Microinjection of α-Calcitonin Gene-Related Peptide into the Hypothalamus Activates Sympathetic Outflow in Rats

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ABSTRACT—Effects of rat α-calcitonin gene-related peptide (α-CGRP) microinjected into various hypothalamic nuclei on plasma levels of catecholamines and arterial blood pressure were investigated in urethane-anesthetized rats. α-CGRP (0.05 and 0.25 nmol) microinjected into the hypothalamic paraventricular nucleus (PVN) increased the plasma level of noradrenaline (NA), but not that of adrenaline (AD), in a dose-dependent manner. A similar increase in plasma level of NA was also observed by α-CGRP (0.05 nmol) microinjected into the preoptic area (POA), anterior hypothalamus (AH), dorsomedial hypothalamus (DMH) and ventromedial hypothalamus (VMH). A significant increase in arterial blood pressure was observed by microinjection of α-CGRP (0.05 nmol) into the PVN, POA, AH and DMH, and the most prominent increase was caused by its microinjection into the PVN. Microinjection of the same dose of this peptide into the VMH, lateral hypothalamic area and posterior hypothalamus was without effect. The increase in plasma level of NA induced by α-CGRP (0.05 nmol) into the PVN was not affected by bilateral adrenalectomy. Electrical stimulation of the PVN elicited increases in plasma levels of both NA and AD. This increase in NA was abolished by chemical sympathectomy with 6-hydroxydopamine (50 mg/kg, i.v., 3 days before experiments). These results suggest that activation of the PVN by electrical stimulation elicits both sympathetic and adrenomedullary outflow. α-CGRP microinjected into the PVN selectively activates the sympathetic outflow.

Keywords: α-Calcitonin gene-related peptide, Noradrenaline, Hypothalamus, Paraventricular nucleus, Sympathetic and adrenomedullary system

Rat α-calcitonin gene-related peptide (α-CGRP) is a 37-residue neuropeptide which was identified by alternative processing of mRNA from the rat calcitonin gene (1, 2). Succeeding studies in rats identified a second CGRP, β-CGRP, which differs from α-CGRP by a single amino acid (3, 4). Immunohistochemical studies revealed that CGRP is widely distributed in both the central and peripheral nervous systems (2). In the brain, both α- and β-CGRP are present, but α-CGRP is predominant (4, 5).

In 1983, Fisher et al. reported that intracerebroventricular administration of CGRP increases the mean arterial blood pressure, heart rate and plasma level of noradrenaline (NA) in rats (6). In contrast to these central effects of CGRP, centrally applied bombesin increases both NA and adrenaline (AD), and the increase of AD is more predominant than that of NA (7–9). These findings suggest a possibility that sympathetic and adrenomedullary outflows are separately controlled in the brain. It is therefore interesting to determine the exact location of the central regulatory mechanisms of sympathetic and adrenomedullary outflows.

Nguyen et al. and Brown and Gray reported that CGRP microinjected into the central amygdaloid nucleus affects cardiovascular and sympatho-adrenomedullary systems (10, 11). On the other hand, it is generally accepted that hypothalamic nuclei such as the paraventricular nucleus (PVN) is the higher center for central regulation of sympathetic functions. Furthermore, CGRP is abundantly contained in the hypothalamus (12, 13). However, there is still a paucity of information about the possible roles of hypothalamic α-CGRP in the regulation of sympatho-adrenomedullary outflow. We therefore investigated the effects of α-CGRP microinjected into various
nuclei of the hypothalamus on plasma levels of catecholamines and arterial blood pressure.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 300–350 g were maintained in a room at 22–24°C under a constant day-night rhythm for more than 1 week and given food (laboratory chow, CE-2, Clea Japan, Inc., Tokyo) and water ad libitum. Under urethane anesthesia (1.2 g/kg, i.p.), the femoral vein was cannulated for infusion of physiological saline (1.6 ml/hr), and the femoral artery was cannulated for measuring arterial blood pressure and collecting blood samples. After these procedures, the animal was placed in a stereotaxic apparatus. The arterial catheter was connected to a pressure transducer (Nihon Kohden, Ltd., Tokyo), and arterial blood pressure was recorded. For surgical adrenalectomy, bilateral adrenal glands were extirpated 3 hr before administration of rat α-CGRP and the animals were treated with hydrocortisone (5 mg/kg, i.m.). Sympathectomy was chemically performed by a single administration of 6-hydroxydopamine (6-OHDA) (50 mg/kg) dissolved in saline containing 0.5% ascorbic acid. The 6-OHDA solution was injected into the dorsal penic vein under light ether anesthesia 3 days before the experiment.

