Actions of amylin on subfornical organ neurons and on drinking behavior in rats

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Riediger, Thomas, Matthias Rauch, and Herbert A. Schmid. Actions of amylin on subfornical organ neurons and on drinking behavior in rats. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R514–R521, 1999.—Amylin, a peptide hormone secreted by pancreatic β-cells after food intake, contributes to metabolic control by regulating nutrient influx into the blood, whereas insulin promotes nutrient efflux and storage. We now report that amylin activates neurons in the subfornical organ (SFO), a structure in which the lack of a functional blood-brain barrier and the presence of a high density of amylin receptors may render it accessible and sensitive to circulating amylin. In an in vitro slice preparation of the rat SFO, 73% of 78 neurons were excited by superfusion with rat amylin (10−8–10−7 M); the remainder were insensitive. The threshold concentration for the excitatory response of amylin was <10−8 M and thus similar in potency to a previously reported excitatory effect of ANG II on the same neurons. The excitatory effect of amylin was completely blocked by coapplication of the selective amylin receptor antagonist AC-187 (10−6–10−5 M) but was not affected by losartan (10−5 M). Subcutaneous injections of 40 nmol of amylin significantly increased water intake in euvhydrated rats, as did an equimolar dose of ANG II, which is a well-described SFO-mediated effect of circulating ANG II. These results point to the SFO as a sensory central nervous target for amylin released systemically in response to metabolic changes. Furthermore, we suggest that amylin release during food intake may stimulate prandial drinking.

AMYLIN is a 37-amino acid peptide that is cosecreted with insulin by pancreatic β-cells in response to food intake (9, 22, 34, 39, 41). The most potent actions of amylin in vivo are inhibition of food intake (6, 7, 28), gastric emptying, stimulated pancreatic enzyme secretion, and stimulated glucagon secretion (12), all pointing to a coordinated role in controlling nutrient influx into the blood (in contrast to the role of insulin, which promotes nutrient efflux and storage). Previous data have shown that amylin also stimulates lactate efflux from muscle primarily by stimulating glycogenolysis (26), which is followed by an increased gluconeogenesis in the liver with the consequent release of glucose into the blood (4, 42, 43).

Recent data indicate that the anorectic and glucagonostatic actions and the inhibitory effect on gastric emptying (41, 44) are centrally mediated. Other central actions of amylin may include effects on memory and pain perception (27, 39). High-affinity binding sites for amylin have been described in many brain areas (8, 36, 38), with particularly high concentrations in sensory circumventricular organs (CVO), brain regions lacking a functional blood-brain barrier (BBB).

Amylin may play a role in fluid and electrolyte homeostasis, as evidenced by a stimulation of plasma renin activity (40) and inhibition of urine production and sodium excretion, but it is yet to be determined whether the responses are mediated via peripheral or central structures. An action of amylin on solute flux in microperfusion experiments (13) and on cAMP production in kidney membranes indicates that a direct peripheral effect is possible.

The aim of our study was to investigate possible effects of amylin on neurons in the subfornical organ (SFO) as a brain region where this peptide might act to influence fluid and electrolyte homeostasis. Because activation of SFO neurons by blood-borne ANG II is believed to represent the cellular basis for the stimulation of water intake by ANG II (14, 15, 24), we compared the effects of ANG II and amylin on the activity of identical SFO neurons in an in vitro slice preparation and the effects of both peptides on water intake in rats in vivo.

MATERIALS AND METHODS

Male adult Wistar rats (170–230 g) were decapitated, and their brains were quickly removed and superfused with ice-cold artificial cerebrospinal fluid (aCSF) of the following composition (in mM): 124 NaCl, 5 KCl, 1.2 NaH2PO4, 1.3 MgSO4, 1.2 CaCl2, 26 NaHCO3, and 10 glucose, equilibrated with 95% O2 and 5% CO2, pH 7.4, 290 mosmol/kg. The brain was trimmed to a square block containing the entire hypothalamus, from which a coronal section was cut by hand at the level of the anterior commissure. A slice of the body of the fornix containing the entire SFO was cut by hand and preincubated in aCSF at 35°C for 2 h before recording. The SFO slice was transferred to the recording chamber and fixed to the bottom of the chamber with a small metal weight. The gold-plated recording chamber was made from solid brass and, when perfused with aCSF, contained a fluid volume of ~0.7 ml. The chamber was constantly perfused with aCSF at a rate of 1.6 ml/min. The aCSF entering the recording chamber was prewarmed to the same temperature as the solution already present in the chamber. The temperature was kept constant at 37.0°C by means of a Peltier element. Extracellular recordings were made from SFO neurons using glass-coated platinum-iridium electrodes. The SFO could easily be identified by its protrusion into the third ventricle and the lateral blood vessels lining the organ on both sides. ANG II and amylin (from Sigma, Deisenhofen, Germany and Amylin Pharmaceuticals, San Diego, CA, respectively) were added to the aCSF shortly before application. Both drugs...
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RESULTS

