Effect of calcitonin on the activity of ANG II-responsive neurons in the rat subfornical organ.

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Schmid, Herbert A., Matthias Rauch, and Julia Koch. Effect of calcitonin on the activity of ANG II-responsive neurons in the rat subfornical organ. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R1646-R1652, 1998.—In addition to the well-documented ability of calcitonin to lower blood calcium levels, blood-borne calcitonin may also affect neurons located outside the blood-brain barrier, e.g., in the subfornical organ (SFO), where numerous receptors for this peptide have been described. In an in vitro preparation of the rat SFO, calcitonin activated 61% of 36 neurons, only 1 neuron was inhibited, and the remainder were unresponsive. All but two of the neurons excited by 10⁻⁷ M calcitonin were also stimulated by 10⁻⁷ M ANG II. The threshold concentration for the excitatory effects of calcitonin was 10⁻⁹ M and was thus similar to ANG II. Like ANG II, subcutaneous injection of calcitonin stimulated water intake, although to a lower extent. These results suggest that blood-borne calcitonin could stimulate drinking by its excitatory effect on neurons in the SFO. Calcitonin, which is released during food intake, might be involved in prandial drinking, which is presently considered an acquired behavior.

drinking; thirst; osmoregulation; electrophysiology; thyroid; angiotensin II

CALCITONIN IS A 32-AMINO ACID peptide hormone that is produced by parafollicular cells of the thyroid gland and released into the general circulation in response to a rise in the plasma level of calcium (2, 3) and also in response to (or even in anticipation of) food intake (20, 26, 27) as a condition in which plasma calcium levels may increase. Calcitonin is predominantly known for its role in body calcium homeostasis, and, together with parathyroid hormone and calciferol, it ensures the tight control of plasma calcium levels. In the periphery it reduces osteoclastic activity, increases the calcium excretion via the kidneys, and slows the motility of the gastrointestinal tract postprandially (3). Calcitonin has not been shown to be involved in body fluid homeostasis, although a connection between calcitonin and the renin-angiotensin system is suggested by data showing that peripheral calcitonin can cause an increase in plasma renin activity; however, this increase was not associated with an increased blood pressure (5).

Central intracerebroventricular applications of calcitonin have been shown to cause analgesia (4), decreased food intake (17), and a decrease in gastric acid secretion and gastrointestinal motility (2, 3). These and other central effects of calcitonin are thought to be due to different types of calcitonin receptors (10) primarily located inside the blood-brain barrier (BBB) that are most likely stimulated by a brain-intrinsic form of calcitonin (22), because peripheral calcitonin does not cross the BBB in significant amounts (6).

In the rat subfornical organ (SFO) and other circumventricular organs, central nervous structures located on the blood side of the BBB, a high density of calcitonin receptors was demonstrated by in vivo or in vitro receptor autoradiography (10, 21, 30), suggesting that blood-borne calcitonin might indeed act on neurons in the central nervous system. However, nothing is known so far about the possible physiological functions of these receptors, which are accessible to blood-borne calcitonin.

One of the best-investigated functions of the SFO is the increase in water intake in response to elevated plasma levels of ANG II (13). It has been amply documented that blood-borne ANG II can reach and activate neurons in the SFO via the open BBB by acting on ANG II receptors of the AT₁ type that are abundant in the SFO and thus stimulate drinking (7, 11, 13).

The aim of this study was to obtain a first characterization of the effects of calcitonin on the activity of neurons in a circumventricular organ using an in vitro slice preparation of the SFO. The well-described SFO-mediated dipsogenic action of ANG II allowed a first assessment of the possible physiological function of blood-borne calcitonin acting on brain structures by comparison of the actions of peripherally applied calcitonin and ANG II on water intake.

MATERIALS AND METHODS

Male adult Wistar rats (180–200 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% O₂-5% CO₂, pH 7.4, at 290 mosmol/kg. The brain was trimmed 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 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 rat calcitonin (both from Sigma, Deisenhofen, Germany) were added to the aCSF shortly before application. Both drugs were stored in frozen aliquots (~−24°C) and, during an experiment, were kept on ice.
until use. After a stable recording from a single neuron had been established, its responsiveness was tested by switching to a perfusion solution containing the drug under consideration. The recorded action potentials were amplified and displayed on a storage oscilloscope (Gould) and were, after passing a window discriminator (World Precision Instruments, New Haven, CT), analyzed with custom-made software (Spike2 from Cambridge Electronic Design, Cambridge, UK) on a personal computer.