α-CGRP administration

Three hours after the animal was placed in a stereotaxic apparatus, a stainless steel cannula (0.35 mm outer diameter) or a pulled glass micropipette (50–75 μm outer diameter) was inserted into the lateral cerebral ventricle or several nuclei of the hypothalamus according to the rat brain atlas of König and Klippel (14). Artificial cerebrospinal fluid (CSF) (15) or CSF containing α-CGRP was then microinjected into these brain regions over 60 sec. With a Hamilton syringe, 10 and 0.5 μl of the test solutions were administered intraventricularly and intrahypothalamically, respectively. The coordinates of the sites of microinjection were as follows: paraventricular nucleus (PVN), (in mm: AP 5.6, L 0.3, H 2.9); preoptic area (POA), (AP 6.8, L 0.5, H 2.8); anterior hypothalamus (AH), (AP 6.0, L 0.5, H 2.3); lateral hypothalamus (LH), (AP 4.8, L 1.6, H 2.2); dorsomedial hypothalamus (DMH), (AP 4.8, L 0.5, H 2.2); ventromedial hypothalamus (VMH), (AP 4.8, L 0.5, H 1.5) and posterior hypothalamus (PH), (AP 3.3, L 0.3, H 2.5) (AP, anterior from the interaural line; L, lateral from the midline; H, above the interaural line). α-CGRP was purchased from The Peptide Institute, Inc., Osaka.

Electrical stimulation of the PVN

For electrical stimulation of the PVN, a bipolar platinum electrode (0.15 mm/pole) was used; and square-wave pulses of 0.5 mA, 2 msec duration, at 2–10 Hz were applied for 10 min by means of an electronic stimulator (Model 3201, Nihon Kohden, Ltd.).

Measurement of plasma catecholamines

Blood samples (400 μl) were collected through an arterial catheter. Catecholamines in the plasma were extracted by the method of Anton and Sayre (16) with slight modifications and were assayed electrochemically by high performance liquid chromatography (HPLC). The modifications were as follows: plasma (180 μl) was transferred to a centrifuge tube containing 30 mg of activated aluminium oxide, 2 ml of double distilled water and 1 ml of 1.5 M disodium EDTA, and then the preparation was immediately shaken for 5 min. After several washings with 4-ml aliquots of ice-cold double distilled water, catecholamines adsorbed onto aluminium oxide were eluted with 300 μl of 4% of acetic acid containing 0.1 mM disodium EDTA. A pump (880 PU; Japan Spectroscopic Co., Ltd., Tokyo) and an electrochemical detector (ECD-100, Eicom Co., Ltd., Kyoto) were used with HPLC. Analytical conditions were as follows: detector, 0.7 volt potential; column, Cosmosil-packed column (ODS, 4.6×150 mm, Nacalai Tesque, Inc., Kyoto); mobile phase, 0.1 M phosphate buffer pH 3.5, 20 mM EDTA, 4 mM 1-octane sulfate sodium (Nacalai Tesque, Inc.) containing 16% methanol. The amount of catecholamines in each sample was calculated by using the peak height ratio relative to 3,4-dihydroxy benzylamine, an internal standard.

Histology

After the experiments, the brain was removed and fixed in 10% formalin. Frozen sections sliced at 30 μm were stained with cresyl violet for microscopic study of the location of both the microinjection and electrical stimulation sites.

Statistical analyses

All values were expressed as the mean ± S.E., and significant differences from the basal or corresponding values of vehicle-treated group were represented by *, which indicates P<0.05 (paired or unpaired Student's t-tests as appropriate).

