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Serotonergic mechanisms involved in calcitonin potentiation of κ-opioid receptor-mediated effects on adrenal secretion

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Abstract

Calcitonin can selectively modulate the effects of opioids on the rat hypothalamic–pituitary–adrenal axis and increase the release of corticosterone induced by a κ-opioid receptor agonist. Considerable evidence supports the involvement of opioid and serotonergic systems in the analgesic effect of calcitonin. In this study, the involvement of hypothalamic serotonergic pathways in the calcitonin potentiation of the effect of (*trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl) cyclohexyl]-benzeneacetamide methane sulphonate (U-50,488H) on the secretion of corticosterone was examined. The correlation between the calcitonin-induced potentiation of the pituitary–adrenal response to U-50,488H and changes in serotonin turnover was evaluated. Our results show that the increase in the release of corticosterone induced by treatment with calcitonin + U-50,488H was not evident when the turnover of serotonin was decreased by inhibition of its synthesis with *m*-hydroxybenzylhydrazine (NSD 1015) or by blockade of its metabolism with *trans*-2-phenylcyclopropylamine (tranylcypromine). Although other factors can not be discarded, from the present data it can be suggested that the serotonergic system plays an important role in the interaction calcitonin-κ-opioid receptor agonist in the hypothalamic–pituitary–adrenal axis. © 1997 Elsevier Science B.V.

Keywords: Hypothalamic–pituitary–adrenal axis; Corticosterone release; Calcitonin, 5-HT (5-hydroxytryptamine, serotonin) turnover; κ-Opioid receptor

1. Introduction

There is evidence that opioids may be important in the control of the hypothalamic–pituitary–adrenal axis, although the nature of the opioidergic control of the axis might be different depending on the species. In humans, only an inhibitory action of morphine has been demonstrated (Pechnick, 1993), whereas in rats, acute administration of morphine and related opioid receptor agonists produces an increased hypothalamic–pituitary–adrenal axis activity, which results in an enhanced secretion of corticosterone and β-endorphin (Buckingham and Cooper, 1984; Martínez et al., 1990; Pechnick, 1993). These neuroendocrine effects involve the activation of hypothalamic μ-, δ- and κ-opioid receptors (Gonzálvez et al., 1991; Milanés et al., 1991; Alcaraz et al., 1993a,b).

Several lines of evidence have suggested that the polypeptide calcitonin may exert important effects in the central nervous system (CNS). These effects include analgesia (Spampinato et al., 1984; Welch and Dewey, 1990; Martín et al., 1992), alteration of prolactin secretion (Clementi et al., 1983) and behavioural changes (Clementi et al., 1984a). Furthermore, calcitonin administration has been reported to increase hypothalamic–pituitary–adrenal axis activity in humans, and it was proposed that its analgesic effect might be mediated by an increase in the release of β-endorphin from the anterior pituitary (Laurian et al., 1986). Studies from other authors have also suggested a role of opioid mechanisms in the analgesic effect of calcitonin (Collin et al., 1989; Martín et al., 1992), but non-opioid mechanisms have also been postulated (Clementi et al., 1989).

Previous studies from our laboratory have demonstrated that calcitonin can selectively modulate the neuroendocrine effects of opioids on the hypothalamic–pituitary–adrenal axis, through κ- but not through μ-opioid receptors. Thus, calcitonin increased the release of corticosterone induced by the highly selective κ-opioid receptor agonist U-50,488H, a response which was fully antagonized by the

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selective κ-opioid receptor antagonist nor-binaltorphimine (Milanés et al., 1993). This potentiation was seen with doses of U-50,488H that produced no effects on the axis when administered alone. Calcitonin did not modify the actions of morphine (a preferential μ-opioid receptor agonist) on the hypothalamic–pituitary–adrenal axis, since plasma corticosterone levels were unchanged after the injection of the opioid. These data agree with those of in vitro studies showing that calcitonin increased the inhibitory effects of κ-opioid receptor agonists on the guinea-pig ileum, whereas it produced no changes in the effects of [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol] enkephalin (DAMGO), a selective μ-opioid receptor agonist (Martín et al., 1993).

Several lines of evidence support the involvement of opioid and serotonergic systems in the analgesic effect of calcitonin, although the mechanism of this action has not been elucidated (Clementi et al., 1984b, 1985; Colado et al., 1994; Martín et al., 1996).

