Lateral hypothalamic injection of GABA_A antagonist induces gastric vagus-mediated hypocalcemia in the rat

KATSUHIKO SHIRAMINE, SHUJI AOU, AND TETSURO HORI
Department of Physiology, Faculty of Medicine, Kyushu University 60, Fukuoka 812–82, Japan

Shiramine, Katsuhiro, Shuji Aou, and Tetsuro Hori. Lateral hypothalamic injection of GABA_A antagonist induces gastric vagus-mediated hypocalcemia in the rat. Am. J. Physiol. 273 (Regulatory Integrative Comp. Physiol. 42): R1492–R1500, 1997.—The involvement of lateral hypothalamic area (LHA) neurons in the regulation of blood calcium homeostasis was investigated in unanesthetized rats. The microinjection of the γ-aminobutyric acid A receptor antagonist bicuculline methiodide (BM, 4–40 ng 0.5 μl1.5 min) into the LHA decreased the blood concentration of ionized calcium. Total serum calcium also decreased after the BM injection. This hypocalcemic effect was eliminated by a bilateral vagotomy of the gastric branches. An intraventricular injection of atropine methyl bromide (a muscarinic antagonist), nadolol (a β-adrenergic blocker), or ranitidine (a histamine H2 blocker) suppressed the BM-induced hypocalcemia, whereas phenoxybenzamine (an α-adrenergic blocker) proved to be ineffective. Although the intralateral injection of BM increased the serum gastrin, which is known to have a hypocalcemic effect, neither secretin nor somatostatin (gastrin-release inhibitors) blocked the hypocalcemic response. These results suggest that the hypocalcemia observed after the excitation of LHA neurons was mediated by muscarinic, β-adrenergic, and histamine H2 receptors through the gastric vagus.

calcium homeostasis; stomach; histamine

ALTHOUGH CALCIUM HOMEOSTASIS is well known to be regulated by calciotropic hormones acting peripherally on bone, kidney, and intestine, little is yet known about its central regulation mechanisms. It has recently been shown that calcitonin and parathyroid hormone (PTH) alter the activity of hypothalamic neurons through their actions on respective receptors (15, 26). Whereas an intracerebroventricular injection of calcitonin reduces blood calcium levels (6), an injection of PTH reverses the urethan-induced hypocalcemia in a dose-dependent manner (15). Moreover, the electrical stimulation of the lateral hypothalamic area (LHA), paraventricular nucleus (PVN), or ventromedial nucleus of the hypothalamus (VMH) decreases the concentration of blood calcium in anesthetized rats (1, 13). These findings, taken together, suggest that the hypothalamus is involved in the regulation of calcium homeostasis.

Furthermore, we found that the calcium-lowering effects of the electrical stimulation of the LHA and VMH were specifically blocked by a bilateral gastric vagotomy, whereas PVN stimulation-induced hypocalcemia was abolished by a vagotomy of the thyroid-parathyroid branches (13). The immobilization stress-induced hypocalcemia was thus eliminated by VMH lesions (1, 2) as well as by a gastric vagotomy but not by a vagotomy of the thyroid-parathyroid branches (14). In addition, pretreatment with a muscarinic antagonist (atropine), inhibitors of gastric release (secretin and galanin), or antagonists of histamine H2 receptor (cimetidine and ranitidine) also blocked the stress-induced hypocalcemia (3, 14). Moreover, not only a gastrectomy but also a fundectomy or antrectomy abolished the hypocalcemic effect of immobilization (3). These findings suggest that the vagally mediated releases of gastrin and histamine in the stomach may therefore participate in the calcium-lowering process during immobilization stress.

Although hypocalcemia is observed after the electrical stimulation of LHA in the anesthetized rat (13), it remains to be determined whether hypocalcemia is induced in an unanesthetized condition either by the activation of LHA neurons or by stimulation of nerve fibers passing through the site. Using both pharmacological and surgical treatments, we undertook the present study 1) to elucidate whether or not the excitation of LHA neurons decreases the blood calcium concentration and 2) to investigate its peripheral mechanisms in awake and freely behaving rats.

MATERIALS AND METHODS

Animals. Female Wistar rats weighing 210–270 g were used because female rats have been shown to be more sensitive than male rats to attempts to alter the blood calcium in experimental manipulations (16). The rats were housed in groups of three or four in our closed colony and were maintained on a 12:12-h light-dark cycle (light period, 0800–2000) in a constant air-conditioned environment of 23 ± 1°C and 60 ± 10% relative humidity. They had free access to laboratory chow and tap water.

