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Neuronal mechanism of the inhibitory effect of calcitonin on *N*-methyl-D-aspartate-induced aversive behavior

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Abstract

To elucidate the mechanism of antinociceptive effects of calcitonin, we investigated whether receptor antagonists for various neurotransmitter receptors alter the inhibitory effect of calcitonin on intrathecally injected *N*-methyl-D-aspartate-induced aversive behavior in mice. Neither naloxone, an opioid receptor antagonist, phentolamine and benextramine, α-adrenoceptor antagonists, nor ritanserin, a 5-HT_{2A} receptor antagonist, inhibited the calcitonin-induced anti-aversive effects. Pindolol and (−)-propranolol, non-selective antagonists of β-adrenoceptors and 5-HT_1 receptors, 1-(2-methoxyphenyl)-4-[4-(2-phethalimido) butyl]-piperazine hydrobromide (NAN-190), a 5-HT_{1A} receptor antagonist, 3-tropanyl-3,5-dichlorobenzoate (MDL72222) and metoclopramide, 5-HT_3 receptor antagonists, significantly inhibited the calcitonin-induced anti-aversive effects. (−)-Bicuculline, a $GABA_A$ receptor antagonist, phaclofen and 5-aminovaleric acid, $GABA_B$ receptor antagonists, also attenuated the calcitonin-induced anti-aversive effects. These results suggest that β-adrenoceptor, 5-HT_{1A}, 5-HT_3, $GABA_A$ and $GABA_B$ receptors, but not α-adrenoceptor, opioid nor 5-HT_{2A} receptors, are involved in the inhibitory effect of calcitonin on intrathecally injected *N*-methyl-D-aspartate-induced aversive behavior in mice.

Keywords: Calcitonin; NMDA (*N*-methyl-D-aspartate); Aversive behavior; β-Adrenoceptor; 5-HT receptor; GABA receptor

1. Introduction

Calcitonin has been shown to have analgesic activities against pain due to malignancy (Hindley et al., 1982) and migraine (Patti et al., 1986), and against phantom limb pain (Jaeger and Maier, 1992). With regard to the mechanisms of calcitonin-induced analgesia, it has been hypothesized that the hormone influences generation of pain-inducing or pain-exacerbating substances (Pecile et al., 1975). Calcitonin has been reported to inhibit the synthesis of both prostaglandins and thromboxanes in vitro (Ceserani et al., 1979), and to increase plasma β-endorphin and adrenocorticotropic hormone (ACTH) levels (Üstdal et al., 1989; Franceschini et al., 1989; Laurian et al., 1986) in humans. However, it has been reported that calcitonin-induced increases in β-endorphin levels in plasma and cerebrospinal fluid (Fabbri et al., 1981), and plasma ACTH levels (Franceschini et al., 1989) lacked significance. Further, we have shown that in mice calcitonin in combination with acetic acid has no effect on plasma prostaglandin E_2 or β-endorphin levels, although it increased the ACTH level (Maeda et al., 1994a). Therefore, the relationship between calcitonin-induced analgesia and endocrine substances is still ambiguous.

Calcitonin binding sites have been demonstrated in human and rat brain homogenates (Fischer et al., 1981a,b; Nakamuta et al., 1981; Rizzo and Goltzman, 1981). Calcitonin binding sites are located mainly in the hypothalamic region and midbrain (Goltzman and Mitchell, 1985), in well-defined brainstem areas involved in pain-regulating mechanisms. Furthermore, by autoradiography, the calcitonin binding sites were shown to reside in the hypothalamus, mesencephalic reticular formation, periaqueductal gray, locus coeruleus, and the raphe obscurus (Henke et al., 1983; Olgiati et al., 1983; Fabbri et al., 1985). Calcitonin binding has also been reported to occur in the spinal cord (Goltzman and Mitchell, 1985). These findings suggest that the sites of action of calcitonin-induced

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antinociception may be somewhere in these regions. For example, it has been reported that injection of calcitonin into the periaqueductal gray matter, but not into the hypothalamus, induced antinociception (Fabbri et al., 1985). Systemic injection of calcitonin has been reported to produce no analgesic effect, suggesting that these effects are not produced peripherally (Morton et al., 1986). With regard to the spinal calcitonin binding sites, it has been reported that intrathecal injection of calcitonin does not produce analgesia in rats (Wiesenfeld-Hallin and Persson, 1984), while others have reported that spinal calcitonin does indeed produce antinociceptive effects when administered via this route (Spampinato et al., 1984; Guidobono et al., 1986; Nabeshima et al., 1993).