RESULTS

Arterial blood pressure changes induced by intracerebroventricular and intrahypothalamic administrations of α-CGRP

Intracerebroventricular administration of α-CGRP (2 nmol) rapidly and markedly reduced mean arterial blood pressure from 109±11 to 75±6 mmHg (n=3), and this
Fig. 1. Representative illustrations of arterial blood pressure before and after administration of α-CGRP. A: α-CGRP (2 nmol) was intracerebroventricularly administered. B: α-CGRP (0.05 nmol) was microinjected into the unilateral PVN.

Table 1. Changes in mean arterial blood pressure induced by microinjection of α-CGRP

| Site of microinjection | Dose of α-CGRP (nmol) | N | Mean arterial blood pressure (mmHg) before | Mean arterial blood pressure (mmHg) after |
|------------------------|------------------------|---|------------------------------------------|------------------------------------------|
| PVN                    | Vehicle                | 6 | 108 ± 4                                  | 113 ± 4                                  |
| PVN                    | 0.05                   | 5 | 105 ± 4                                  | 120 ± 5                                  |
| POA                    | 0.05                   | 6 | 102 ± 5                                  | 111 ± 4                                  |
| AH                     | 0.05                   | 6 | 101 ± 4                                  | 110 ± 5                                  |
| DMH                    | 0.05                   | 7 | 115 ± 7                                  | 122 ± 7                                  |
| VMH                    | 0.05                   | 4 | 105 ± 10                                 | 114 ± 9                                  |
| PH                     | 0.05                   | 3 | 107 ± 2                                  | 110 ± 4                                  |
| LH                     | 0.05                   | 6 | 98 ± 4                                   | 102 ± 6                                  |

α-CGRP was dissolved in artificial cerebrospinal fluid (CSF) and administered in a volume of 0.5 μl over 60 sec. Changes in mean arterial blood pressure were shown in each group. *, mean arterial blood pressure before microinjection of α-CGRP; †, mean arterial blood pressure 10 min after the start of microinjection; ‡, significantly different from the basal values (P<0.05). N, number of animals. PVN, paraventricular hypothalamic nucleus; POA, preoptic area; AH, anterior hypothalamic nucleus; DMH, dorsomedial hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus; PH, posterior hypothalamus; LH, lateral hypothalamus.

Fig. 2. The effect of α-CGRP (0.05 nmol) microinjected into various regions of the hypothalamus on plasma levels of catecholamines. α-CGRP dissolved in 0.5 μl of artificial CSF was administered over 60 sec. Arrow indicates the microinjection of α-CGRP. Sites of microinjections: A, PVN (n=5); B, POA (n=6); C, AH (n=6); D, DMH (n=7); E, VMH (n=4); F, LH (n=6); G, PH (n=3). •, noradrenaline; ○, adrenaline. The other abbreviations are the same as described in Table 1. *significantly different from the basal values (P<0.05).
hypotensive effect continued over 60 min (Fig. 1A). After repeated intravenous administration of α-CGRP (1 nmol) for several times, the hypotensive effect induced by intracerebroventricularly administered α-CGRP (2 nmol) was completely abolished or rather reversed to a hypertensive effect (data not shown). Microinjection of 0.05 nmol α-CGRP into the PVN induced a marked and significant increase in mean arterial blood pressure, and the level was gradually returned to the basal value within 30 min, as shown in Fig. 1B and Table 1. Microinjection of α-CGRP (0.05 nmol) into the POA, AH and DMH also induced slight but significant increases in mean arterial blood pressure. On the other hand, α-CGRP into the VMH, LH and PH was without effect (Table 1).

