Possible Involvement of Descending Serotonergic Systems in Antinociception by Centrally Administered Elcatonin in Mice

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To elucidate the mechanisms of analgesic action of elcatonin, a synthetic analog of eel calcitonin, the effect of centrally injected elcatonin on acetic acid-induced writhing behavior was examined in mice. Intracisternal or intracerebroventricular injection of elcatonin significantly inhibited acetic acid-induced writhing behavior, while the intrathecal injection of elcatonin did not inhibit it. The inhibitory effect of intracisternal elcatonin was significantly attenuated by subcutaneous pretreatment with methysergide and ($\pm$)-propranolol or by intrathecal pretreatment with methysergide, ($\pm$)-propranolol, 1-(2-methoxyphenyl)-4-[4-(2-phthalimido) butyl]-piperazine hydrobromide (NAN-190) and granisetron, but not with ($\pm$)-atenolol or butoxamine. Further, the depletion of spinal 5-hydroxytryptamine (5-HT, serotonin) by pretreatment with 5,7-dihydroxytryptamine (5,7-DHT) significantly attenuated the inhibitory effect of intracisternally injected elcatonin on acetic acid-induced writhing behavior. These results suggest that the inhibitory descending serotonergic systems may be involved, through 5-HT_{1A} and 5-HT₃ receptors, in the production of an antinociceptive effect by centrally injected elcatonin.

Key words elcatonin; antinociception; serotonergic system; 5-HT_{1A} receptor; 5-HT₃ receptor; writhing behavior

Calcitonin (CT) is a 32-amino acid hormone secreted from the C cells of the thyroid gland and involved in the regulation of calcium homeostasis in blood and in the metabolism of bone calcium. In humans and rats, CT binding sites^{1–4}) and the expression of CT receptor mRNA⁵) have been demonstrated in the CNS (central nervous system), including in several nuclei of the hypothalamus and brain stem, suggesting that CT has distinct functions in the CNS. When injected centrally in various species of animals, CT exerts several pharmacological activities, such as the inhibition of food and water intake,^{6,7}) changes in the secretion of anterior pituitary hormones including prolactin,^{8,9}) growth hormone,¹⁰) ACTH and β -endorphin,¹¹) a decrease in amphetamine-induced locomotor activity,^{12,13}) and the inhibition of gastric acid secretion.¹⁴)

CT has also been reported to produce an analgesic effect on the pain of osteoporosis^{15,16}) and advanced cancers^{17,18}) in man and on experimental pain in animals,^{19,20}) but the mechanisms underlying the effect are not clear. Although there are some possibilities such as the involvement of opioid systems,^{21–23}) serotonergic systems^{24–27}) and anti-inflammatory effects,^{28,29}) these hypotheses are still in controversy. In the present study, to elucidate the analgesic mechanisms of elcatonin, a synthetic analog of eel calcitonin,³⁰) we investigated possible mechanisms of an inhibitory effect by centrally injected elcatonin on acetic acid-induced writhing behavior.

MATERIALS AND METHODS

Animals Male ddY mice weighing 16–22 g were maintained on a 12 h light/dark cycle (lights on at 8:00 a.m.) and fed *ad libitum*. All experiments followed the ethical guidelines for the investigation of experimental pain in conscious animals.³¹)

Writhing Test Mice were intraperitoneally administered 15 ml/kg of 1.2% acetic acid to produce a typical writhing reaction that is characterized by a wave of contraction of the abdominal musculature followed by extension of the hind limbs. They were placed individually in observation contain-

ers. The animals that demonstrated no writhing reaction up to 6 min after acetic acid administration were removed from the experiment. The animals were intracisternally, intracerebroventricularly or intrathecally injected with elcatonin or the vehicle 6 min after the administration of acetic acid, then the number of writhes was counted 10 to 25 min after the injection of acetic acid.

Methods of Injections The method of intracisternal injection was described previously.³²) Intracerebroventricular and intrathecal injections were carried out following the methods of Haley and McCormick³³) and Hylden and Wilcox,³⁴) respectively.