Excitatory amino acids act as transmitters in the central projection of nociceptive information (Frenk et al., 1988; Wilcox, 1988). When excitatory amino acids are injected intrathecally into mice, they induce aversive responses such as scratching and tail-biting (Sakurada et al., 1990). In previous studies, we reported that i.c.v. injection of calcitonin inhibited the aversive responses induced by intrathecally injected *N*-methyl-D-aspartate, kainic acid, and quisqualic acid (Maeda et al., 1994b; Nabeshima et al., 1993). In the present study, to elucidate the mechanism of this inhibitory effect of calcitonin on intrathecally injected *N*-methyl-D-aspartate-induced aversive behavior in mice, we investigated the effects of antagonists of various neurotransmitter receptors on the calcitonin-induced anti-aversive effects.

2. Materials and methods

2.1. Animals

Male *ddY* mice (Nihon SLC Co., Shizuoka, Japan) weighing 22–27 g were used. Animals were given food and water ad libitum and were kept under controlled environmental conditions (23 ± 4°C, 55 ± 5% humidity) with a 12-h light-dark cycle (9 a.m. to 9 p.m. light) for at least 5 days before experiments.

2.2. Drugs

Salmon calcitonin (Sandoz, Basel, Switzerland) was dissolved in acetic acid buffer (pH 4.28). *N*-Methyl-D-aspartate (Sigma, St. Louis, MO, USA), morphine hydrochloride (Takeda Chem. Ind., Osaka, Japan), naloxone hydrochloride (Sigma), phentolamine mesylate (Research Biochemicals International, MA, USA), benextramine tetrahydrochloride (Research Biochemicals Int.), (−)-propranolol hydrochloride (Research Biochemicals Int.), metoclopramide monohydrochloride (Sigma), (−)-bicuculline methiodide (Research Biochemicals Int.), phaclofen (Research Biochemicals), and 5-aminovaleric acid (Research Biochemicals Int.) were dissolved in 0.9% saline. Pindolol (Sigma) was dissolved in saline containing 0.3% carboxymethylcellulose. 1-(2-Methoxyphenyl)-4-[4-(2-phethalimido) butyl]-piperazine hydrobromide (NAN-190; Research Biochemicals Int.) and 3-tropanyl-3,5-dichlorobenzoate (MDL72222; Research Biochemicals Int.) were dissolved in a small amount of dimethyl sulfoxide (DMSO), and then diluted in saline. Ritanserin was dissolved in distilled water with a small amount of lactic acid, and then the pH was adjusted to about 4.0 with sodium bicarbonate.

Each animal was injected with *N*-methyl-D-aspartate in a volume of 5 μl into the intervertebral space between L5 and L6 (i.t. injection). Calcitonin was injected intracerebroventricularly (i.c.v.) in a volume of 10 μl, 20 min before the injection of *N*-methyl-D-aspartate. The control group received injection with vehicle only (acetic acid buffer, pH 4.28). The i.c.v. and i.t. injections were carried out following the methods of Haley and McCormick (1957) and Hylden and Wilcox (1980), respectively.

Ritanserin (s.c.) was administered 60 min, phentolamine (i.p.) and NAN-190 (i.p.) 45 min, pindolol (i.p.), (−)-bicuculline (i.p.), and phaclofen (i.c.v.) 30 min, metoclopramide (i.p.) 25 min, calcitonin (i.c.v.), benextramine (i.p.), MDL72222 (i.p.) and AVA (i.c.v.) 20 min, (−)-propranolol (i.p.) and morphine (i.c.v.) 15 min, and naloxone (s.c.) 5 min before *N*-methyl-D-aspartate injection, respectively.