In general, 10 ml of aCSF containing calcitonin or ANG II were superfused per stimulus. The concentration of ANG II (10^{-7} M) was chosen according to previous experiments that showed that this concentration induced clearly visible responses with minimum desensitization. In the experiments investigating possible interferences of calcitonin with the central renin-angiotensin system, the converting enzyme inhibitor captopril (10^{-5}–10^{-4} M, Sigma) or the ANG II receptor antagonist losartan (10^{-6}–10^{-5} M; a gift from Merck Sharp & Dohme, West Point, PA) was coapplied with an effective dose of calcitonin. From the continuously recorded ratemeter counts, the average discharge rate of each neuron was evaluated for 60 s before the stimulus. This value (referred to as “control”) was subtracted from all subsequent changes in firing rate, and the results were expressed in “%change of control.” If the averaged change of discharge rate during the entire response time was larger than ±20%, the neuron was considered sensitive to the applied substance. Furthermore, the effects of both agents had to be reversible to be included in this study to avoid possible false positive responses. To induce synaptic blockade, the slice was perfused with the low-Ca^{2+} (0.3 mM), high-Mg^{2+} (9.0 mM) aCSF for at least 10 min before the agonist superfusion began, and superfusion was maintained for at least 5 min after the agonist infusion had been stopped.

Water intake was investigated in male Wistar rats (180–200 g) using an automated system (Omnitech, Columbus, OH) that monitored locomotor activity and food and water intake one time every minute and stored data directly on a computer using Integra software (Omnitech). Animals were adapted to the cages (40 × 40 cm) for at least 12 h before the experiment started, and their food and water intake as well as their locomotor activity was continuously recorded. Rats were fed on ground rat chow. Grinding of the chow was necessary for precise registration of food intake, because it prevented the removing of pellets from the food container that rested on an electric balance. Water bottles were fixed upside down on a holder that was mounted on an electric balance (sensitivity 0.1 g) and were connected via tubing to a drinking spout. Access to food was blocked 1 h before the experiment started to avoid interference with prandial drinking. All drugs were dissolved in sterile saline and were injected subcutaneously (200 µl per rat) with small syringes (Omnican, 29 gauge, 0.5 ml; Braun, Melsungen, Germany) at the end of the activity phase (9–10 AM). ANG II was injected in a known effective concentration (0.2 mg/kg, i.e., 2 × 10^{-8} M per rat), and a similar concentration was used for calcitonin (1.4 mg/kg, i.e., 4 × 10^{-8} M per rat). In the experiments investigating possible interferences of calcitonin with the renin-angiotensin system, 200 µl losartan (30 mg/kg, i.e., 6.5 × 10^{-2} M per rat) was subcutaneously injected 30 min before the injection of calcitonin or saline.

Various plasma parameters were determined from the blood of eight male rats. The animals were decapitated 60 min after the injection of calcitonin (200 µl, 4 × 10^{-8} M), and the plasma was separated by rapid centrifugation for 10 min, with 4,000 U/min, at 4°C (Sigma centrifuge) in heparinized (heparin, 20 µl/ml blood; Promonta, Hamburg, Germany) tubes. Total plasma calcium was determined with a flame photometer (Eppendorf FCM 6341), and plasma osmolality was measured with an osmometer (Westor 5500, Logan, UT). Mean values in the text are given with the standard error of the mean. Statistical significance was evaluated using Mann-Whitney rank sum test (Sigma Stat), and differences were regarded as significant with P < 0.05.

RESULTS

Electrophysiological study. In this study, only those neurons were included that could be tested for their responsiveness to ANG II as well as calcitonin, both peptides being superfused in the same concentrations and usually for the same time (6 min). Superfusion of calcitonin at 10^{-7} M caused an excitation in 61% (n = 36) of all neurons tested, only 1 neuron (3%) was inhibited, and the remainder were unresponsive. Cells recorded from the central medial portion of the SFO were activated by calcitonin with a similar frequency (12 out of 17 neurons tested) as were cells recorded from the central lateral portions (8 out of 15 neurons tested).