The objective of the present study was to examine the possible involvement of hypothalamic serotonergic pathways in the calcitonin potentiation of U-50,488H-induced pituitary–adrenal secretion. In order to do this, two experimental schedules were followed: (1) the possible correlation between the calcitonin-induced potentiation of the pituitary–adrenal response to U-50,488H and changes in serotonin content and turnover were examined and (2) the calcitonin-induced potentiation of the neuroendocrine effects of U-50,488H was studied in rats whose serotonergic synthesis was inhibited and in rats whose serotonergic metabolism was blocked. Serotonin turnover rates were measured as the accumulation of the 5-hydroxytryptamine (5-HT) precursor 5-hydroxytryptophan (5-HTP) after inhibition of aromatic amino acid decarboxylase, and by evaluating the hypothalamic changes in the content of 5-HT and its metabolite 5-hydroxy-indolacetic acid (5-HIAA) after inhibition of monoamine oxidase. Plasma levels of corticosterone (as a marker of pituitary–adrenal activity) were evaluated, simultaneously with 5-HT analysis, after the same treatments.

2. Materials and methods

2.1. Animals and drugs

Male Sprague–Dawley rats (200–250 g) were housed four to five per cage under a 12 h light/dark cycle (light: 08.00–20.00 h), in a room with controlled temperature (22 ± 1°C) and humidity (50 ± 10%) and food and water available ad libitum.

The following drugs were used: salmon-calcitonin (Rhône-Poulenc Rorer S.A., Madrid, Spain); U-50,488H (*trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl) cyclohexyl]-benzeneacetamide methane sulphonate; Upjohn, Kalamazoo, MI); tranylcypromine (*trans*-2-phenylcyclopropylamine) HCl (Sigma Chemical Co); NSD 1015 (*m*-hydroxybenzylhydrazine; Sigma Chemical Co). Drugs were prepared fresh before use and were dissolved in sterile 0.9% NaCl (saline). Drug injections were given in volumes of 0.10–0.15 ml/100 g body weight.

2.2. Experimental protocol

In the first set of experiments, we tested the effect of calcitonin administered alone on the activity of the hypothalamic–pituitary–adrenal axis. Rats were given calcitonin at the dose of 2.5 IU/kg i.p. and 15 min later received saline i.p. Rats were killed 30 min after saline injection. Control rats were administered saline and saline again 15 min later and were killed 30 min after the last injection. To check the influence of U-50,488H on the hypothalamic–pituitary–adrenal axis, rats were injected with saline i.p. 15 min before administration of U-50,488H (1 mg/kg i.p.) and were killed 30 min after the κ-opioid receptor agonist injection. To check the interaction between calcitonin and the κ-opioid receptor agonist and its influence on the hypothalamic–pituitary–adrenal axis activity, groups of rats were injected with calcitonin (2.5 IU/kg i.p.) 15 min before U-50,488H (1 mg/kg i.p.). Animals were killed 30 min after U-50,488H administration. The drugs were administered following the schedule shown in Table 1.

In the second and third set of experiments, we investigated the influence of either the inhibition of 5-HT synthesis or the blockade of 5-HT metabolism on the effect of the calcitonin-U-50,488H interaction on the activity of the hypothalamic–pituitary–adrenal axis. We used an inhibitor of aromatic amino acid decarboxylase, NSD 1015 (100 mg/kg i.p.) and a monoamine oxidase inhibitor, tranylcypromine (15 mg/kg i.p.). Doses of U-50,488H and calcitonin that do not produce any effect on the hypothalamic–pituitary–adrenal axis were selected (Alcaraz et al., 1993b; Milanés et al., 1993). Drug administration followed the schedule shown in Table 2.

2.3. Analysis of 5-HT, precursor and metabolite

Rats were killed by decapitation and the brains were quickly removed and stored at −80°C. The effects of calcitonin (2.5 IU/kg, i.p.), U-50,488H (1 mg/kg, i.p.) and those of their interaction on the hypothalamic concen-

Table 1
Administration schedule of calcitonin (CT), U-50,488H and saline in the first set of experiments

	After 15 min	After 30 min
Saline	saline	sacrifice
CT	saline	sacrifice
Saline	U-50,488H	sacrifice
CT	U-50,488H	sacrifice

Table 2
Administration schedule of calcitonin (CT), U-50,488H, NSD 1015, tranylcypromine and saline in the second and third set of experiments