Neurotoxin Experiments A week before the experiment, 5,7-dihydroxytryptamine (5,7-DHT) at a dose of 60 μ g was administered intracerebroventricularly in a volume of 5 μ l following pretreatment with desipramine 25 mg/kg i.p. to protect the catecholaminergic neurons.

Measurements of Monoamine Content HPLC with an electrochemical detector was used to determine the content of 5-HT, noradrenaline and dopamine in the whole lumbar cord and brain stem of the 5,7-DHT-treated or vehicle-treated mice. Tissues were homogenized in 0.1 N HClO₄ containing 10 mM Na₂S₂O₅ and 10 mM disodium EDTA. The homogenates were centrifuged at 30000 $\times g$ for 15 min at 4 °C, and the pH of the supernatants was adjusted to 3.0 with 1 M CH₃COONa. After filtration through a 0.45 μ m pore size membrane filter, a 20 μ l aliquot was injected onto the HPLC column (Eicompak MA-5 ODS, 150 mm, 4.6 mm outer diameter, 5 μ m particle size) protected with a precolumn (35 mm, 4.6 mm outer diameter, 5 μ m particle size). The mobile phase for the measurement of the 5-HT, noradrenaline and dopamine content consisted of the following: 0.044 M citric acid–0.039 M sodium acetate buffer (pH 3.5) containing 1 mM sodium 1-octanesulfonate, 0.013 mM disodium EDTA and 17% methanol. The elution rate was set at 1 ml/min and electrochemical detection was performed at a potential of 750 mV. Quantitative determinations were made with a CR4A Shimadzu integrator using appropriate standards.

Drugs Elcatonin ([Asu^{1,7}] eel calcitonin), a synthetic

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analog of eel calcitonin (a gift from Asahi Chemical Industry, Osaka, Japan), was diluted in 0.1 mM sodium acetate buffer containing 0.02% bovine serum albumin (pH 5.5) to achieve appropriate doses. ($\pm$)-Propranolol hydrochloride (Nacalai Tesque, Kyoto, Japan), granisetron hydrochloride (a gift from Nissin Flour Milling, Saitama, Japan), naloxone hydrochloride (Sigma, St. Louis, MO, U.S.A.), phentolamine hydrochloride (a gift from Novartis Pharma, Tokyo, Japan) and desipramine (Nacalai Tesque) were dissolved in 0.9% saline. Methysergide hydrogenmaleate (a gift from Novartis Pharma) was dissolved in a small amount of 1 N methanesulphonic acid and then diluted with 0.9% saline. 1-(2-Methoxyphenyl)-4-[4-(2-phthalimido) butyl]-piperazine hydrobromide (NAN-190; Research Biochemicals, Inc., Natick, MA, U.S.A.), ($\pm$)-atenolol (Research Biochemicals, Inc.), and butoxamine hydrochloride (Sigma) were dissolved in a small amount of dimethyl sulfoxide (DMSO) and then diluted with 0.9% saline. 5,7-dihydroxytryptamine creatinine sulfate (5,7-DHT; Sigma) was dissolved in 0.9% saline containing 0.2% ascorbic acid.

Statistical Analysis Results were expressed as the mean $\pm$ S.D. The statistical significance was calculated using a one-way analysis of variance (ANOVA) test followed by Dunnett's or Tukey's multiple comparisons test, or the Kruskal-Wallis test followed by Dunn's multiple comparisons test, Student's *t*-test or Mann-Whitney's *U*-test.

RESULTS

Inhibitory Effects of Centrally Injected Elcatonin on Acetic Acid-Induced Writhing Behavior Intracisternally injected elcatonin at doses of 0.04, 0.1 and 0.2 IU/mouse significantly decreased the number of writhes induced by intraperitoneally administered 1.2% acetic acid. Intracerebroventricularly injected elcatonin at doses of 0.1 and 0.2 IU/mouse also significantly decreased the number of writhes. However, intrathecally injected elcatonin at all doses used did not change the number of writhes significantly, although it showed a slight tendency to decrease them (Fig. 1).