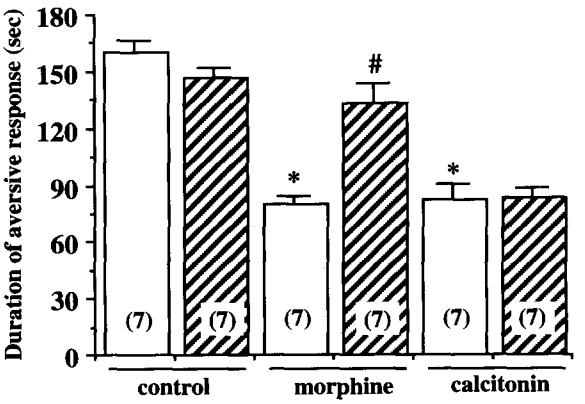

Fig. 1. The effects of naloxone on morphine- and calcitonin-induced inhibition of *N*-methyl-D-aspartate-induced aversive behavior. Calcitonin (0.1 IU/mouse i.c.v.), morphine (1 nmol/mouse i.c.v.), and naloxone (1 mg/kg s.c.) were administerd 20, 15 and 5 min, respectively, before injection of *N*-methyl-D-aspartate (1 nmol/mouse i.t.). The duration of *N*-methyl-D-aspartate-induced aversive behavior was observed over a 3-min period as described in the Materials and methods section. Numbers in parentheses are the number of animals used. Each column represents the mean ± S.E. Open columns: vehicle, s.c.; hatched columns: naloxone, s.c. $^{*}P < 0.05$ vs. vehicle-injected control group. $^{\#}P < 0.05$ vs. vehicle-injected morphine-treated group.

2.3. Behavioral study

The duration of aversive behavior, i.e. scratching and tail-biting, induced by i.t. injection of *N*-methyl-D-aspartate was recorded for a 3-min period commencing with placement of the animal in an observation chamber following drug injection as described previously (Maeda et al., 1994b).

2.4. Statistics

Results are expressed as mean ± S.E. Data were analyzed by the Scheffe *F*-test. *P* values of < 0.05 were regarded as significant.

3. Results

As shown in Fig. 1, *N*-methyl-D-aspartate (1 nmol/mouse i.t.) produced aversive behaviors such as scratching and tail-biting lasting for 160.4 ± 13.8 s. Calcitonin (0.1 IU/mouse, i.c.v.) and morphine (0.1 nmol/mouse, i.c.v.) significantly inhibited this *N*-methyl-D-aspartate-induced aversive behavior. Naloxone (1 mg/kg s.c.), an opioid receptor antagonist, significantly antagonized morphine-induced, but not calcitonin-induced inhibition of *N*-methyl-D-aspartate-induced aversive behavior.

As shown in Fig. 2A and B, neither phentolamine (10 and 20 mg/kg i.p.) nor benextramine (10 and 20 mg/kg i.p.), both α-adrenoceptor antagonists, had any effect on *N*-methyl-D-aspartate-induced aversive behavior. Further, these compounds failed to affect the inhibitory effect of calcitonin on this behavior.

On the other hand, both pindolol (10 and 20 mg/kg i.p.; Fig. 2C) and (−)-propranolol (10 and 15 mg/kg i.p.; Fig. 2D), non-selective β-adrenoceptor and 5-HT_1 receptor antagonists, significantly and dose-dependently antagonized the inhibitory effect of calcitonin on *N*-methyl-D-aspartate-induced aversive behavior, although both compounds themselves had no effect on this behavior.