Effects of intrahypothalamically-applied α-CGRP on the plasma levels of NA and AD

Microinjection of vehicle alone and blood sampling for 5 times over a 60-min period did not affect the plasma levels of both NA and AD. Plasma levels of NA and AD before administration of vehicle were 410 ± 45 and 230 ± 28 pg/ml, respectively. Microinjection of 0.05 nmol α-CGRP into the PVN, POA, AH, DMH and VMH induced marked increases in plasma levels of NA (Fig. 2, A–E). Plasma levels of AD were not affected by this treatment. Levels of NA 10 min after the administration of α-CGRP into the PVN, POA, AH, DMH and VMH were 936 ± 130, 839 ± 112, 805 ± 92, 1055 ± 130 and 908 ± 112 pg/ml, respectively. On the other hand, microinjection of α-CGRP into the LH and PH was without effect (Fig. 2, F and G). Sites of microinjection (represented unilaterally) used in the data analyses are plotted in Fig. 3. Then, the dose-response relationship was examined by microinjection of α-CGRP into the PVN. The level of NA 10 min after the administration of 0.25 nmol α-CGRP was not significantly different from that of 0.05 nmol α-CGRP (Fig. 4). However, the elevated level by 0.25 nmol of α-CGRP was not reverted to the control level within 60 min. The level of AD was not affected even by this larger dose of α-CGRP. A smaller dose of α-CGRP (0.005 nmol) was without effect on the plasma levels of both NA and AD.

The effect of adrenalectomy on the α-CGRP-induced increase in plasma level of NA

It has been reported that adrenal glands secrete both NA and AD (17–19). Adrenalectomy was therefore performed to examine whether or not the α-CGRP-induced increase in plasma level of NA was due to its release from the adrenal medulla and/or the sympathetic nerve terminals. Three hours after the bilateral adrenalectomy, AD was not detected in the plasma. In these adrenalectomized animals, α-CGRP (0.05 nmol) microinjected into the PVN still induced a marked increase in the plasma level of NA, and this increase was almost the same as that obtained in the non-treated animals (Fig. 5). This treatment with α-CGRP elevated mean arterial blood pressure from 107 ± 5 to 118 ± 5 mmHg (P < 0.05).

Fig. 3. Diagram of coronal sections of the rat brain showing each microinjection site as shown in Fig. 2. Closed circles represent effective sites in increasing plasma noradrenaline levels, open circles represent ineffective sites. Numbers in each section give the distance of the plate from the interaural line. Plates adapted from König and Klippel (14). The site in plate 6860 lies between 6790 and 7020, 6570 lies between 6570 and 6790, 6060 lies between 5910 and 6280, 5660 lies between 5340 and 5780, 4890 lies between 4620 and 4890 and 3290 lies between 3290 and 3430. The abbreviations are the same as described in Table 1.

Effects of electrical stimulation of the PVN on arterial blood pressure and plasma catecholamine levels

Electrical stimulation of the PVN at 2 Hz (0.5 mA, 2 msec for 10 min) induced a slight increase in the plasma level of AD without affecting that of NA and blood pressure. When the PVN was stimulated at 10 Hz, marked increases in plasma levels of both NA and AD were observed (Fig. 6). Levels of NA and AD reached to
811±123 and 1176±180 pg/ml 10 min after the start of stimulation, and then they reverted to the respective basal values (423±29 and 328±69 pg/ml). These responses were associated with a rapid increase in arterial blood pressure. Mean arterial blood pressure before and 10 min after the start of stimulation at 10 Hz were 101±4 and 119±3 mmHg (n=6) (P<0.05).

We reported that 6-OHDA (50 mg/kg, i.v., 3 days before experiments) decreased the content of NA in sympathetically innervated organs, such as the stomach, to less than 15% of the controls without affecting the contents of catecholamines in the brain and the adrenal glands (20). In the present study, the basal plasma level of AD 3 days after treatment with 6-OHDA was very high as reported in our recent paper (9); 899±182 pg/ml for the 6-OHDA treated group (n=8) and 223±14 pg/ml for the non-treated group (n=68), respectively. On the other hand, the basal plasma level of NA was not affected. In these chemically sympathectomized animals, electrical stimulation of the PVN at 10 Hz induced a marked increase in the plasma level of AD without affecting that of NA (Fig. 7). During electrical stimulation, the mean arterial blood pressure elevated from 84±4 to 104±7 mmHg (P<0.05).
DISCUSSION