Effects of Various Antagonists on the Inhibition of Acetic Acid-Induced Writhing Behavior by Intracisternally Injected Elcatonin The effects of antagonists at various neurotransmitter receptors on the inhibitory effect by intracisternally injected 0.1 IU/mouse elcatonin on acetic acid-induced writhing behavior were studied. When methysergide, a non-subtype-selective 5-HT receptor antagonist, at a dose of 10 mg/kg was subcutaneously pretreated 6 min before the administration of acetic acid, that is, 12 min before intracisternal injection of elcatonin, the inhibitory effect of elcatonin was significantly attenuated. Pretreatment with ($\pm$)-propranolol, a non-selective antagonist at the β -adrenoceptors and 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2C} receptors, also attenuated the inhibitory effect of elcatonin significantly. On the other hand, subcutaneous pretreatment with naloxone (1 and 5 mg/kg), an opioid receptor antagonist, or phentolamine (10 and 20 mg/kg), an α -adrenoceptor antagonist, did not affect the inhibitory effect of elcatonin (Fig. 2).

In the next series of experiments, the effects of the intracisternal co-administration of antagonists with elcatonin were studied. The inhibitory effect of elcatonin (0.1 IU/mouse) was not significantly affected by the co-administra-

Fig. 1. Effects of Intracisternally (a), Intracerebroventricularly (b) and Intrathecally (c) Injected Elcatonin on Acetic Acid-Induced Writhing Behavior

In the writhing test, the numbers of writhing behavior were counted during the period from 10 to 25 min after the intraperitoneal administration of acetic acid. Elcatonin was injected 6 min after the administration of acetic acid. The number in each column shows that of mice used in each group. All data are given as the mean $\pm$ S.D. ** denotes a significant difference from vehicle-treated mice at $p < 0.01$ (ANOVA test followed by Dunnett's multiple comparisons test).

tion of methysergide (30 nmol/mouse), ($\pm$)-propranolol (30 nmol/mouse) or naloxone (100 nmol/mouse) (Fig. 3).

In order to examine the involvement of spinal neurotransmitter receptors in the inhibitory effect of intracisternally injected elcatonin on acetic acid-induced writhing behavior, intrathecal pretreatments with several antagonists were studied. When methysergide at a dose of 30 nmol/mouse was intrathecally pretreated 6 min before acetic acid administration, the inhibitory effect of intracisternal elcatonin (0.1 IU/mouse) was significantly attenuated. Likewise, intrathecal injections of ($\pm$)-propranolol (30 nmol/mouse), NAN-190 (2 nmol/mouse), a selective 5-HT_{1A} antagonist, and granisetron (5 nmol/mouse), a selective 5-HT₃ antagonist, all significantly decreased the inhibition by elcatonin (Fig. 4). How-

Fig. 2. Effects of Subcutaneously Injected Methysergide (a), Propranolol (b), Naloxone (c) and Phentolamine (d) on the Inhibition of Acetic Acid-Induced Writhing Behavior by Intracisternally Injected Elcatonin

Methysergide (10 mg/kg), propranolol (10 mg/kg), naloxone (1 and 5 mg/kg) and phentolamine (10 and 20 mg/kg) were subcutaneously injected 6 min before the administration of acetic acid, and elcatonin (0.1 IU/mouse) was intracisternally injected 6 min after the administration of acetic acid. The numbers in or above the columns show those of the mice used. All data are given as the mean $\pm$ S.D. *, ** and *** denote significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively (ANOVA test followed by Tukey's multiple comparisons test).

ever, the intrathecal pretreatment with ($\pm$)-atenolol (30 and 300 nmol/mouse), a selective β_1 -adrenoceptor antagonist, or butoxamine (30 and 300 nmol/mouse), a selective β_2 -adrenoceptor antagonist, did not affect the effect of elcatonin (Figs. 5a and b). Intrathecal pretreatment with naloxone or phentolamine also did not significantly influence the effect of elcatonin (Figs. 5c and d).