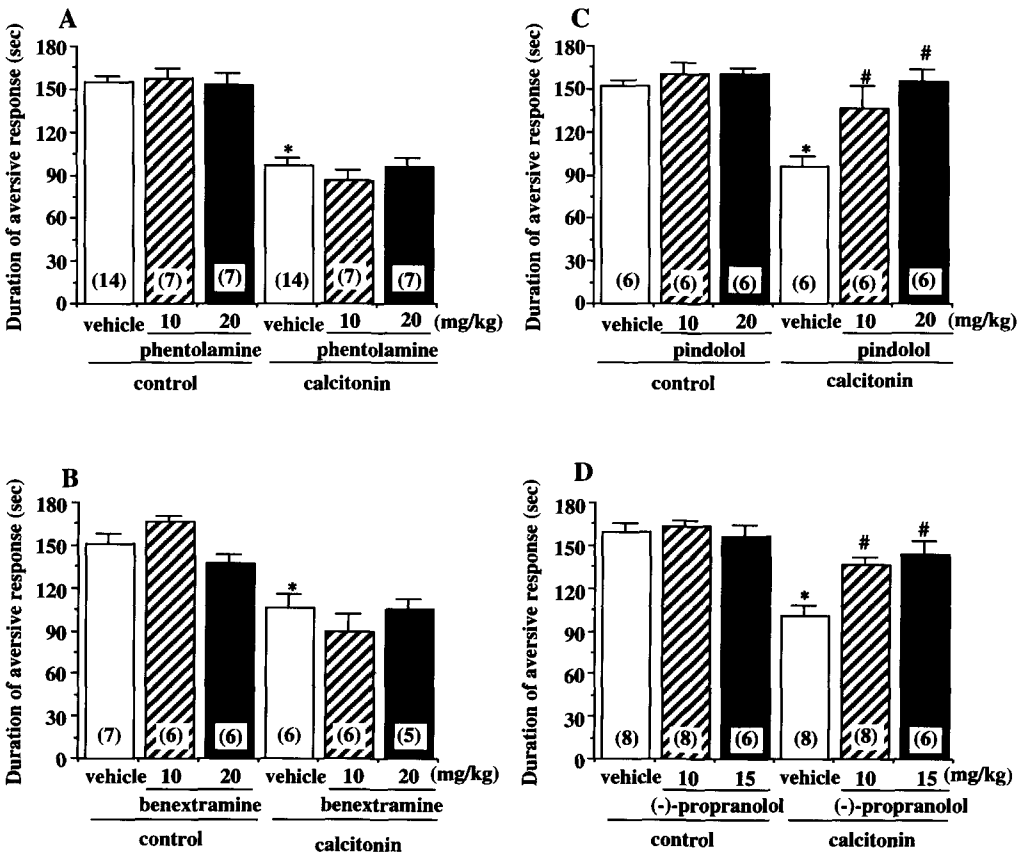

Fig. 2. The effects of phentolamine (A), benextramine (B), pindolol (C) and (−)-propranolol (D) on calcitonin-induced inhibition of *N*-methyl-D-aspartate-induced aversive behavior. Phentolamine (10 and 20 mg/kg i.p.), benextramine (10 and 20 mg/kg i.p.), pindolol (10 and 20 mg/kg i.p.) and (−)-propranolol (10 and 15 mg/kg i.p.) were administered 45, 20, 30 and 15 min, respectively, before injection of *N*-methyl-D-aspartate (1 nmol/mouse i.t.). Calcitonin (0.1 IU/mouse i.c.v.) was injected 20 min before *N*-methyl-D-aspartate (1 nmol/mouse i.t.) injection. The duration of *N*-methyl-D-aspartate-induced aversive behavior was observed over a 3-min period as described in the Materials and methods section. Numbers in parentheses are the number of animals used. Each column represents the mean ± S.E. $^{*}P < 0.05$ vs. vehicle-injected control group. $^{\#}P < 0.05$ vs. vehicle-injected calcitonin-treated group.

As shown in Fig. 3A, while NAN-190 (1.5 and 3 mg/kg i.p.), a 5-HT_{1A} receptor antagonist, had no effect on *N*-methyl-D-aspartate-induced aversive behavior, it significantly and dose-dependently antagonized the inhibitory effect of calcitonin on this response to *N*-methyl-D-aspartate.

Ritanserin (1 and 2 mg/kg s.c.), a 5-HT_{2A} receptor antagonist, neither affected *N*-methyl-D-aspartate-induced aversive behavior nor the inhibitory effect of calcitonin on this behavior (Fig. 3B).

As shown in Fig. 3C and D, MDL72222 (1 and 2 mg/kg i.p.) and metoclopramide (0.5 and 1 mg/kg i.p.), 5-HT_3 receptor antagonists, significantly and dose-dependently antagonized the inhibitory effect of calcitonin on *N*-methyl-D-aspartate-induced aversive behavior, although neither compound itself had any effect on this response to *N*-methyl-D-aspartate.

(−)-Bicuculline (1, 2 and 3 mg/kg i.p.), a $GABA_A$ receptor antagonist, significantly and dose-dependently antagonized the inhibitory effect of calcitonin on *N*-methyl-D-aspartate-induced aversive behavior, although the compound itself had no effect on this behavior (Fig. 4A).

Phaclofen (50 and 100 nmol/mouse i.c.v.) and 5-aminovaleric acid (0.5 and 1 μl/mouse i.c.v.), $GABA_B$ receptor antagonists, both significantly and dose-dependently antagonized the inhibitory effect of calcitonin on *N*-methyl-D-aspartate-induced aversive behavior, although neither compound itself had any effect on this response to *N*-methyl-D-aspartate (Fig. 4B and C).