Figure 1 shows a continuous ratemeter recording of a spontaneously active rat SFO neuron in which superfusion of calcitonin caused an excitatory effect that was dose dependent with a threshold concentration <10^{-8} M. The inset of Fig. 1 displays the averaged dose response relationship of five neurons (tested with the identical protocol as the original recording in Fig. 1) examined in a range between 10^{-11} and 10^{-7} M, indicating 10^{-9}–10^{-8} M as the average threshold concentration for the calcitonin-induced excitation. The similarity of the effects of calcitonin and ANG II is shown in the example in Fig. 2, which is a continuous ratemeter recording of an SFO neuron in which superfusion with identical concentrations of ANG II and calcitonin caused excitatory responses of similar magnitude, although in this case with a slightly shorter response to ANG II compared with calcitonin, which is due to a slightly smaller (7 ml instead of the usual 10 ml) application volume of the drug. Representative segments of the original spike recordings are shown in the top trace of Fig. 2, in the presence and absence of both peptides. The average signal-to-noise ratio under our recording conditions was 13:1 (n = 36). Comparing the excitatory responses of those neurons on which ANG II and calcitonin had been applied for the same time and in the same concentration (10^{-7} M), we found that the mean ANG II-induced excitation was 2.8 ± 0.3 impulses/s and thus not different from the calcitonin-induced excitation of 2.8 ± 0.3 impulses/s (n = 16). Similarly, no difference could be observed in the peak (bin width 30 s) response (4.6 ± 0.5 impulses/s for ANG II vs. 4.1 ± 0.4 impulses/s for calcitonin) and the onset of the excitatory effect (64 ± 10 s after ANG II vs. 70 ± 8 s after calcitonin). None of these effects were statistically different from each other except that the duration of the calcitonin response (759 ± 45 s) was significantly longer (P < 0.01, paired t-test) than the response of ANG II (542 ± 37 s). As shown in Table 1, all but two of the neurons that were excited by calcitonin could also be excited by superfusion with ANG II at the same
concentration (10^{-7} M). Superfusion of ANG II (10^{-7} M) caused an excitation of 83% of all cells tested (n = 36), and the remainder were unresponsive.

To exclude the possibility that the strong excitatory effect of calcitonin is caused by a local, SFO-intrinsic production of ANG II, possibly by stimulating the central renin-angiotensin system, the effect of calcitonin on SFO neurons was investigated after blocking the angiotensin-converting enzyme with captopril or after blocking the AT_{1} receptors with losartan.

Superfusion of losartan (10^{-6} M) alone had either no effect (see example in Fig. 3) or caused a small inhibitory effect (-26 ± 2%) on the spontaneous activity of 6 out of 13 SFO neurons, possibly indicating angiotensinergic, excitatory interactions among neurons in the rat SFO. However, this dose of losartan did not affect (inhibit) the calcitonin-induced excitation (Fig. 3), although in this concentration it completely abolished the ANG II-induced excitation in each of four neurons tested. Superfusion with captopril (10^{-5} M), which effectively blocked ANG I-induced excitation at this concentration (not shown), had no effect on the calcitonin-induced excitation. Superfusion with captopril alone at this concentration resulted in a small inhibitory effect (-6 ± 2%) in three out of nine neurons, and the remaining cells were not affected.
The excitatory effect of calcitonin could still be evoked after superfusion of six responsive neurons with a solution containing 0.3 mM Ca\(^{2+}\) and 9.0 mM Mg\(^{2+}\), which is known to block synaptic transmission. Superfusion of the six neurons with this blocking solution increased the spontaneous activity in four neurons, decreased it in one, and did not change it in one other neuron. Irrespective of the change in baseline activity, calcitonin was still able to increase the activity (1.8 ± 0.1 impulses/s before synaptic blockade) in each of the neurons tested (2.9 ± 0.8 impulses/s during synaptic blockade). The example in Fig. 4 shows a spontaneously active neuron in which blocking solution increased the baseline activity and induced a burstlike firing pattern. Despite these changes in spontaneous activity, calcitonin was still able to cause a reversible activation of this neuron during superfusion with blocking solution.

Drinking experiments. Subcutaneous injection of calcitonin (4 × 10\(^{-4}\) M, 200 µl) caused 12 out of 19 rats to drink water within the 2-h time period after the injection (Fig. 5A). Similarly, injection of ANG II (2 × 10\(^{-4}\) M, 200 µl) caused 16 out of 20 rats to drink water (Fig. 5B). Only 6 out of 33 rats that received a subcutaneous injection of 200 µl of saline consumed water within the observation period (not shown). The average amount of water consumed by all rats, including those that did not drink at all, was 0.8 ± 0.2 ml 2 h after calcitonin and 2.8 ± 0.4 ml 2 h after ANG II, and thus was significantly different from the control rats, which drank 0.09 ± 0.05 ml (Fig. 6). The onset of the drinking response was more rapid after the injection of ANG II compared with calcitonin, resulting in a highly significant water intake already after 30 min. The water intake in response to calcitonin was significant only after 60 min compared with the controls, which received the same amount of isotonic saline at the same time. Injection of calcitonin together with ANG II in the indicated concentrations resulted in an increase in water intake after 120 min (3.5 ± 0.8 ml; n = 8) that was not significantly higher than the water intake seen after ANG II alone (Fig. 6).

