos after intraperitoneal injection of
saline (control), amylin (5 μg/kg), and amylin after pretreatment
with the amylin receptor antagonist AC-187 (500 μg/kg). Amylin
stimulated strong c-fos expression in the AP/nucleus of the solitary
tract (NTS), which was blocked by AC-187.](http://ajpregu.physiology.org/doi/fig/10.1152/ajpregu.00815.2001)
Fig. 7. Top: immunohistochemical staining of 20-μm-thick coronal sections of rat AP processed for cGMP after subcutaneous injection of saline (control) and amylin (784 μg/kg). Bottom: same treatment described for top after preadministration of the NOS inhibitor N-monomethyl-L-arginine (L-NMMA, 100 mg/kg ip). Amylin stimulated a strong cGMP formation in AP neurons and their fibers. Blockade of NO release by L-NMMA did not affect amylin-induced cGMP formation.

Fig. 8. Top: immunohistochemical staining of 20-μm-thick coronal sections of rat subfornical organ (SFO) processed for cGMP after subcutaneous injection of saline (control) and amylin (784 μg/kg). Bottom: same treatment described for top after preadministration of the NOS inhibitor L-NMMA (100 mg/kg ip). Amylin stimulated a strong cGMP formation in SFO neurons and their fibers. Blockade of NO release by L-NMMA prevented amylin-induced cGMP formation.
the cGMP-IR neurons. Especially, the latter observation suggests that amylin-stimulated cGMP production of AP neurons should be mediated by a guanylyl cyclase that is not identical to the soluble guanylyl cyclase that is activated by NO. Although the structural basis of amylin receptors has recently been revealed by the discovery of receptor activity-modifying proteins (4, 25, 27), a receptor-intrinsic guanylyl cyclase activity, as is known for the atrial natriuretic factor receptor (11) and present in the AP (15, 29), has not been described for the amylin receptor.

In our electrophysiological experiments, 8-BrcGMP consistently excited AP neurons. This contrasts with the exclusively inhibitory effects of this cGMP analog on SFO neurons. Most of those AP neurons that were excited by 8-BrcGMP were also activated by amylin. Together with the immunohistochemical evidence, these studies suggest that cGMP mediates the excitatory effect of amylin in the AP. Mechanistically, cGMP may directly increase neuronal activity and/or affect other second messenger systems such as cAMP by activating cGMP-dependent ion channels or altering the activity of cytoplasmic phosphodiesterases (12, 44).

The distribution of amylin-sensitive AP neurons determined by electrophysiological recordings corresponded closely to that found after in vivo stimulation with amylin for cGMP and c-Fos immunoreactivity in the caudal, middle, and anterior regions of the AP. In addition, however, c-Fos-IR neurons were also found in the NTS, a structure within the blood-brain barrier. Because of the strong axonal interconnections of the AP with the NTS (16, 46, 49), NTS neurons are most likely activated secondarily in vivo after amylin injection. Our most recent observation demonstrating that amylin-induced c-fos expression in the NTS is suppressed in AP-lesioned rats (unpublished) supports this suggestion.

Other brain sites that are part of an ascending pathway conveying information from the brain stem to the forebrain include the lateral parabrachial nucleus, the central nucleus of the amygdala, and the bed nucleus of the stria terminalis. As reported earlier (39), peripheral application of amylin induces a strong c-fos expression in these sites that is absent in AP-lesioned animals. Although not included in our present report, we could confirm these results. This indicates that activation in these brain sites is driven by synaptic interaction and not by a direct action of amylin on neurons located in these nuclei. Thus it appears that neurons of...
the AP are the primary receptive targets for circulating amylin, while the NTS, the lateral parabrachial nucleus, the central nucleus of the amygdala, and the bed nucleus of the stria terminalis may function as important relay and/or integrative centers. This is also suggested by our most recent data indicating that amylin infusion directly into the AP effectively suppresses food intake (unpublished observations). Together with our present finding of a direct excitatory effect of amylin on AP neurons, this observation corroborates earlier studies showing that amylin, in contrast to the satiety effects of bombesin and cholecystokinin, inhibits food intake independently of afferent vagal transmission (10, 18–20, 22, 37).

In summary, our present results provide functional and histological evidence that the anorectic hormone amylin, which is released in response to food intake and is known to suppress feeding via an AP-dependent mechanism, potently activates AP neurons. We propose that the excitatory effect of amylin is the neurophysiological correlate for its anorectic action. We further hypothesize that the amylin-induced activation of AP neurons is driven by direct (NO-independent) intracellular formation of cGMP, since the membrane-permeating analog 8-BrcGMP excited amylin-sensitive neurons in the AP.

**Perspectives**

To further elucidate the neuronal mechanisms that are involved in the amylin-mediated suppression of feeding, future studies should address whether manipulations of intracellular cGMP signaling in the AP (guanylate cyclase inhibitors/stimulators and 8-BrcGMP) affect food intake. Similarly, it would be of interest whether blockade of guanylate cyclase in vitro inhibits the excitatory effect of amylin on AP neurons. Together with immunohistological evidence for the colocalization of c-Fos and cGMP after amylin injection, such studies would help to directly prove our hypothesis that amylin excites AP neurons via formation of cGMP.

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