4. Discussion

In agreement with previous studies (Braga et al., 1978; Candeletti et al., 1985; Fabbri et al., 1981, 1985; Spampinato et al., 1984), naloxone (1 mg/kg) failed to

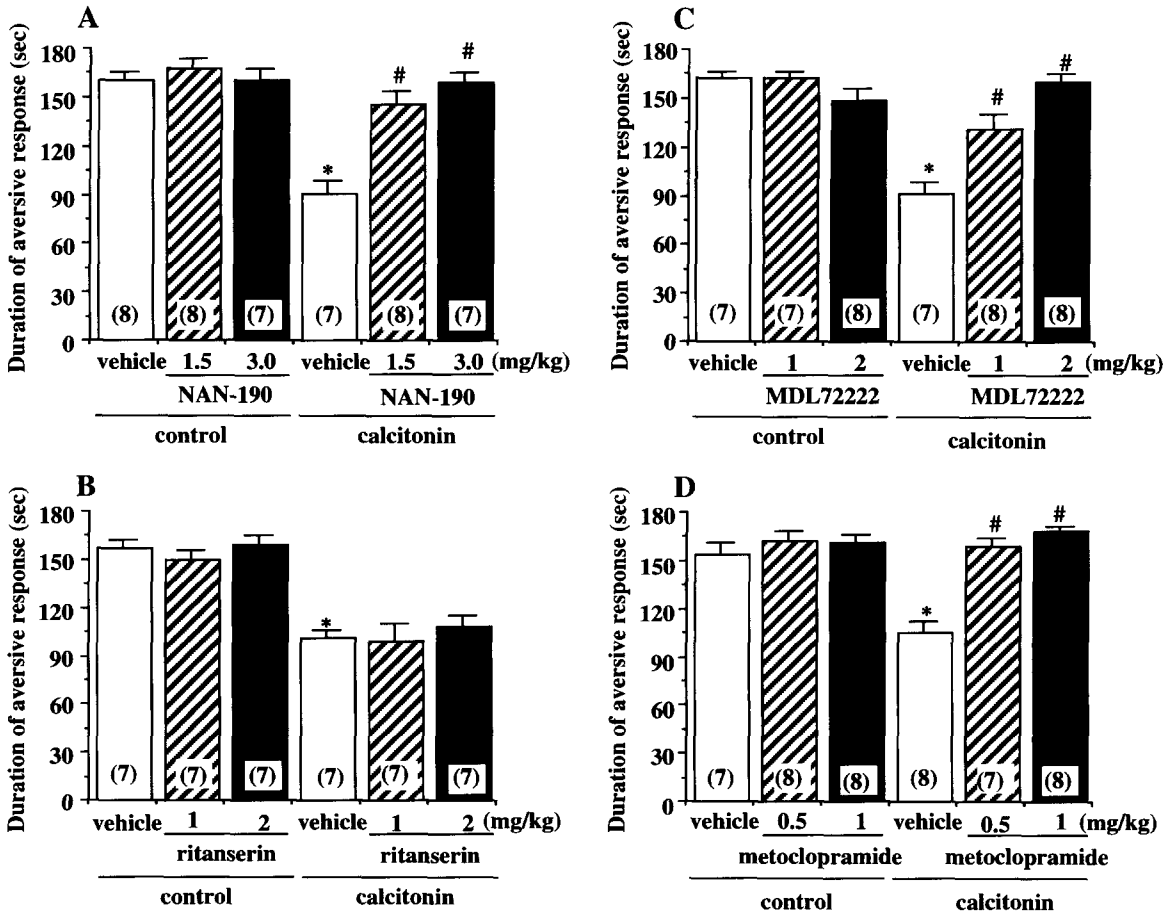

Fig. 3. The effects of NAN-190 (A), ritanserin (B), MDL 72222 (C) and metoclopramide (D) on calcitonin-induced inhibition of *N*-methyl-D-aspartate-induced aversive behavior. NAN-190 (1.5 and 3.0 mg/kg i.p.), ritanserin (1 and 2 mg/kg s.c.), MDL 72222 (1 and 2 mg/kg i.p.), metoclopramide (0.5 and 1 mg/kg i.p.) and calcitonin (0.1 IU/mouse i.c.v.) were administered 45, 60, 20, 25 and 20 min, respectively, before injection of *N*-methyl-D-aspartate (1 nmol/mouse i.t.). The duration of *N*-methyl-D-aspartate-induced aversive behavior was observed over a 3-min period as described in the Materials and methods section. Numbers in parentheses are the number of animals used. Each column represents the mean ± S.E. $^{*}P < 0.05$ vs. vehicle-injected control group. $^{\#}P < 0.05$ vs. vehicle-injected calcitonin-treated group.