Surgery. The animals were anesthetized with pentobarbital sodium (30–50 mg/kg ip) and placed in a stereotaxic apparatus. A stainless steel guide cannula (0.5 mm OD) was unilaterally (left side) implanted into a position 1 mm above the LHA (anterior-posterior 6.20, lateral 1.8, depth 8.5–9.0) according to the atlas of Paxinos and Watson (19). The stainless steel cannula was anchored firmly to screws in the skull by dental cement. Thereafter, some animals underwent a selective vagotomy 7 days after implantation of the cannula. While rats were under pentobarbital sodium anesthesia (30–50 mg/kg ip), the vagal nerves were cut bilaterally at either the gastric, celiac, or hepatic branches. In the sham-operated rats, the vagal nerves were exposed at the respective sites but were not cut. At least 5 days were allowed for postsurgical recovery. Subsequently, 2 days before the experiment, while rats were under ketamine anesthesia (25 mg/kg), a Silastic catheter (1.0 mm OD, 0.5 mm ID) filled with heparinized (20 IU/ml, Ciba Corning) physiological saline was inserted into either the right jugular vein or the right femoral artery for sampling of blood.

Hypothalamic injection and measurements of blood ionized calcium and pH and arterial blood pH, PCO2, and PO2. All experiments were done with the rats in a freely moving condition. The rats were fasted overnight (with only water available). A stainless steel injection cannula filled with bicuculline methiodide (BM, 4–40 ng) or Ringer solution was
inserted through the guide cannula and placed in a position 1 mm beyond its tip. Because an injection of glutamate (0.05–0.5 M in concentration, 0.5 µl in volume) did not elicit hypocalcemia (our unpublished observation), we used BM to activate LHA neurons with inhibition of γ-aminobutyric acidergic inputs. After 120–150 min, the drug in a fluid volume of 0.5 µl was administrated into the LHA slowly over a 5-min period. We intended to use a relatively large volume (0.5 µl) to cover the entire LHA at the level of VMH to avoid inconsistent responsiveness, as previously reported (24).

Two baseline blood samples (0.15 ml each) were collected 30 min and just before the LHA injection of drugs. If the difference between these two baseline values of the blood concentration of ionized calcium was >3.5%, an additional baseline blood sample was then collected after another 30-min interval. If the baseline levels were still unstable, no further blood sampling was performed. After we confirmed stable basal calcium levels, the blood samples were collected 15, 30, 60, 90, and 120 min after the injection. The concentration of the ionized calcium and pH in the whole blood was measured by ion-selective electrodes (643 Ca²⁺/pH Analyzer, Ciba Corning). Po₂, PCO₂, and pH of arterial blood (0.25 ml each) were measured by a pH-blood gas analyzer (238, Ciba Corning). Each measurement was duplicated, and the average value was used for the data analysis.

Measurements of serum total calcium, blood sodium, potassium, chloride, and serum gastrin. In a separate series of experiments, a 5-ml blood sample was collected 30 min after the LHA administration of either BM or Ringer solution. The concentration of sodium, potassium, and chloride in the whole blood was measured by ion-selective electrodes (644 Na/K/Cl analyzer, Ciba Corning). The concentration of total calcium and magnesium in the serum was then measured by a calcium-magnesium meter (EDTA titration method, Joko). The concentration of serum gastrin was then determined by a radioimmunoassay (Gastrin RIA kit II, Dinabot) with use of blood samples taken from the catheter 15 min after the LHA injection of either BM or Ringer solution. Each measurement was done either done two or three times, and the average value was then used for the data analysis.

Pharmacological treatment. Various pharmacological manipulations were made to identify the peripheral mechanisms of the central BM-induced hypocalcemia. Atropine methyl bromide (0.1 and 0.6 mg/kg, Sigma, St. Louis, MO) and secretin (6 µg/kg, Peptide Institute) were injected intravenously 5 min before BM injection. Phenoxybenzamine (PBZ, 3 mg/kg, Tokyo Chemical), nadolol (2 mg/kg, Sigma), and ranitidine (5 mg/kg, Sigma) were administered intravenously 20 min before the BM injection. These drugs were dissolved in Ringer solution before use and were injected in a fluid volume of 0.1 ml (atropine, ranitidine, and secretin) or 0.2 ml (PBZ and nadolol). Somatostatin (1 µg·h⁻¹·rat⁻¹, Sigma) was dissolved in physiological saline and was then continuously administered for 25 min by intravenous drip infusion starting 5 min before the BM injection.