Electrophysiological study. To allow a direct comparison of amylin and ANG II responsiveness of SFO neurons, only those 78 neurons from 68 SFO preparations that could be successfully tested for their responsiveness to both amylin and ANG II were included in this study. Stimulation was performed for ~6 min using a maximum concentration of 10^{-7} M for both peptides. Superfusion with amylin (10^{-8}–10^{-7} M) excited 73% of all neurons tested (n = 78), and the remaining neurons were insensitive. Amylin, like ANG II, never caused an inhibitory response. Figure 1 shows a continuous ratemeter recording of a spontaneously active rat SFO neuron in which amylin caused a dose-dependent excitatory effect with a threshold concentration of 10^{-9} M. The average threshold concentration for the excitatory responses was between 10^{-9} and 10^{-8} M, as displayed in Fig. 1, inset. ANG II and amylin caused similar excitatory responses when applied in equimolar concentrations to the same neurons (Fig. 2). In the top trace, representative segments of the original spike recording taken at the end of each peptide application and during basal activity are shown. By comparison of the mean effect parameters of all responsive neurons tested with ANG II and amylin in the same concentration (10^{-7} M; n = 14), no significant differences in the mean excitation (2.4 ± 0.3 impulses/s for ANG II vs. 2.4 ± 0.3 impulses/s for amylin), the peak response (3.3 ± 0.3 impulses/s for ANG II vs. 3.6 ± 0.4 impulses/s for amylin; evaluated at a bin width of 30 s), or the onset of the excitation (87 ± 15 s after ANG II vs. 87 ± 13 s after amylin) were detected. Only the mean duration of the amylin-mediated excitation (775 ± 66 s) was significantly longer than the ANG II response (446 ± 34 s, P < 0.001, paired t-test). As summarized in Table 1, a high percentage of neurons (41%, n = 78) were excited by both peptides (10^{-6}–10^{-7} M). Similar to previous studies, 59% of the cells were excited by ANG II and the remainder did not respond.

To confirm that the excitatory action of amylin on SFO neurons is mediated by specific amylin receptors and not due to an amylin-induced increase in SFO-intrinsic ANG II production, we investigated the amylin- and ANG II-induced excitations in the absence and presence of specific antagonists for amylin receptors (AC-187) and ANG II-receptors (losartan, AT_{1}-receptor antagonist).

When superfused alone, AC-187 (10^{-6}–10^{-5} M) exerted no effects on the electrical activity of SFO neurons. In 9 out of 10 neurons, coapplication of amylin and AC-187 in tenfold (n = 6) or 100-fold (n = 3) excess potently blocked the amylin-induced excitations. Only in one case did AC-187 (tenfold excess) not block the amylin-induced excitation, but reduced it significantly. Figure 3 shows an example of a neuron in which AC-187 caused a complete and reversible block of the amylin response. In contrast to AC-187, coapplication of losartan, at a concentration (10^{-8} M) shown to inhibit ANG II-induced responses, was ineffective (n = 3) in
blocking amylin-induced excitations (Fig. 4). Likewise, AC-187 had no effect on the ANG II-induced excitations \((n = 3; \text{Fig. 4})\). In one out of four recordings, superfusion of losartan alone decreased the spontaneous discharge rate, possibly indicating excitatory, angiotensinergic interactions among SFO neurons (30). Drinking experiments. After injection of amylin \((200 \mu l \text{ sc, } 2 \times 10^{-4} M)\), 8 out of 13 rats drank water within the following 60 min (Fig. 5A). Similarly, ANG II \((200 \mu l \text{ sc, } 2 \times 10^{-4} M)\) caused 17 out of 23 rats to drink water within 60 min after injection (Fig. 5B). Only 2 out of 11 control animals receiving injections of saline solution \((200 \mu l)\) consumed water during the observation period (data not shown). The average amount of water consumed by all amylin-treated rats, including those animals that did not drink at all, was 2.1 ± 0.5 ml. This value was not significantly different from the average water consumption of the ANG II-treated animals \((2.5 ± 0.4 ml)\). Both groups of rats drank significantly more water than the control animals receiving saline solution \((0.05 ± 0.03 ml; \text{Fig. 6})\). The course of drinking responses after application of amylin and ANG II was characterized by rapid onset (Fig. 5), with most of the fluid ingested within 30 min after injection. The average time of half-maximal fluid uptake was slightly although significantly shorter \((P < 0.05, \text{Mann-Whitney rank sum test})\) after ANG II injection \((16 ± 2 \text{ min})\) than after amylin injection \((24 ± 4 \text{ min})\).