Effect of 5,7-DHT-Treatment on the Inhibition of Acetic Acid-Induced Writhing Behavior by Intracisternally Injected Elcatonin Seven days after the intracerebroventricular injection of 5,7-DHT at a dose of 60 μ g, the increase in body weight was significantly inhibited compared with vehicle-treated mice. However, there was no difference in motor function or general behavior between the 5,7-DHT-treated group and the vehicle-treated group. The content of 5-HT in the lumbar cord and brain stem was significantly reduced, to 6.8% and 54%, respectively. The contents of noradrenaline and dopamine in the lumbar cord and brain stem were unchanged, indicating that the noradrenergic and dopaminergic systems were not affected by this treatment (Table 1). In 5,7-DHT-treated mice, the inhibitory effect by intracisternally injected 0.1 IU/mouse elcatonin on acetic acid-induced writhing response was significantly reduced compared with the vehicle-treated and elcatonin injected mice. The pretreatment with 5,7-DHT by itself did not affect the number of writhes induced by acetic acid (Fig. 6).

DISCUSSION

In the present study, intracisternally and intracerebroventricularly injected elcatonin significantly inhibited the writhing behavior induced by intraperitoneally administered acetic acid. The maximal effective dose was 0.1 IU/mouse in both routes of injections, and the extent of inhibition after the intracisternal injection was slightly greater than that after the intracerebroventricular one, though the difference was not statistically significant. The existence of specific binding sites for CT and the expression of CT receptor mRNA have been demonstrated in the brain of humans and rats,¹⁻⁵ including the hypothalamus, periaqueductal gray matter (PAG), locus coeruleus and raphe nuclei, which are reported to be involved in the transmission and modulation of nociceptive information. As the microinjection of salmon calcitonin (sCT) into the PAG or the medullary raphe regions induced increases in hot-plate latencies in rats¹⁹ and reduced nociceptive responses of dorsal horn neurons in cats,³⁵ elcatonin may act on these regions to exert its antinociceptive effect. In this study, however, intrathecally injected elcatonin did not significantly produce an inhibitory effect on acetic acid-induced writhing behavior, although CT binding sites have been reported in the spinal dorsal horns in rats.^{2,3} In this context, the existence of CT receptor subtypes was suggested.³ Further studies are needed to identify in detail the site where elcatonin binds and acts.

Table 1. Content of 5-HT, Noradrenaline and Dopamine (nmol/g tissue) in the Lumbar Cord and Brain Stem of Mice Treated with Vehicle or 5,7-DHT

	5-HT content		Noradrenaline content		Dopamine content	
	Lumbar cord	Brain stem	Lumbar cord	Brain stem	Lumbar cord	Brain stem
Control	5.35±1.67	9.11±1.87	3.64±0.92	8.11±0.92	0.40±0.06	1.87±0.58
5,7-DHT	0.37±0.23**	4.93±1.08***	3.36±1.61	9.18±1.48	0.39±0.04	1.74±0.27

Results are expressed as the mean±S.D. $n=6-7$. ** and *** denote significant differences from corresponding data of the control group at $p<0.01$ and $p<0.001$, respectively. As a statistical test, Student's t -test was used for NA content in the lumbar cord and brain stem, and for DA content in the brain stem, while Mann-Whitney's U -test was used for 5-HT content in the lumbar cord and brain stem, and for DA content in the brain stem.

Fig. 3. Effects of Intracisternal Co-administration of Methysergide (30 nmol/mouse) (a), Propranolol (30 nmol/mouse) (b) and Naloxone (100 nmol/mouse) (c) with Elcatonin (0.1 IU/mouse) on the Inhibition of Acetic Acid-Induced Writhing Behavior

The numbers in or above the columns show those of the mice used. All data are given as the mean±S.D. *** denotes a significant difference at $p<0.001$ (ANOVA test followed by Tukey's multiple comparisons test).