Histology. At the end of the experiments, 0.5 µl of Pontamine sky blue was injected through the LHA cannula. The rats were deeply anesthetized with an overdose of pentobarbital sodium (60 mg/kg ip) and were transcardially perfused with 10% Formalin. The brain was cut into 120-µm serial frozen sections by a microtome and was stained with Neutral red to identify the site of injection histologically. Only the data obtained from the animals that were found to have the tip of the cannula positioned correctly in the LHA were used for the analyses.

Statistics. Results are expressed as means ± SE. The changes in either the blood calcium or pH levels were calculated by subtracting the blood calcium or pH value just before the injection of BM or vehicle (time 0) from the values observed after injection. Either the unpaired Student's t-test or the one-way or two-way analysis of variance (ANOVA) with post hoc Bonferroni test was used for the statistical analyses. P values <0.05 were considered statistically significant.

RESULTS

Changes in blood levels of ionized calcium and pH after BM injection into LHA. The basal blood concentration of ionized calcium of the awake rat 30 min before and just before the intra-LHA injection of BM or vehicle ranged from 1.30 to 1.45 mM. There was no significant difference in basal level (time 0) among the vehicle-injected control group (1.39 ± 0.01 mM, n = 5) and the BM-injected groups (4, 12, 30, and 40 ng: 1.37 ± 0.01, 1.40 ± 0.004, 1.40 ± 0.02, and 1.37 ± 0.02 mM, respectively; n = 4 or 5). Although intra-LHA injection of 4 ng BM did not affect the concentration of blood ionized calcium, the injection of 12, 30, and 40 ng of BM significantly decreased calcium concentration in a dose-dependent manner [F(4,90) = 3.69, P < 0.001, 2-way ANOVA; Fig. 1A]. The blood calcium level significantly decreased 15 min after the start of the BM injection (12–40 ng) [F(4,18) = 5.05, P < 0.01, 1-way ANOVA, in comparison with that of the vehicle-injected control.
group], and then the decrease reached a peak 30 min after injection \( [F(4,18) = 12.45, P < 0.001, 1\text{-way ANOVA}] \). Blood calcium returned to the basal level within 60 min.

In association with the hypocalcemia, the blood pH level increased dose dependently after the BM injection \( [F(4,90) = 5.51, P < 0.001; \text{Fig. 1C}] \). The blood pH level reached a peak 15 min after the start of the BM injection \( [F(4,18) = 12.21, P < 0.001, 1\text{-way ANOVA}] \). The increase remained at statistically significant levels 30 min after the injection \( [F(4,18) = 11.95, P < 0.001, 1\text{-way ANOVA}] \) and then returned to the basal level 60 min after the injection.

Changes in arterial blood pH, PCO2, and PO2 after BM injection into LHA. Because the blood concentration of ionized calcium is known to be affected by blood pH, we subsequently sought a theoretical basis for the adjustment of the calcium data for pH 7.4. To determine the nature of the BM injection-induced alkalosis, we measured pH, PO2, and PCO2 of the arterial blood of the BM (30 ng)- and vehicle-injected groups. The arterial blood pH and PCO2 of the BM-injected group were significantly different from those of the vehicle-injected rats \( [\text{pH}, F(1,50) = 11.06, P < 0.001; \text{PCO}_2, F(1,50) = 5.22, P < 0.001; 2\text{-way ANOVA}; \text{Fig. 2A}] \). Fifteen minutes after the BM injection, the arterial blood pH increased significantly to 7.54 \pm 0.01 Torr \( (P < 0.05) \), and PCO2 decreased significantly to 27.86 \pm 0.91 Torr \( (P < 0.05) \) in comparison with the values of the vehicle-injected group \( (\text{pH}, 7.48 \pm 0.02; \text{PCO}_2, 34.75 \pm 1.89 \text{Torr}) \).

Arterial blood pH and PCO2 returned to the basal levels 30 min after the injection. When PO2 and pH of the BM-injected group at 0, 15, and 30 min after BM injection were plotted on the acid-base chart (29), the values at 15 and 30 min were within the area of acute hypocapnia, indicating respiratory alkalosis (Fig. 2B).