2nd set of experiments			
	after 15 min	after 30 min	
Saline	saline + NSD 1015	sacrifice	
CT	saline + NSD 1015	sacrifice	
Saline	U-50,488H + NSD 1015	sacrifice	
CT	U-50,488H + NSD 1015	sacrifice	
3rd set of experiments			
	after 15 min	after 15 min	after 30 min
Tranylcypromine	saline	saline	sacrifice
Tranylcypromine	CT	saline	sacrifice
Tranylcypromine	saline	U-50,488H	sacrifice
Tranylcypromine	CT	U-50,488H	sacrifice

trations of 5-HT, its precursor 5-HTP and its metabolite 5-HIAA were studied.

The concentrations of 5-HT and 5-HIAA in the hypothalamus of saline- and drug-treated rats were measured by the high-performance liquid chromatography (HPLC) technique.

In order to analyse the possible modification of the synthesis and metabolism of 5-HT after the administration of calcitonin and the opioid receptor agonist, we used NSD 1015 and tranylcypromine. The rate of 5-HTP accumulation after aromatic amino acid decarboxylase inhibition by NSD 1015 (Pileblad and Carlsson, 1986) and the rate of 5-HT accumulation after tranylcypromine inhibition of monoamine oxidase (Marshall and Grahame-Smith, 1971) were determined.

The hypothalamus was dissected out and homogenized in perchloric acid (0.2 M) containing sodium metabisulphite (0.1%), cysteine (0.1%) and EDTA (0.01%) (Green et al., 1992). Homogenates were centrifuged at 15000 *g* for 20 min at 4°C. Aliquots of the supernatant were taken for analysis of 5-HTP, 5-HT and 5-HIAA by HPLC with electrochemical detection. The mobile phase for 5-HTP, 5-HT and 5-HIAA analysis consisted of KH_2PO_4 (0.05 M), octanesulphonic acid (1.5 mM), EDTA (0.1 mM) and methanol (15%), and the pH was adjusted to 2.85 with phosphoric acid. The mobile phase was filtered and degassed. The flow rate was 1 ml/min and the working electrode potential was set at 0.7 V. The HPLC system consisted of a pump (Waters 510) linked to an automatic sample injector (Waters 712 WISP), a stainless steel reversed phase column (Resolve C18, 5 μm, 3.9 mm × 15 cm) with a precolumn (Resolve C18) and an amperometric detector (Waters M460). The current produced was monitored by using an integrator (Waters M745).

2.4. Plasma corticosterone radioimmunoassay

On the experimental days, the rats were killed by decapitation between 10.30–11.00 h (to avoid circadian variations in plasma levels of corticosterone) and trunk blood was collected into iced tubes containing 5% EDTA and centrifuged (2500 rpm; 4°C; 15 min). The plasma was stored at −20°C until it was assayed for corticosterone.

The plasma levels of corticosterone were estimated with a commercially available kit for rats ([^{125}I]-corticosterone radioimmunoassay, ICN, Biomedicals, USA). The sensitivity of the assay was 0.4 ng/ml. The inter- and intra-assay coefficients of variations were 6.5 and 4.4%, respectively. The antibody cross-reacted 100% with corticosterone and less than 0.5% with other steroids.

2.5. Data analysis

The results are expressed as the means of *n* experiments ± S.E.M. Statistical analysis was performed by a one-way analysis of variance (ANOVA), followed by the Newman–Keuls post hoc test, using a computer program. Differences were considered significant if $P < 0.05$.