To exclude the possibility that the amylin-induced water intake was mediated by an increased production of peripheral renin leading to an elevation of ANG II in the blood, we coapplied amylin with the peptidergic and BBB-impermeable ANG II antagonist Sar-Ile and with the nonpeptidergic and BBB-permeable ANG II antagonist losartan. In contrast to losartan, that significantly reduced the fluid uptake in response to amylin to 26%, Sar-Ile had no effect on the average amylin-induced water consumption (Fig. 6). Both antagonists completely blocked ANG II-dependent water intake (Fig. 6).

### Table 1. Numbers of SFO neurons responsive to ANG II and amylin

<table>
<thead>
<tr>
<th>Amylin ((10^{-7} M))</th>
<th>Excited</th>
<th>No effect</th>
<th>Total ANG II</th>
</tr>
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<tbody>
<tr>
<td>ANG II ((10^{-7} M))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excited</td>
<td>32</td>
<td>14</td>
<td>46</td>
</tr>
<tr>
<td>No effect</td>
<td>26</td>
<td>6</td>
<td>32</td>
</tr>
<tr>
<td>Total amylin</td>
<td>58</td>
<td>20</td>
<td>78</td>
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SFO, subfornical organ.

**DISCUSSION**

This study presents the first electrophysiological data describing an effect of the pancreatic polypeptide amylin on neurons in the central nervous system. It shows that amylin excites the majority of neurons in the rat SFO, which are accessible to blood-borne hormones and might therefore be responsible for centrally mediated effects of peripheral amylin. The responses were dose dependent and reversible and could be blocked by the peptidergic amylin receptor antagonist AC-187, a truncated form of salmon calcitonin (36). Thus the amylin-induced excitations were very likely due to the activation of amylin receptors, which have been shown to be highly expressed in the SFO (36).

Although a direct excitatory postsynaptic action of amylin and ANG II on the same neurons has not been shown in this study, such an action is most likely, based on the results of a previous study (35) and the facts that both peptides caused exclusively excitatory effects on the majority of neurons.

The findings that amylin and ANG II had exclusively excitatory effects on the majority of SFO neurons and that blood-borne ANG II is known to stimulate water intake by activating neurons in the SFO (14, 24) led to the hypothesis that peripherally applied amylin might also stimulate water intake in vivo. This could be confirmed by showing that the same dose of amylin and
ANG II increased water intake. Drinking after subcutaneously injected amylin was not mediated by activation of the peripheral renin-angiotensin system (RAS) (40), because blocking of ANG II receptors with an effective concentration of the peptidergic antagonist Sar-Ile had no effect on amylin-induced water intake. The nonpeptidergic ANG II receptor antagonist losartan, however, reduced amylin-induced water intake. This seemingly contradictory result can be explained by the fact that losartan, in contrast to Sar-Ile, is able to cross the BBB in significant amounts (2, 17) and can additionally affect ANG II receptors located inside the BBB. Therefore, we propose that the angiotensinergic pathways that transmit neuronal information from the SFO to the median preoptic nucleus and other hypothalamic and extrahypothalamic nuclei involved in water intake (18, 19) are interrupted by peripherally applied losartan but not by Sar-Ile, which can only act on receptors outside the BBB.

The possibility that amylin might exert its excitatory effects on SFO neurons by activating the SFO-intrinsic RAS, resulting in a local production of ANG II (20, 30), could be excluded by showing that the effects of amylin persisted after blocking of ANG II receptors with

Fig. 2. Continuous ratemeter recording of a spontaneously active neuron from rat SFO. Superfusion of equimolar concentrations of ANG II and amylin for indicated times evoked similar excitatory responses on same neuron. Representative segments of original recordings of action potentials of this neuron in presence of ANG II (1), after washout (2), and in presence of amylin (3) are shown in top trace.

Fig. 3. Recording from a single neuron of rat SFO showing that amylin-induced excitation was completely inhibited by receptor antagonist AC-187. After-washout responsiveness against amylin was fully retained.
ANG II receptors of the AT_1 type, which are known to be responsible for the strong excitatory effect of ANG II on SFO neurons, can be blocked by Sar-Ile as well as losartan (10).