There was no significant difference in the arterial blood PCO2 between the BM- and vehicle-injected groups (data not shown).

Changes in blood level of ionized calcium adjusted for pH 7.4, total calcium level, and other blood electrolytes after BM injection into LHA. In the case of respiratory alkalosis, the blood Ca2+ levels can be well adjusted for pH to evaluate pH-independent changes in blood Ca2+ with use of the following equation (5)

\[
\text{adjusted } [\text{Ca}^{2+}] = \text{required } [\text{Ca}^{2+}] \cdot [1 + 0.53 (\text{required pH} - \text{adjusted pH})]
\]

The data thus adjusted for pH 7.4 revealed a dose-dependent hypocalcemia after the BM injection \( [F(4,90) = 2.05, P < 0.05, 2\text{-way ANOVA}] \). The pH-adjusted change in ionized calcium (\( \Delta\text{Ca}^{2+}_{\text{adj}} \)) reached a minimum 30 min after the injection \( [F(4,18) = 4.74, P < 0.01, 1\text{-way ANOVA}; \text{Fig. 1B}] \) and then recovered gradually.

To further characterize the BM-induced hypocalcemia, we measured the serum levels of total calcium and magnesium (Table 1). The total calcium level of the BM (30 ng)-injected group was significantly lower at 30 min

Table 1. Effect of BM injection into LHA on serum levels of calcium, magnesium, sodium, potassium, and chloride

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>BM (30 ng)</th>
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<tr>
<td>Calcium</td>
<td>5.12 ± 0.05</td>
<td>4.93 ± 0.07*</td>
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<tr>
<td>Magnesium</td>
<td>1.82 ± 0.05</td>
<td>1.81 ± 0.07</td>
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<tr>
<td>Na</td>
<td>139 ± 0.47</td>
<td>140 ± 0.49</td>
</tr>
<tr>
<td>K</td>
<td>3.78 ± 0.05</td>
<td>3.53 ± 0.10*</td>
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<tr>
<td>Cl</td>
<td>101 ± 0.59</td>
<td>100 ± 0.70</td>
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Injection values are means ± SE (in meq/l) measured 30 min after bicuculline methiodide (BM) injection. LHA, lateral hypothalamic area. *P < 0.05.
after BM injection than that of the vehicle-injected group (P < 0.05), whereas the serum magnesium levels did not differ between two groups (Table 1). Blood concentrations of sodium and chloride of the BM-injected group did not differ from those of the vehicle-injected group either. However, the blood potassium level of the BM (30 ng)-injected group was significantly lower than that of the vehicle-injected group (P < 0.05).

Histological examination. The results of the histological examination are summarized in Fig. 3. The hypocalcemic effect of the BM injection was observed when the cannula tips were located within the LHA. The injection of the vehicle into comparable sites in the LHA had no hypocalcemic effect. The sites of BM injection in the animals used to examine the effects of selective vagotomy or pharmacological pretreatments were also located within this area (See Figs. 5, 7, and 9).

Effects of selective subdiaphragmatic vagotomy on hypocalcemia induced by BM injection into LHA. To determine the involvement of the vagal nerves in BM injection-induced hypocalcemia, we examined the effects of a selective vagotomy (Figs. 4 and 5). A gastric vagotomy almost completely abolished the hypocalcemia induced by the BM injection (Fig. 4A, left). $\Delta Ca^{2+}$ of the gastric-vagotomized group 30 min after the injection (−0.008 ± 0.010 mM, n = 4) was significantly smaller than that of the sham-operated group (−0.072 ± 0.005 mM, n = 4, P < 0.01). A gastric vagotomy also attenuated the BM-induced alkalosis (Fig. 4C, left). Although the pH did not change significantly ($\Delta pH$, −0.003 ± 0.011) 30 min after the BM injection in the

**Fig. 3.** Location of cannula tips in vehicle-injected rats (left) and BM-injected rats (right). Symbols represent different levels of decreases in blood ionized calcium levels 30 min after LHA stimulation in comparison with basal calcium levels measured before stimulation. (× ≥ −0.03 mM, ◆ ≥ −0.07 mM). Drawings were made on the basis of the atlas of Paxinos and Watson (19), with slight modifications. Numbers at left bottom, rostrocaudal distance (mm) from interaural line.