3. Results

3.1. Effects of calcitonin, U-50,488H, NSD 1015 and tranylcypromine on the hypothalamic 5-HT content and turnover

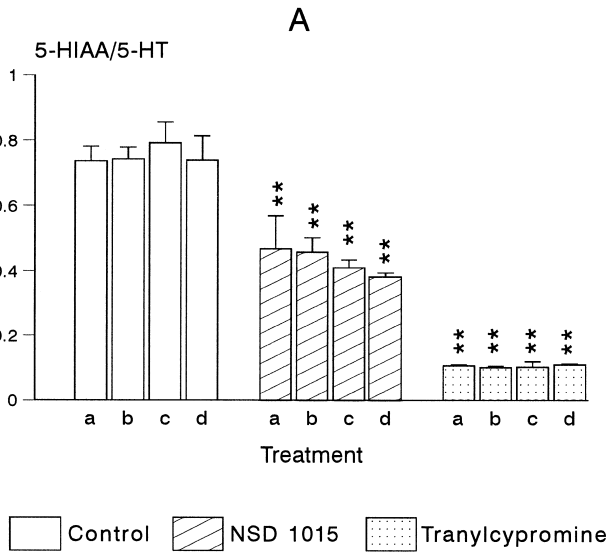
Table 3 shows the effect of calcitonin, U-50,488H and their interaction on hypothalamic 5-HT and 5-HIAA content. Neither calcitonin nor U-50,488H significantly changed the concentration of 5-HT or 5-HIAA in the hypothalamus. Calcitonin pre-treatment produced no modifications of the effects of the κ-opioid receptor agonist on 5-HT or 5-HIAA levels. Similarly, no significant alteration was observed in the 5-HIAA/5-HT ratio (Fig. 1A).

NSD 1015 administered 30 min before the animals were killed induced a marked accumulation of 5-HTP in the

Table 3
Influence of NSD 1015 (NSD) and tranylcypromine (TCP) on hypothalamic 5-HT accumulation and 5-HT content after calcitonin (CT), U-50,488H or CT + U-50,488H administration. Data are reported as means ± S.E.M. for 5–7 rats per group. Significance was assessed by one-way analysis of variance followed by Newman–Keuls test

Treatment	5-HTP (ng/g)	5-HT (ng/g)	5-HIAA (ng/g)
Saline + saline		516 ± 21	381 ± 29
CT + saline		432 ± 56	323 ± 20
Saline + U-50,488H		537 ± 19	399 ± 22
CT + U-50,488H		473 ± 23	342 ± 29
Saline + saline + NSD	260 ± 11	521 ± 16	236 ± 43[a]
CT + saline + NSD	237 ± 22	517 ± 48	210 ± 20[a]
Saline + U-50,488H + NSD	203 ± 16	560 ± 28	255 ± 29[a]
CT + U-50,488H + NSD	245 ± 8	583 ± 16	223 ± 10[a]
TCP + saline + saline		894 ± 36[a]	96 ± 6[b]
TCP + CT + saline		941 ± 78[a]	91 ± 10[b]
TCP + saline + U-50,488H		937 ± 30[a]	95 ± 7[b]
TCP + CT + U-50,488H		996 ± 27[a]	109 ± 3[b]

[a] $P < 0.01$; [b] $P < 0.001$ versus the corresponding control group not treated with NSD 1015 or TCP.



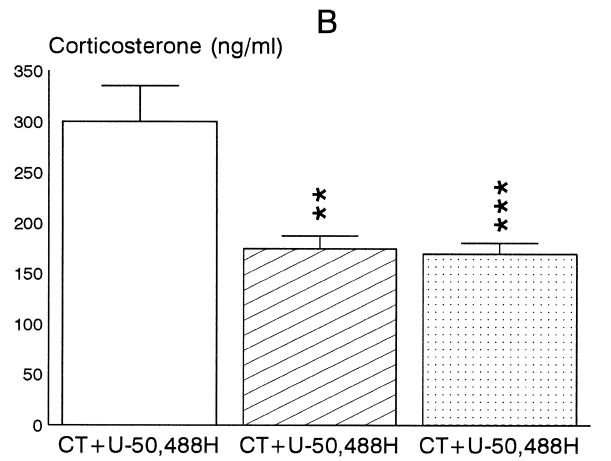


Fig. 1. (A) Bars show the mean ± S.E.M. of the 5-HIAA/5-HT ratio in rats ($n = 5–7$) treated with (a) saline, (b) calcitonin (CT) (2.5 IU/kg i.p.), (c) U-50,488H (1 mg/kg i.p.) and (d) calcitonin (CT) and U50,488H. (B) Bars show the mean ± S.E.M of plasma concentrations of corticosterone in rats ($n = 5–7$) treated with the same doses of calcitonin (CT) and U-50,488H. * significant difference versus its respective control values, (** $P < 0.01$, *** $P < 0.001$).