To exclude the possibility that the dipsogenic action of calcitonin was mediated by an increased production of peripheral renin, leading to an elevation of plasma levels of ANG II, we investigated the dipsogenic action of calcitonin in the presence of losartan applied at a concentration (6.5 × 10\(^{-2}\) M) that is known to abolish ANG II-induced drinking in rats (Fig. 6). Pretreating rats with the same amount of losartan 30 min before the subcutaneous injection of calcitonin did not prevent the dipsogenic action of calcitonin; the observed water intake of 1.0 ± 0.4 ml after 2 h was significantly different from the control value.

Calcitonin effects on plasma calcium and osmotic concentration. The total calcium concentration in the blood plasma of eight rats treated with calcitonin (200 µl, 4 × 10\(^{-4}\) M) was 2.3 ± 0.1 mM 60 min after the injection and, thus, was significantly lower (\(P < 0.001\)) in comparison with the control level of 2.9 ± 0.1 mM determined in eight rats that had received the same volume of vehicle. In the same two groups of animals, those treated with calcitonin revealed a significantly reduced plasma osmolality of 285 ± 1 mosmol/kg in comparison to 289 ± 1 mosmol/kg determined in the controls (\(P < 0.05\)). The injection of losartan (200 µl, 6.5 × 10\(^{-2}\) M) caused no change in plasma calcium levels (3.0 ± 0.1 mM, n = 4) and did not antagonize the reduction in plasma calcium (2.3 ± 0.1 mM; n = 4) caused by calcitonin injections (200 µl, 4 × 10\(^{-4}\) M) when administered 30 min before (\(P < 0.05\)).

Table 1. Numbers of neurons in the subfornical organ responsive to ANG II and calcitonin

<table>
<thead>
<tr>
<th>Calcitonin (10(^{-7}) M)</th>
<th>ANG II (10(^{-7}) M)</th>
<th>Excited</th>
<th>No effect</th>
<th>Inhibited</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>9</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Excited</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Inhibited</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Total calcitonin</td>
<td>22</td>
<td>13</td>
<td>1</td>
<td>36</td>
</tr>
</tbody>
</table>

Fig. 3. Recording from a single neuron of rat SFO showing that calcitonin-induced excitation was not inhibited by doses of losartan and captopril, which have been shown to block the excitatory effects of ANG II and ANG I effectively.
DISCUSSION

This study shows that calcitonin has a strong excitatory effect on the majority of neurons in the SFO of rats. The excitatory effect of calcitonin was dose dependent and reversible and was primarily observed on SFO neurons that could in addition be activated by ANG II. The similarity of the effects of ANG II and calcitonin on largely identical neurons in the rat SFO led to the prediction that peripheral calcitonin, like ANG II, should also increase water intake. This hypothesis could be confirmed experimentally by showing that subcutaneously applied calcitonin caused a significantly increased water intake in water-sated rats. These results suggest that blood-borne calcitonin may directly affect central neurons and that the physiologi-
rical functions should be similar to the functions of ANG II on SFO-mediated effects.

The SFO-mediated water intake in response to ANG II and the excitatory effect on SFO neurons have been shown to be mediated by the AT₁-receptor subtype (7, 11). The persistence of the excitatory action of calcitonin on SFO neurons in vitro, when ANG II effects had been abolished by an effective dose of the AT₁ antagonist losartan or by the converting-enzyme inhibitor captopril, indicates that the neuronal effect of calcitonin was not mediated in one way or another by ANG II. Peripheral application of calcitonin and of the related peptide amylin has been shown to enhance plasma renin activity in rats and humans without affecting the blood pressure (5). However, for the present study, the presumption that calcitonin effects, secondary to the stimulation of renin release and subsequently enhanced formation of ANG II, might account alone for its dipsogenic action is also excluded by the persistence of the calcitonin-induced drinking after AT₁ receptor blockade with losartan.

Plasma levels of calcitonin in rats (and humans) under control conditions are 40–100 pg/ml (i.e., 1–3 × 10^{-11} M) (3, 18–20) and are thus similar to the plasma levels of ANG II in the same species (1–10 × 10^{-11} M) (12, 15). The plasma levels of calcitonin are positively correlated with the Ca^{2+} concentration in the plasma, differ with age and sex of the animals, and increase in response to several physiological factors such as pregnancy and lactation (3) and notably with (and even in anticipation of) food intake, which has been shown to increase plasma levels of calcitonin to 600–1,000 pg/ml (i.e., 2–3 × 10^{-10} M) (20, 27).