**Fig. 4.** Effects of selective vagotomy (Vagx) on LHA stimulation-induced hypocalcemia and alkalosis. A: $\Delta Ca^{2+}$ from prestimulation basal level 30 min after LHA stimulation. B: changes in $\Delta Ca^{2+}$ 30 min after stimulation. C: $\Delta pH$ 30 min after stimulation in comparison with prestimulation level. No. of rats in each group indicated in bars. *P < 0.05, **P < 0.01.

**Fig. 5.** Location of cannula tips in sham-operated rats and Vagx rats. Symbols represent different levels of decreases in blood ionized calcium levels 30 min after LHA stimulation in comparison with basal calcium levels measured before stimulation. (× ≥ −0.03 mM, −0.04 ≤ ◆ ≥ −0.07 mM, ◆ ≤ −0.08 mM).
Effects of pharmacological treatments on BM injection-induced hypocalcemia. Because gastric vagal activation has been shown to promote the release of acetylcholine, gastrin, and histamine, we investigated the effects of various treatments with their antagonists and release inhibitors on BM injection-induced hypocalcemia. There was no significant difference in the basal level of blood Ca\textsuperscript{2+} between the drug-pretreated groups (ranging from 1.35 ± 0.01 to 1.41 ± 0.02 mM) and the vehicle-pretreated groups (ranging from 1.37 ± 0.01 to 1.41 ± 0.01 mM).

Intravenous injection of atropine methyl bromide (0.1 and 0.6 mg/kg), which does not cross the blood-brain barrier, suppressed the induction of the BM-induced hypocalcemia in a dose-dependent manner (Figs. 6 and 7). The ΔCa\textsuperscript{2+} of rats pretreated with atropine (0.1 mg/kg, −0.039 ± 0.009 mM, n = 9, P < 0.05; 0.6 mg/kg, −0.018 ± 0.010 mM, n = 11, P < 0.005) showed significant differences from that of the vehicle-pretreated control rats (−0.072 ± 0.009 mM, n = 5 and −0.070 ± 0.012 mM, n = 7, respectively). Atropinization also blocked changes in the blood pH induced by a BM injection (0.1 mg/kg, 0.019 ± 0.006 vs. 0.038 ± 0.012, P < 0.01; 0.6 mg/kg, −0.003 ± 0.013 vs. 0.034 ± 0.007, P < 0.05). Although there was no significant difference in the pH-adjusted ΔCa\textsuperscript{2+} between the groups pretreated with 0.1 mg/kg of atropine (−0.042 ± 0.006...
Dmia was completely blocked (\(0.048 \pm 0.004 \text{ mM}\)) and vehicle (\(-0.048 \pm 0.004 \text{ mM}\)), the 0.6 mg/kg treatment with atropine suppressed the BM-induced decrease in pH-adjusted \(\text{Ca}^{2+}\) (0.6 mg/kg, \(-0.026 \pm 0.006 \text{ mM}\) vs. vehicle, \(-0.049 \pm 0.007 \text{ mM}\); \(P < 0.05\); Fig. 6).

It has been shown that the gastric vagus nerves contain adrenergic fibers (7). We thus investigated the possible involvement of adrenergic mechanisms. PBZ (3 mg/kg iv), an \(\alpha\)-adrenergic antagonist, failed to affect the BM-induced hypocalcemia and alkalosis. When the \(\beta\)-adrenergic antagonist nadolol (2 mg/kg iv), which does not easily cross the blood-brain barrier, was given, the degree of hypocalcemia was significantly suppressed (\(\Delta\text{Ca}^{2+}\), \(-0.013 \pm 0.013 \text{ mM}\), \(P < 0.05\); \(\Delta\text{Ca}_{adj}^{2+}\), \(0.010 \pm 0.011 \text{ mM}\), \(P < 0.005\); \(n = 12\)) in comparison with the vehicle-pretreated group (\(\Delta\text{Ca}^{2+}\), \(-0.059 \pm 0.011 \text{ mM}\); \(\Delta\text{Ca}_{adj}^{2+}\), \(-0.041 \pm 0.005 \text{ mM}\); \(n = 7\)) without affecting alkalosis (\(\Delta\text{pH}\) of nadolol-pretreated group, \(0.033 \pm 0.005\); \(\Delta\text{pH}\) of vehicle-pretreated group, \(0.043 \pm 0.012\)). When the rats were pretreated with both nadolol (2 mg/kg iv) and atropine (0.6 mg/kg iv), hypocalcemia was completely blocked (\(\Delta\text{Ca}^{2+}\) of rats pretreated with nadolol and atropine, \(0.002 \pm 0.006 \text{ mM}\), \(P < 0.0001\); \(\Delta\text{Ca}_{adj}^{2+}\), \(-0.006 \pm 0.008 \text{ mM}\), \(P < 0.05\); \(n = 5\) vs. \(\Delta\text{Ca}^{2+}\) of rats pretreated with vehicle, \(-0.083 \pm 0.003 \text{ mM}\); \(\Delta\text{Ca}_{adj}^{2+}\), \(-0.053 \pm 0.013 \text{ mM}\); \(n = 3\); Fig. 6).