There are some controversies about the effect of centrally administered CGRP on arterial blood pressure. In conscious rats, intracerebroventricularly administered CGRP increased (6) or induced no change (21) in arterial blood pressure. In the present study performed under urethane anesthesia, intracerebroventricularly administered CGRP induced a marked decrease in arterial blood pressure. However, after desensitization of peripheral CGRP receptors by repeated intravenous bolus administration of α-CGRP, intracerebroventricular administration of this peptide did not induce any significant decrease or rather induced an increase in arterial blood pressure. Peripherally administered α-CGRP has intense hypotensive and vasodilator actions (6, 21–24). Furthermore, 8% leakage of α-CGRP from the lateral brain ventricle to the peripheral circulation was demonstrated in rats (25). The decrease in arterial blood pressure induced by intracerebroventricularly administered α-CGRP in the present study is therefore probably due to its leakage into the systemic circulation. Moreover, discrepancies in cardiovascular responses caused by intracerebroventricular administration of α-CGRP might be attributable to the different experimental conditions, i.e., with or without anesthesia.

To examine the effects of α-CGRP on the sympatho-adrenomedullary outflow, we microinjected a small dose of α-CGRP into the hypothalamus. Leakage of this peptide into the systemic circulation is therefore considered to be negligible, and non-specific activation of the sympatho-adrenomedullary system by peripheral α-CGRP-induced sudden fall in blood pressure is excluded.

CGRP-like immunoreactive fibers and neurons are observed in the PVN, POA, DMH and AH (12, 13). Furthermore, CGRP dense binding sites are observed in the PVN, AH and DMH, and moderate binding sites in the POA, VMH and LH (26). Based on these reports, α-CGRP was microinjected into these nuclei of the hypothalamus. Microinjection of α-CGRP into the PVN, POA, AH, DMH and VMH induced increases in the plasma levels of NA without affecting those of AD. There now exists considerable evidence suggesting that the PVN is the controlling center of the sympathetic nervous system (27, 28). The PVN directly projects to the intermediolateral nucleus in the spinal cord and activates the sympathetic nervous system (29–35). In the present study, the increase in plasma level of NA by administration of α-CGRP into the PVN was also observed in bilaterally adrenalectomized animals. It is thus evident that this increase of NA by α-CGRP is due to its release from sympathetic nerve terminals.

Recently, 8-hydroxy-2-(di-n-propylamino) tetralin (5-HT1A receptor agonist) injected into the PVN of rats has been shown to induce a significant increase in the plasma level of AD without affecting that of NA (36). On the other hand, chemical stimulation of neuronal cell bodies in the PVN with glutamate increases plasma levels of both NA and AD (37). In the present study, electrical stimulation of the PVN induced increases in plasma levels of both NA and AD. The increase in the plasma level of NA induced by electrical stimulation probably originates from sympathetic nerve terminals, since chemical sympathectomy with 6-OHDA completely abolishes this increase in NA. The present results together with the reported evidence suggest a possibility that the PVN has two systems; one selectively activates sympathetic outflow and the other selectively activates adrenomedullary outflow. It is likely that electrical stimulation of the PVN activates both sympathetic and adrenomedullary outflows, and α-CGRP in the PVN selectively activates sympathetic outflow.

The VMH projects to the periaqueductal gray – medullary reticular formation – intermediolateral column circuit, thereby activating sympathetic outflow (38). Recently, the PVN has been suggested to be a main hypothalamic sympathetic outflow station (28, 38). The PVN receives
projections from various parts of the hypothalamus, such as the POA, AH, VMH, DMH and LH, and may integrate hypothalamic autonomic responses (28, 39, 40). In the present study, however, mechanisms of sympathetic activation induced by microinjection of α-CGRP into the POA, AH, DMH and VMH remains to be elucidated.

In conclusion, there probably exists two different sympato-adrenomedullary activating systems in the PVN; one activates adrenomedullary outflow and the other activates sympathetic outflow. α-CGRP selectively activates the sympathetic activating system in the PVN. The roles of other hypothalamic nuclei in central regulation of sympathetic outflow are the subjects of ongoing investigations.

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