Because the subcutaneous administration of methysergide significantly attenuated the antinociceptive effect of intracisternally injected elcatonin in the mouse writhing test, it is suggested that 5-HT receptors are involved in the production of the antinociception. This result is consistent with several

findings from experiments using methods other than those in the present study in which the pretreatment with 5-HT receptor antagonists and the lesion of serotonergic neurons by a neurotoxin significantly attenuated the antinociceptive effect of CT.²⁴⁻²⁷ However, as the intracisternal co-administration of elcatonin with methysergide did not affect the antinociceptive effect of elcatonin, it is suggested that the 5-HT receptors located near the binding sites of elcatonin are not involved.

In the production of an antinociceptive effect by supra spinally injected elcatonin, the descending monoaminergic systems are possibly involved.³⁵ In order to examine this possibility, the effects of several intrathecally injected antagonists on the inhibition of acetic acid-induced writhing behavior by intracisternally injected elcatonin were studied. Intrathecal injection of methysergide, NAN-190 or granisetron significantly decreased the antinociceptive effect by elcatonin, suggesting that 5-HT receptors, especially 5-HT_{1A} receptors and 5-HT₃ receptors, in the spinal cord are involved in the production of the antinociceptive effect by intracisternal elcatonin.

In the 5,7-DHT-treated mice, the antinociceptive effect of intracisternal elcatonin was significantly reduced. As the content of 5-HT in the lumbar cord was depleted to 6.8% compared with vehicle-treated mice, most of the serotonergic neurons projecting to the spinal cord are thought to be degenerated in the 5,7-DHT-treated mice. Consequently, it is suggested that the descending serotonergic systems originating from the brain stem to the spinal cord may contribute to the production of the inhibitory effect of intracisternal elcatonin on acetic acid-induced writhing behavior.

It has been demonstrated that electrical stimulation of, or microinjection of morphine into, the PAG or nucleus raphe magnus (NRM) produces the antinociception³⁶ and the inhibition of spinal dorsal horn neurons.^{37,38} Those effects are partly mediated through the release of 5-HT in the spinal dorsal horn.^{39,40} Several behavioral and electrophysiological studies have revealed the involvement of 5-HT_{1A} and 5-HT₃ receptors in the antinociception induced by activation of the PAG or NRM following local electrical stimulation or by microinjection of opiates.^{41,42} 5-HT_{1A} receptors are negatively coupled to adenylyl cyclase, and their activation decreases neuronal excitability, whereas 5-HT₃ receptors are ligand-gated cation channels, and their activation increases that. It is speculated that 5-HT is released in the spinal dorsal horn from bulbospinal neurons by stimulation of the PAG or NRM, and that the released 5-HT acts, on one hand, on 5-HT_{1A} receptors which are located on dorsal horn neurons to inhibit them,⁴¹ and on the other hand, on 5-HT₃ receptors which are located on GABAergic or glycinergic interneurons

Fig. 4. Effects of Intrathecally Injected Methysergide (a), Propranolol (b), NAN-190 (c) and Granisetron (d) on the Inhibition of Acetic Acid-Induced Writhing Behavior by Intracisternally Injected Elcatonin

Methysergide (30 nmol/mouse), propranolol (30 nmol/mouse), NAN-190 (2 nmol/mouse) and granisetron (5 nmol/mouse) were intrathecally injected 6 min before the administration of acetic acid, and elcatonin (0.1 IU/mouse) was intracisternally injected 6 min after the administration of acetic acid. The numbers in or above the columns show those of the mice used. All data are given as the mean $\pm$ S.D. *, ** and *** denote significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. As statistical tests, an ANOVA test followed by Tukey's multiple comparisons test was used in (a), (b) and (d), and an ANOVA test followed by Dunnett's multiple comparisons test was used in (c).