On the basis of the similarities in plasma concentrations of both peptides and their similar effect on neuronal activity in vitro, it can be speculated that both peptides should cause similar SFO-mediated effects in vivo. The data of the drinking studies indicate at least the possibility that blood-borne calcitonin might act on the SFO to stimulate water intake, although it might also, or even primarily, be involved in other SFO-mediated functions, like salt or calcium appetite or release of arginine vasopressin.

The smaller and, compared with ANG II, delayed drinking response might partly be explained by a slightly higher sensitivity of rat SFO neurons to equimolar doses of ANG II under identical conditions (Rauch and Schmid, unpublished observations) and by a possible direct renal effect of calcitonin, leading to an increased excretion of calcium, sodium, potassium, and chloride ions (1, 3) (unpublished observations), which in summary cause a significant decrease in plasma osmolality, which might diminish the SFO-mediated dipsogenic effect of calcitonin by inhibiting osmosensory structures.

A reduction in food intake is a well-established response to centrally as well as peripherally applied calcitonin (8, 29). The reduction in water intake observed after central applications of calcitonin intracerebroventricularly or into the lateral hypothalamus (25, 29) is presumably a consequence of the reduced food intake (17). Electrophysiological studies investigating the effect of calcitonin on the electrical activity of thalamic and hypothalamic neurons revealed primarily inhibitory effects on neuronal activity (4, 9, 25, 28). The strong inhibitory effect on the electrical activity of neurons in these studies is in sharp contrast to the almost exclusively excitatory effect of calcitonin on SFO neurons in our experiments and adds to the specificity of the observed effect. In other words, centrally (icv or intrahypothalamic), unlike peripherally, applied calcitonin should not cause the same stimulatory effect on water intake as centrally applied ANG II, which is known to stimulate many hypothalamic neurons located inside the BBB and possibly mediating dipsogenic responses (11, 13). Furthermore, it has been shown that peripherally applied calcitonin does not cross the BBB in significant amounts (6) and that the physiologically relevant ligand for calcitonin receptors located inside the BBB might be different from the calcitonin that is released from the thyroid and that circulates in the blood (14, 22). All these pieces of evidence taken together suggest that the newly described SFO-mediated effects of calcitonin should clearly be separated from the so-far-known “central” effects of calcitonin (3).

Receptors for calcitonin have been described in various regions of the rat brain (16), with particularly high concentrations in circumventricular organs (30) including the SFO, which showed strong labeling after intravenous application of radioiodinated calcitonin (21). The calcitonin receptors in the SFO showed no cross-reactivity with ANG II (21), ACTH, and parathyroid hormone (30), thus underlining the specificity of these receptors. Recent autoradiographic and molecular cloning studies characterized three different receptor subtypes (C1a, C1b, and C3) of calcitonin or calcitonin-like receptors in the rat brain, each subtype with a slightly different distribution pattern in the rat brain (10, 23, 24).

Despite the fact that so far no receptor-specific agonists or antagonists are available for each of these (and possibly other and as yet unidentified) receptor subtypes, all three of these and other related receptor subtypes are expressed in the SFO in high quantities (10, 24). However, it is of minor importance for the physiological relevance of the action of calcitonin in the SFO which receptor subtype is primarily responsible for the almost exclusively excitatory neuronal response. Physiologically it is also of minor importance for the described SFO-mediated effects whether the brain-intrinsic ligand for calcitonin receptors is actually calcitonin or a structurally related peptide (22), because the calcitonin receptors located in the SFO are definitely accessible to blood-borne calcitonin which originates mainly from the thyroid gland.

Perspectives

In summary, it can be concluded that physiological conditions that cause a significant rise in plasma levels of calcitonin, e.g., food intake and elevated levels of calcium, during pregnancy and lactation, when there is a strong calcium demand (3), should result in an activation of neurons in the SFO, and thus it may cause
thirst and possibly other SFO-mediated responses like salt appetite or release of vasopressin. It is tempting to speculate that prandial drinking, which is so far regarded to be a learned behavior, might actually be caused by circulating peptides. Blood-borne calcitonin might act on structures outside the BBB to cause a reduction in food intake or an altered appetite for calcium-containing food. These important questions have to be addressed in future physiological experiments, and the specific brain site of action for circulating calcitonin has to be characterized by direct microapplication and ablation studies.

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