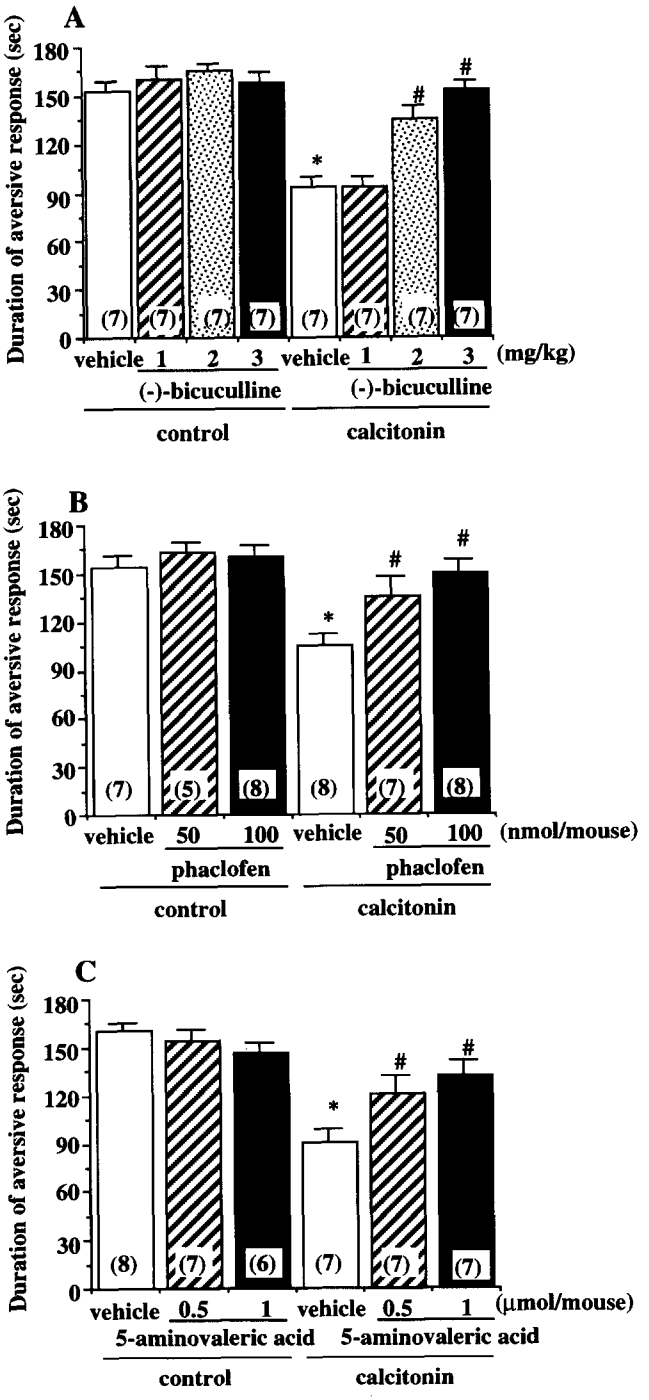

Fig. 4. The effects of (−)-bicucuclline (A), phaclofen (B) and 5-aminovaleric acid (C) on calcitonin-induced inhibition of *N*-methyl-D-aspartate-induced aversive behavior. (−)-Bicuculline (1, 2 and 3 mg/kg i.p.), phaclofen (50 and 100 nmol/mouse i.c.v.), 5-aminovaleric acid (0.5 and 1 μmol/mouse i.c.v.) and calcitonin (0.1 IU/mouse i.c.v.) were administered 30, 30, 20 and 20 min, respectively, before injection of *N*-methyl-D-aspartate (1 nmol/mouse i.t.). The duration of *N*-methyl-D-aspartate-induced aversive behavior was observed over a 3-min period as described in the Materials and methods section. Numbers in parentheses are the number of animals used. Each column represents the mean ± S.E. $^{*}P < 0.05$ vs. vehicle-injected control group. $^{\#}P < 0.05$ vs. vehicle-injected calcitonin-treated group.

block the inhibitory effect of calcitonin on the aversive behavior induced by intrathecally injected *N*-methyl-D-aspartate, while it antagonized the anti-aversive effects of morphine. Therefore, it is suggested that opioid mechanisms may not be involved in the anti-aversive effects of calcitonin.