to induce the release of inhibitory amino acids,^{42,43} consequently dorsal horn neurons are inhibited. These findings, together with our results, suggest that intracisternally injected elcatonin may produce an inhibitory effect on acetic acid-induced writhing behavior through mechanisms similar to those mentioned above. This suggestion is supported by findings that the microinjection of sCT into the PAG or the medullary raphe regions induced the antinociception¹⁹) and the inhibition of nociceptive responses of dorsal horn neurons.³⁵⁾

When ($\pm$)-propranolol was subcutaneously or intrathecally pretreated, the antinociceptive effect by elcatonin was decreased. ($\pm$)-Propranolol has high binding affinities for 5-HT_{1A}, 5-HT_{1B} and 5-HT_{2C} receptors^{44,45}) in addition to β -adrenoceptors. Furthermore, the intrathecal injection of neither ($\pm$)-atenolol, a selective β_1 -adrenoceptor antagonist, nor butoxamine, a selective β_2 -adrenoceptor antagonist, influenced the antinociceptive effect of elcatonin. Therefore, it is likely that the attenuation by ($\pm$)-propranolol of the antinociceptive effect by intracisternal elcatonin is attributed to antagonism at the 5-HT receptors in the spinal cord rather than that at the β -adrenoceptors. However, we cannot exclude the possibility that the membrane-stabilizing effect of ($\pm$)-propranolol may influence the antinociceptive action of elcatonin. Subcutaneously or intrathecally injected phentolamine did not influence the antinociceptive effect of elcatonin, suggesting that α -adrenoceptors are not involved in the effect.

This result is consistent with the finding that phentolamine does not antagonize the antinociceptive effect of CT injected intracerebroventricularly.^{24,27)} From these results with several adrenoceptor antagonists, descending noradrenergic systems may not be involved in the mediation of the antinociception by intracisternal elcatonin.

Naloxone did not significantly influence the antinociceptive effect of elcatonin by any route of injection. Although evidence against^{19,46,47)} or for^{21–23)} the involvement of endogenous opioids in CT-induced antinociception has been reported, our current results suggest that opioid receptors may not be involved in the inhibitory effect of acetic acid-induced writhing behavior by centrally injected elcatonin.

In summary, the results from the present experiments suggest that intracisternal elcatonin activates the inhibitory descending serotonergic systems from the brain stem to the spinal dorsal horn, and the released serotonin stimulates 5-HT_{1A} and 5-HT₃ receptors in the latter region to inhibit acetic acid-induced writhing behavior. However, the central opioidergic and inhibitory descending noradrenergic systems are unlikely to be involved in the inhibition of writhing behavior by intracisternal elcatonin.

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Fig. 5. Effects of Intrathecally Injected Atenolol (a), Butoxamine (b), Naloxone (c) and Phentolamine (d) on the Inhibition of Acetic Acid-Induced Writhing Behavior by Intracisternally Injected Elcatonin

Atenolol (30 and 300 nmol/mouse), butoxamine (30 and 300 nmol/mouse), naloxone (100 nmol/mouse) and phentolamine (30 nmol/mouse) were intrathecally injected 6 min before the administration of acetic acid, and elcatonin (0.1 IU/mouse) was intracisternally injected 6 min after the administration of acetic acid. The numbers in or above the columns show those of the mice used. All data are given as the mean $\pm$ S.D. *, **, and *** denote significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. As statistical tests, the Kruskal-Wallis test followed by Dunnett's multiple comparisons test was used in (a), (b) and (c), and ANOVA followed by Tukey's multiple comparisons test was used in (d).

Fig. 6. Effect of Depletion of 5-HT by 5,7-DHT on the Inhibition of Acetic Acid-Induced Writhing Behavior by Intracisternally Injected Elcatonin

5,7-DHT (60 μ g/mouse) was intracerebroventricularly injected 7 days before the experimental day.

Elcatonin (0.1 IU/mouse) was intracisternally injected 6 min after the administration of acetic acid. The numbers in or above the columns show those of the mice used. All data are given as the mean $\pm$ S.D. *, **, and *** denote significant differences at $p < 0.01$ and $p < 0.001$, respectively (Kruskal-Wallis test followed by Dunnett's multiple comparisons test).

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