Pretreatment with the histamine \(\text{H}_2\) receptor antagonist ranitidine (5 mg/kg iv) blocked the hypocalcemic effect of BM injection (\(\Delta\text{Ca}^{2+}\), \(-0.031 \pm 0.007 \text{ mM}\); \(n = 11\), \(P < 0.05\); \(\Delta\text{Ca}_{adj}^{2+}\), \(-0.006 \pm 0.008 \text{ mM}\), \(P < 0.05\); \(n = 5\)), but it did not affect the alkalosis (\(\Delta\text{pH}\), \(0.036 \pm 0.010\); Figs. 8 and 9).

In contrast, secretin (6 µg/kg iv), an inhibitor of gastrin release, did not affect \(\Delta\text{Ca}^{2+}\) (\(-0.070 \pm 0.006 \text{ mM}\); \(n = 5\), \(P < 0.05\); \(\Delta\text{Ca}_{adj}^{2+}\), \(-0.040 \pm 0.011 \text{ mM}\); \(n = 5\)), or \(\Delta\text{pH}\) (0.052 ± 0.013). Somatostatin (1 µg/h), the most potent inhibitor of gastrin release, which was infused intravenously for 25 min starting 5 min before BM injection, also failed to eliminate hypocalcemia (\(\Delta\text{Ca}^{2+}\), \(-0.047 \pm 0.011 \text{ mM}\); \(\Delta\text{Ca}_{adj}^{2+}\), \(-0.034 \pm 0.013 \text{ mM}\)) and alkalosis (\(\Delta\text{pH}\), 0.023 ± 0.007; \(n = 7\); Figs. 8 and 9).

Serum gastrin after BM injection into LHA. The concentration of gastrin in the serum significantly increased after the BM injection into the LHA (186.0 ± 25.4 pg/ml, \(n = 9\)) in comparison with that of the

Fig. 10. Effect of BM injection in LHA on serum gastrin level. No. of rats in each group indicated in each bar. * \(P < 0.05\).

Fig. 8. Effects of histamine \(\text{H}_2\) blocker (ranitidine) and gastrin-release inhibitors (secretin and somatostatin) on LHA stimulation-induced hypocalcemia and alkalosis. A: \(\Delta\text{Ca}^{2+}\) 30 min after LHA stimulation from prestimulation basal level. B: \(\Delta\text{Ca}_{adj}^{2+}\) 30 min after stimulation. C: \(\Delta\text{pH}\) 30 min after stimulation in comparison with prestimulation level. Each bar represents mean ± SE. Open bars, pretreated vehicle; hatched bars, drug pretreated. No. of rats in each group indicated in each bar. * \(P < 0.05\).

Fig. 9. Location of cannula tips in rats pretreated with vehicle, histamine \(\text{H}_2\) blocker, or gastrin-release inhibitors. Symbols represent different levels of decreases in blood ionized calcium levels 30 min after LHA stimulation in comparison with basal calcium levels measured before stimulation. (\(\times \leq -0.03 \text{ mM}\), \(-0.04 \leq \triangle \leq -0.07 \text{ mM}\), \(\bullet \leq -0.08 \text{ mM}\)).

Fig. 11. Location of cannula tips in rats pretreated with vehicle, histamine \(\text{H}_2\) blocker, or gastrin-release inhibitors. Symbols represent different levels of decreases in blood ionized calcium levels 30 min after LHA stimulation in comparison with basal calcium levels measured before stimulation. (\(\times \leq -0.03 \text{ mM}\), \(-0.04 \leq \triangle \leq -0.07 \text{ mM}\), \(\bullet \leq -0.08 \text{ mM}\)).
vehicle-injected group (97.0 ± 20.1 pg/ml, n = 7, P < 0.05; Fig. 10).