Aanonsen and Wilcox (1987) reported that norepinephrine inhibits *N*-methyl-D-aspartate (i.t.)-induced aversive responses, and that phentolamine reverses the norepinephrine-induced inhibition, suggesting that norepinephrine neurons modulate *N*-methyl-D-aspartate-induced aversive behavior. With regard to calcitonin-induced analgesia, Guidobono et al. (1985) have shown that propranolol reduced the analgesic effect of calcitonin in rats. In the present study, neither phentolamine nor benextramine, both α-adrenoceptor antagonists, inhibited anti-aversive effects of calcitonin (Fig. 2A and B), while pindolol and (−)-propranolol, non-selective β-adrenoceptor and 5-HT_1 receptor antagonists, antagonized the inhibitory effect of calcitonin on *N*-methyl-D-aspartate-induced aversive behavior (Fig. 2C and D). These results indicate the involvement of 5-HT receptors and β- but not α-adrenoceptors in the anti-aversive effects of calcitonin.

With regard to the involvement of 5-HT receptors in the effects of calcitonin, it has been demonstrated that calcitonin-induced analgesia is antagonized by methysergide, a non-selective 5-HT receptor antagonist, in the hot plate test (Clementi et al., 1984). In contrast, other investigators failed to demonstrate the involvement of serotonergic systems in calcitonin-induced analgesia (Candeletti et al., 1985; Guidobono et al., 1986). The i.t. injection of 8-hydroxy-2-(di-*n*-propylamino)tetralin, a 5-HT_{1A} receptor agonist, increases nociceptive threshold in the hot plate test, and inhibits the aversive response induced by i.t. injection of *N*-methyl-D-aspartate (Alhaider and Wilcox, 1993). Conversely, i.t. injection of RU 24969, a 5-HT_{1B} receptor agonist, has no effect on the nociceptive threshold in the hot plate test or on the aversive behavior induced by *N*-methyl-D-aspartate (Mjellem et al., 1992). The i.t. injection of NAN-190, a selective 5-HT_{1A} receptor antagonist (Glennon et al., 1988a,b; Millian et al., 1991), increases the aversive response induced by *N*-methyl-D-aspartate (Mjellem et al., 1993), suggesting that stimulation of 5-HT_{1A} receptors, but not of 5-HT_{1B} receptors, modulates spinal antinociception. In the present study, we demonstrated that calcitonin-induced anti-aversive effects were inhibited by NAN-190 (Fig. 3A). Therefore, 5-HT_1 receptors, especially 5-HT_{1A} receptors, may be involved in the calcitonin-induced amelioration of the aversive effects of *N*-methyl-D-aspartate. In contrast to the obervation made by Mjellem et al. (1993) that i.t. injection of NAN-190 potentiates *N*-methyl-D-aspartate (i.t.)-induced aversive behavior, *N*-methyl-D-aspartrate (i.t.)-induced

aversive behavior in mice did not change by the treatment with NAN-190 (i.p.) alone. This discrepancy may be due to the different route of injection of NAN-190 and different concentration of the agent in the spinal cord. Since the identification of NAN-190 as a selcetive 5-HT_{1A} receptor antagonist has been recently questioned (Fletcher et al., 1993), further experiments with other selective antagonists should be carried out to confirm the involvement of 5-HT_{1A} receptors in the calcitonin-induced inhibition of *N*-methyl-D-aspartate-induced aversive behavior.

It has been reported that i.t. injection of α-methyl-5-HT, a selective 5-HT_2 receptor agonist, induces an aversive response (Wilcox and Alhaider, 1990), suggesting that 5-HT_2 receptors are involved in the excitation of spinal nociceptive processing. However, ritanserin, a 5-HT_{2A} receptor antagonist, neither inhibited *N*-methyl-D-aspartate-induced aversive behavior, nor potentiated the anti-aversive effect of calcitonin (Fig. 3B), suggesting that 5-HT_{2A} receptors are not involved in the effects of calcitonin.