hypothalamus (Table 3). In saline-treated rats (control), reflecting the physiological situation, this compound could not be detected. Previous injection of calcitonin, U-50,488H or calcitonin plus U-50,488H into NSD 1015-treated rats did not significantly alter the 5-HTP accumulation. Administration of NSD 1015 did not modify the hypothalamic 5-HT content, as compared with that of the saline-injected control group. However, this compound significantly ($P < 0.01$) reduced the concentration of the metabolite 5-HIAA and the 5-HIAA/5-HT ratio, indicating a decrease in 5-HT turnover (Fig. 1A). In rats pre-treated with calcitonin, U-50,488H or calcitonin plus the κ-opioid receptor agonist U-50,488H, administration of NSD 1015 produced no modifications in hypothalamic 5-HT levels, but significantly decreased the 5-HIAA concentration ($P < 0.01$) (Table 3) and the turnover of 5-HT ($P < 0.01$) (Fig. 1A) in all three groups, as compared with the respective groups of rats that did not receive NSD 1015.

Tranylcypromine also led to a marked accumulation ($P < 0.01$) of 5-HT in the hypothalamus of control rats (Table 3), whereas both 5-HIAA (Table 3) production and the 5-HIAA/5-HT ratio (Fig. 1A) were significantly ($P < 0.001$; $P < 0.01$, respectively) reduced. None of these effects were modified by administration of calcitonin, U-50,488H or calcitonin plus the κ-opioid receptor agonist to tranylcypromine-pre-treated rats.

3.2. Effects of calcitonin, U-50,488H, NSD 1015 and tranylcypromine on the activity of the hypothalamic–pituitary–adrenal axis

Calcitonin administered at the dose of 2.5 IU/kg i.p. did not produce significant changes in the release of corticosterone (188 ± 15 ng/ml), when compared with that of the saline control group (126 ± 9 ng/ml). Administration of U-50,488H (1 mg/kg i.p.) did not alter plasma corticosterone levels 30 min after injection (165 ± 27 ng/ml). To test the influence of calcitonin on the effect of U-50,488H, a group of rats was pre-treated with calcitonin (2.5 IU/kg i.p.) and 15 min later the rats were given U-50,488H (1 mg/kg i.p.). Plasma corticosterone concentration was measured 30 min after κ-opioid receptor agonist administration. Treatment with calcitonin + U-50,488H markedly increased ($P < 0.01$) the corticosterone release (300 ± 35 ng/ml) (Fig. 1B) when compared with control values (126 ± 9 ng/ml).

Administration of NSD 1015 produced no significant alteration in plasma corticosterone concentration (183 ± 34 ng/ml), when compared with that of the control group treated with saline. NSD 1015 treatment did not alter the effects on corticosterone secretion of either calcitonin (185 ± 17 ng/ml) or U-50,488H (173 ± 14 ng/ml). In contrast, NSD 1015 fully blocked ($P < 0.01$) the increase in corticosterone release that was seen after the calcitonin + U-50,488H treatment (Fig. 1B).

Administration of tranylcypromine did not modify the release of corticosterone (188 ± 11 ng/ml), as compared with that of the saline-treated group. Tranylcypromine pre-treatment induced no changes in the neuroendocrine effects of either calcitonin (144 ± 11 ng/ml) or U-50,488H (146 ± 7). In contrast, tranylcypromine led to a marked increase ($P < 0.001$) in the corticosterone release that was seen after the calcitonin and U-50,488H interaction (Fig. 1B).

4. Discussion

The interaction between calcitonin and the serotonergic system has been previously studied. Bourgoin et al. (1988) demonstrated that calcitonin is able to release 5-HT from

rat spinal cord in vitro. This effect of calcitonin has not been confirmed in in vivo assays (Colado et al., 1994). Nevertheless, when serotonergic activity was modified by very distinct procedures (Clementi et al., 1984b, 1985), the analgesic effect induced by calcitonin was reduced.

Several studies have focused on the possible involvement of the hypothalamic–pituitary–adrenal axis in the analgesic action of calcitonin, although the mechanisms underlying this effect are not well understood. In the present work, we examined the possible involvement of hypothalamic serotonergic pathways in the calcitonin-induced potentiation of the neuroendocrine effects of a selective kappa opioid agonist, U-50,488H (Von Voigtlander et al., 1983), at the hypothalamic–pituitary–adrenal axis level.