Reported plasma concentrations of circulating amylin under resting conditions in rats are 5–100 pM (9, 39, 44) and are similar to plasma concentrations reported for ANG II in the same species (1–100 pM) (23, 29). Plasma concentrations of amylin are positively correlated with plasma insulin levels and increase two- to threefold after food intake in rats and humans (9). Under pathophysiological conditions, e.g., in diabetic and insulin-resistant rats or in patients with pancreatic tumors, plasma levels for amylin can be elevated up to 600-fold (9, 39). On the basis of the similar plasma concentrations of amylin and ANG II and our findings that equimolar concentrations of both hormones cause thirst and activate neurons in the SFO equipotently, we propose that blood-borne amylin may stimulate water intake by activating SFO neurons (35). Whether or not amylin and calcitonin activate SFO neurons via the same receptors circulating ANG II, suggest that this pancreatic peptide might trigger prandial drinking.

A dipsogenic action of amylin would furthermore complement the recently reported stimulatory effect of amylin on renal water retention and sodium retention (13). That study also suggested that amylin might contribute to renal hypertension by increasing the extracellular fluid volume. Interestingly, an activation of SFO neurons is known to increase blood pressure and stimulate the release of vasopressin from the neurohypophysis (10, 24). Thus amylin and ANG II may act in concert, although via separate receptors, to influence blood pressure and hydromineral balance via the SFO. Opposite effects on blood pressure, however, are observed after acute intravenous applications of high doses of ANG II and amylin (16, 39), whereas subcutaneous application of amylin has no direct effect on blood pressure (45). Possibly relevant for evaluating physiological effects of amylin on blood pressure regulation in the future are data on ANG II, indicating that this hormone is physiologically more relevant for a slowly developing effect on blood pressure than for acute rises (16). On the basis of these considerations, we speculate that elevated plasma levels of amylin might contribute to hypertension often observed in insulin-resistant type II diabetes patients (32).

A dipsogenic effect of peripherally applied amylin was not observed in studies investigating control mechanisms of food intake in rats after central (5) or peripheral application of amylin (1, 6, 21). This seemingly contradictory finding is most likely explained by the strong and long-lasting anorectic effect of amylin and the tightly coupled decrease in water intake, which probably masks a short-term dipsogenic effect of amylin, as observed in our study.

Although receptors for amylin have been described in many areas of the brain (36) and amylin may be able to cross the BBB by an aluminum-sensitive, saturable transport mechanism (3), our data on its dipsogenic effect in vivo favor a direct action of circulating amylin on SFO neurons. This view is supported by the fact that the highest concentrations of amylin receptors in the brain are found in sensory CVOs, notably the SFO (36), and by the comparable effects of amylin and ANG II on neuronal activity and water intake. The presence of immunoreactive amylin in many parts of the central nervous system (37) suggests that amylin might also act as a neuromodulator affecting central nervous structures located inside the BBB. These actions, however, have to be clearly separated from the effects of amylin on SFO neurons, which are accessible to blood-borne hormones.

In a recent study, we showed that calcitonin, a peptide hormone structurally related to amylin that is released from the thyroid gland in response to food intake and high calcium concentrations, also activates ANG II-responsive neurons in the SFO via different receptors and causes an increase in water intake after subcutaneous injection (35). Whether or not amylin and calcitonin activate SFO neurons via the same receptors...
or separate receptors showing a different pharmacology (36) is still an open question. The fact that AC-187 is able to block amylin- as well as calcitonin-induced excitations of SFO neurons suggests a pharmacologically very similar receptor subtype (33). Independent of the involved receptor subtypes, our data showing that the majority of SFO neurons are activated by amylin and calcitonin indicate two possible physiological implications. First, there are other blood-borne hormones besides ANG II that also activate SFO neurons and thus might likewise be important in the regulation of salt and fluid balance. Second, the SFO, besides its well-established function in osmoregulation, might also play a role in calcium homeostasis and glucose regulation, which are physiological processes known to be influenced by these peptides. Although the SFO has additionally been implicated in the control of food intake (25), recent evidence points to the area postrema as the major central nervous target site mediating the anorectic effects of peripherally applied amylin (22).

**Perspectives**

In conclusion, we suggest that neurons in the SFO could be activated by blood-borne amylin, which is elevated under various physiological (e.g., food intake) and pathophysiological (e.g., type II diabetes and insulin resistance) conditions. Although lesion studies are necessary to show that the SFO is indeed responsible for the dipsogenic effect of peripherally applied amylin, the strong excitatory effect of amylin on ANG II-responsive neurons in the SFO qualifies the SFO as the most likely central nervous target for mediating this effect. Neurons in the area postrema are currently studied to identify the cellular mechanism that could explain how blood-borne amylin might reduce food intake and gastric emptying (22, 44) by acting on this brain structure. Activation of neurons in sensory CVOs might be a mechanism by which blood-borne amylin can inform regulatory control centers in the brain about changes in glucose homeostasis that then trigger adequate physiological (e.g., prandial thirst, slowing of gastric emptying), behavioral (e.g., anorectic), or pathophysiological (e.g., diabetes-associated hypertension) responses.

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