ICS 205-930, a 5-HT_3 receptor antagonist, blocks the increase in the nociceptive threshold in tail flick and hot plate tests produced by intrathecal injection of 5-HT (Glaum et al., 1988,1990), but 2-methyl-5-HT, a 5-HT_3 receptor agonist, blocks the aversive response induced by i.t. injection of *N*-methyl-D-aspartate (Alhaider et al., 1991; Wilcox and Alhaider, 1990). Moreover, zacopride, another 5-HT_3 receptor antagonist, inhibits the increase in tail flick and hot plate latencies produced by i.t. injection of 2-methyl-5-HT (Alhaider et al., 1991). These results suggest that 5-HT_3 receptors are involved in spinal nociception. In the present study, both metoclopramide and MDL 72222, known 5-HT_3 antagonists (Smith et al., 1988; Costall et al., 1988; Glaum et al., 1990), inhibited the anti-aversive effect of calcitonin (Fig. 3C and D), suggesting that not only 5-HT_{1A} but also 5-HT_3 receptors may be involved in the inhibitory effect of calcitonin on the aversive behavior induced by intrathecal injection of *N*-methyl-D-aspartate. It has been reported that calcitonin-induced analgesic effects in tail flick and hot plate tests are antagonized by administration of 5,7-dihydroxytryptamine, a 5-HT neurotoxin, into the nucleus raphe dorsalis (Clementi et al., 1985), and that calcitonin enhances the release of 5-HT in spinal cord slices in vitro (Bourgoin et al., 1988). Therefore, the observed anti-aversive effects of calcitonin may be due to stimulation of descending 5-HT neurons and subsequent activation of 5-HT_{1A} and 5-HT_3 receptors in the spinal cord.

Muscimol, a $GABA_A$ receptor agonist, produces antinociception in various nociceptive tests; in tail flick and hot plate tests after p.o. administration (Spaulding et al., 1980; Liebman and Pastor, 1980; Hill et al., 1981; Sawynok and LaBella, 1982), in the pinch test after i.c.v. administration (Liebman and Pastor, 1980), in the tail flick test after rostral ventromedial medulla administration (Heinricher and Kaplan, 1991) or i.t. administration (Hammond and Drower, 1984), and it inhibits the aversive response induced by i.t. injection of *N*-methyl-D-aspartate (Aanonsen and Wilcox, 1989). Thus, $GABA_A$ receptors appear to play a modulatory role in nociception. In the present study, bicuculline, a $GABA_A$ receptor antagonist, blocked the inhibitory effect of calcitonin on the *N*-methyl-D-aspartate-induced aversive behavioral response (Fig. 4A), suggesting the involvement of $GABA_A$ receptors in the antinociceptive effects of calcitonin. Alhaider et al. (1991) suggested that raphe-spinal neurons release 5-HT in the spinal cord, and that this released 5-HT activates 5-HT_3 receptors on GABAergic interneurons, inducing GABA release by these cells which may in turn inhibit nociceptive transmission at a site postsynaptic to the terminals of primary afferent fibers when *N*-methyl-D-aspartate is used as a nociceptive stimulus. With regard to our present findings along with this hypothesis proposed by Alhaider et al. (1991), we postulate that calcitonin may produce antinociceptive effects by activating the descending 5-HT neurons. Subsequent activation by released 5-HT of 5-HT_{1A} and/or 5-HT_3 receptors on GABAergic neurons in the spinal cord may result, mediated via $GABA_A$ receptors, in the observed inhibition of the nociceptive response to *N*-methyl-D-aspartate. Further studies are, however, required to confirm this hypothesis.

Baclofen, a $GABA_B$ receptor agonist, produces analgesic effects in various nociceptive tests when administered systemically (Liebman and Pastor, 1980; Hill et al., 1981; Sawynok and LaBella, 1982; Giuliani et al., 1988), intracerebroventricularly (Liebman and Pastor, 1980), and intrathecally (Hammond and Drower, 1984; Sawynok and Dickson, 1985; Wilson and Yaksh, 1978). In the present study, phaclofen and 5-aminovaleric acid, both $GABA_B$ receptor antagonists, blocked the inhibitory effect of calcitonin on *N*-methyl-D-aspartate-induced aversive behavior (Fig. 4B and C), suggesting that $GABA_B$ receptors in the brain are also involved in the anti-aversive effects of calcitonin.

In conclusion, the inhibitory effect of calcitonin on *N*-methyl-D-aspartate-induced aversive behavior was antagonized by pindolol, (−)-propranolol, NAN-190, MDL72222, metoclopramide, (−)-bicuculline, phaclofen and 5-aminovaleric acid, but not by naloxone, phentolamine, benextramine or ritanserin. These results suggest that β-adrenoceptor, 5-HT_{1A}, 5-HT_3, $GABA_A$ and $GABA_B$ receptors, but not α-adrenoceptor, opioid nor 5-HT_{2A} receptors, are involved in the inhibitory effect of calcitonin on intrathecally injected *N*-methyl-D-aspartate-induced aversive behavior in mice.

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