**DISCUSSION**

The present study demonstrated that an intra-LHA injection of BM induced hypocalcemia, and this effect was eliminated either by a gastric vagotomy or pretreatment with either muscarinic, β-adrenergic, or histaminergic H2 receptor antagonist. The decrease in blood calcium levels (0.05–0.1 mM, 0.2–0.4 mg/dl) shown in the present study did not appear to be a great change; similar hypocalcemic responses have been shown after immobilization or an injection of hypocalcemic agents (1, 3, 13, 14, 20) that may be sufficient enough to trigger the calcium-regulating responses (~60–70% of the maximum response of PTH) as well as other physiological and psychological responses (2, 14).

The blood concentration of ionized calcium is known to depend on the blood pH (21), which increases with the development of alkalosis. In the present study, the BM injection into the LHA resulted in an increase in blood pH that preceded a decrease in the blood calcium. In the case of respiratory alkalosis, the blood ionized calcium levels can be adjusted for blood pH (5). In the present study, arterial blood gas analyses revealed that the changes in pH were caused by respiratory alkalosis; we therefore calculated the blood level of Ca2+ adjusted for pH 7.4. The blood level of the Ca2+ significantly decreased 30 min after the BM injection in a dose-dependent manner. The serum level of total calcium also significantly decreased. We thus conclude that the hypocalcemic response is not a result of the associated respiratory alkalosis.

Blood concentrations of sodium, chloride, and magnesium did not change after the BM injection, although hypocalcemia and hypokalemia did occur. This indicates that the effect of BM injection into the LHA on the peripheral blood electrolytes is selective and not caused by any nonspecific mechanisms such as dilution caused by fluid shift or water retention. Although the mechanisms for the induction of hypocalcemia remain unknown, this is the first demonstration that the neuronal activation of LHA elicits hypocalcemia. It has been shown that the hypothalamus may be involved in potassium metabolism through renal electrolyte regulation and/or the production of endogenous Na+-K+ adenosinetriphosphatase inhibitors in the hypothalamus (23). Several clinical investigations have also suggested that brain injury induces hypocalcemia through the peripheral catecholaminergic systems (22). Further study is necessary, however, to elucidate the precise mechanisms and physiological significance of the central nervous system, but these findings suggest that it is involved in the control of not only the blood calcium level but also the blood potassium concentration.

The hypocalcemic effect after the BM injection was observed when the cannula tips were located within the area of the LHA, as shown in Fig. 3. In this study, the volume of the BM solution (0.5 µl) was used to cover the entire LHA at the level of VMH. This volume might be relatively large in comparison with some microinjection studies (24, 30), but the effective sites were confined to within the LHA and the effect was greatly attenuated or disappeared when the tips of cannulas were located outside but in the vicinity of the LHA (Fig. 3). The rate of administration (0.1 µl/min) was slow enough to avoid any nonspecific effects. We did not observe any nonspecific effects such as irritability, convulsion, or other aggressive behavior except for digging behavior. The present results therefore suggest that the hypocalcemic effect of the BM injection was induced by neural activation of the LHA, not of other hypothalamic loci. In our preliminary study, we found that a microinjection of a smaller volume of BM into the VMH or PVN (0.3 or 0.2 µl, respectively) also induced hypocalcemia. The effects were also greatly attenuated when the injection sites were in the vicinity of, but outside, these areas (unpublished observation).

In the previous studies in awake rats, we found that the stomach was involved in blood calcium regulation during immobilization stress (3, 14) and that the hypocalcemic effects of electrical stimulation of the LHA in anesthetized rats were abolished by a vagotomy of the gastric branches but not by a vagotomy of the thyroid-parathyroid branches (13). LHA stimulation has been shown to facilitate activities of neurons of the dorsomotor nucleus of the vagus (17, 27) and gastric vagal nerves (27). In the present study, the hypocalcemia induced by the BM injection into the LHA was eliminated by a vagotomy of gastric branches but not by that of the celiac or hepatic branches. Although a nonvagal mechanism cannot be ruled out, these findings suggest that the gastric vagal nerves almost exclusively mediate the hypocalcemic effect of LHA neuronal activation.