The presence of calcitonin binding sites in the hypothalamus, the region that regulates pituitary–adrenal secretion, has been demonstrated (Olgiati et al., 1983; Goltzman and Mitchell, 1985). In addition, the hypothalamus also contains opioid receptors, mainly of the κ type (Mansour et al., 1995) and opioid neurons, which are involved in the regulation of the hypothalamic–pituitary–adrenal axis (Pechnick, 1993). In agreement with previous studies (Alcaraz et al., 1993a,b) the present results indicate that administration of U-50,488H at the dose of 1 mg/kg did not produce any alterations in the release of corticosterone, with higher doses (15 mg/kg) being necessary to enhance hypothalamic–pituitary–adrenal axis activity (Alcaraz et al., 1993b). However, pre-treatment with calcitonin enhanced the response of the hypothalamic–pituitary–adrenal axis to the κ-opioid receptor agonist, as demonstrated by a significant increase in plasma corticosterone levels. Such a potentiation was seen with a subanalgesic dose of calcitonin (2.5 IU/kg) (Martín et al., 1992) that produced no effect on the release of corticosterone when administered alone and is presumably the result of the selective activation of κ-opioid mechanisms, probably at the hypothalamic level, as was demonstrated previously (Milanés et al., 1993). Since calcitonin binding sites have not been demonstrated in the pituitary (Cooper et al., 1977) and the fact that dexamethasone administration suppresses the calcitonin-induced activation of the pituitary–adrenal system (Oberman et al., 1992), it can be suggested that the potentiation of the neuroendocrine effects of U-50,488H might be exerted at the hypothalamic level. In our previous study, we observed that calcitonin given alone at different doses (2.5, 5, 10 or 20 IU/kg i.p.) induced no changes in the pituitary–adrenal secretion, 45 min after its administration. Studies performed with humans have indicated both an increase in adrenocorticotrophin hormone (ACTH) and cortisol secretion (Laurian et al., 1986; Ustdal et al., 1989) and no changes in plasma β-endorphin (Vescovi et al., 1990) after calcitonin administration. So, the mechanism by which calcitonin may regulate pituitary–adrenal secretion is complex and is not yet established.

It is well known that ACTH and β-endorphin secretion is regulated, mainly by corticotrophin-releasing factor (CRF). In addition, the majority of in vitro and in vivo studies demonstrate a stimulatory role for serotonin in CRF release (for revision, see Owens and Nemeroff, 1991). The 5-HIAA/5-HT ratio is generally accepted to reflect the activity of 5-HT neurons and, as expected, our results showed that both NSD 1015 and tranylcypromine are able to reduce the turnover of 5-HT. The treatment with NSD 1015 did not reduce total levels of 5-HT. Although NSD 1015 acts mainly as an inhibitor of aromatic amino acid decarboxylase, the fact that it reduces levels of the metabolite indicates that it also inhibits MAO (Carlsson et al., 1972), so that the decrease in synthesis was compensated for and the final effect was a reduction in turnover.

Interestingly, when 5-HT turnover was decreased, the increase in the release of corticosterone induced by calcitonin-U-50,488H was prevented (Fig. 1). Although other factors cannot be disregarded, from the present data it can be suggested that the serotonergic system is involved in the calcitonin-κ-opioid receptor agonist interaction. This is consistent with previous data obtained in studies of analgesia that have shown a relationship between calcitonin-induced analgesia and the serotonergic system (Colado et al., 1994).

Although most serotonergic neurons are found within the raphe nuclei, there are small neurons containing 5-HT that have been described in the hypothalamus (Ungerstedt, 1971; Beaudet and Descarries, 1979) near the third ventricle in the dorso-medial nucleus, and serotonin histofluorescence has also been reported in some perikarya of the arcuate nucleus (Kent and Sladek, 1978). The presence of modest concentrations of serotonin in the hypothalamus may also be attributed to raphe projections that innervate different hypothalamic nuclei (ventromedial part of the suprachiasmatic nucleus, lateral part of the medial preoptic nucleus, dorsal and ventral premammillary nuclei, parvocellular part of the paraventricular nucleus, anterior wall of the third ventricle, ...) (Sawchenko et al., 1983; Simerly et al., 1984), because the presence of serotonin in the hypothalamus is not limited to a particular hypothalamic structure, and it may originate from the hypothalamus or from other structures such as the raphe. From the present data it cannot be determined if there is a specific hypothalamic structure involved in the calcitonin–opioid receptor–ACTH release relationship.

In summary, these results confirm the importance of the serotonergic system in the increase of opioid effects induced by calcitonin and show that this interaction is not restricted to analgesic effects.

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