Atropinization has been shown to mimic the effect of a vagotomy in many experimental conditions, and atropine methyl bromide, which did not cross the blood-brain barrier, significantly suppressed the hypocalcemic effect of LHA neuronal activation in the present study. This indicates that muscarinic receptors mediate, at least in part, the hypocalcemic effect of an intra-LHA injection of BM through the gastric vagal nerves. In a previous study, we found that atropine methyl bromide at 0.6 mg/kg completely abolished the immobilization-induced hypocalcemia (14). In the present study, however, a slight decrease in the blood calcium levels was still observed after the BM injection in rats pretreated with the same dose of atropine methyl bromide. It is known that the gastric vagal nerves contain not only cholinergic fibers but also catecholaminergic fibers (12) and that a vagotomy reduces the catecholamine levels in the stomach wall by 50% (7). Because nadolol, a peripherally acting β-adrenoceptive blocker, also suppressed the BM-induced hypocalcemia in the present study, the hypocalcemia after the LHA neuronal activation is mediated by β-adrenergic receptor mechanisms as well as muscarinic receptor mechanisms. It is worthwhile to note that atropine methyl bromide suppressed both hypocalcemia and alkalosis, whereas nadolol eliminated only the hypocalcemic response without affecting the alkalosis.
Therefore, different effects of cholinergic and adrenergic inputs to gastric target cells may be involved in this phenomenon.

Gastrin and histamine, which are released from the stomach by vagal stimulation, are known as hypothalamic agents (10, 20). The hypocalcemic effects of both agents are eliminated by gastrectomy (10, 20), thus indicating that the target site of these chemicals is the stomach. Because a histamine $H_2$ antagonist (ranitidine) was shown to block the BM-induced hypocalcemia, the release of histamine as a result of LHA neuronal activation may thus be involved in hypocalcemia. In contrast, somatostatin and secretin, inhibitors of gastrin release, failed to affect BM injection-induced hypocalcemia. It has been reported that a $\beta$-adrenergic agonist may release gastrin from the stomach and that gastrin decreases the blood calcium level through the release of thyrocalcitonin (4) and/or the putative gastric hypocalcemic factor gastrocadin (20). The present results, however, suggest that gastrin is not an essential factor for BM injection-induced hypocalcemia, even though an intra-LHA injection of BM facilitates the release of gastrin.

Although homeostasis of blood calcium has long been thought to be regulated by peripheral mechanisms, especially by calciotropic hormones, the present study demonstrates that LHA neurons also participate in the control of the blood calcium level by changes in the activity of the gastric vagal nerves that involve the mechanisms of muscarinic, $\beta$-adrenergic, and histamine $H_2$ receptors.

Perspectives

The physiological significance of the hypocalcemic action of the LHA currently remains to be clarified. The LHA is well known to be involved in the control of feeding behavior and the related visceral functions, including secretion of gastric acid and insulin during the cephalic phase (9). LHA neurons have been shown to respond to the sight, taste, and/or smell of food (11, 25). The LHA contains a particular group of neurons, designated as glucose-sensitive neurons, the firing rate of which specifically decreases in response to glucose administered either systemically or locally (18). The majority of glucose-sensitive neurons respond to both taste and odor stimuli (11), and the activation of these neurons promotes gastric acid secretion (28). It has been reported that food intake never elicits hypercalcemia but instead induces transient hypocalcemia (0.05 mM decrease) at an early phase of food intake that may be mediated by the secretion of gastrin and histamine (8). These findings together with the present results thus support the hypothesis that the hypocalcemic mechanism of the LHA-gastric vagal axis plays a role in the cephalic control of calcium homeostasis, thereby preventing a postprandial increase in blood calcium concentration.

We thank Dr. B. T. Quinn for critical comments and help in preparing this manuscript.

This study was supported by Grants-In-Aid (05NP0101 and 07557008) for Scientific Research from the Ministry of Education, Science, and Culture of Japan (S. Aou) and a Research Grant for Nervous and Mental Disorders from the Ministry of Health and Welfare of Japan (S. Aou).

Address reprint requests to S. Aou.

Received 29 October 1996; accepted in final form 10 July 1997.

REFERENCES


20. Persson, P., R. Håkansson, J. Axelson, and F. Sundler. Gastrin releases a blood calcium-lowering peptide from the