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Cocaine- and amphetamine-regulated transcript peptide-(55–102) and thyrotropin releasing hormone inhibit hypothalamic dopamine release

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Abstract

Cocaine- and amphetamine-regulated transcript (CART) peptide-(55–102) and thyrotropin releasing hormone (TRH) play an anorectic role in the hypothalamus. Catecholamines are also involved in appetite control and we have previously found that leptin, an adipocyte-derived anorectic hormone, inhibits hypothalamic norepinephrine and dopamine release. We have studied the effect of CART peptide-(55–102) and TRH on basal and depolarization (K^+ 15 mM)-induced norepinephrine and dopamine release from rat hypothalamic neuronal endings (synaptosomes) in vitro. We have found that basal catecholamine release was not modified; both CART peptide-(55–102) and TRH, the former with a higher sensitivity, dose-dependently inhibited depolarization-induced dopamine release, and did not affect the stimulated norepinephrine release. Considering the role played by dopamine in the central mechanisms of reward, these findings suggest that the inhibition of dopamine release could underlie the decreased appetitive behaviour induced by CART peptide-(55–102) and TRH.

Keywords: CART (cocaine- and amphetamine-regulated transcript); TRH (thyrotropin releasing hormone); Dopamine; Norepinephrine; Synaptosome; Hypothalamus

1. Introduction

Cocaine- and amphetamine-regulated transcript (CART) has been first isolated as mRNA produced in the rat striatal neurons after psychostimulant drug treatment, with the predicted translation products either 129 or 116 aminoacids in length (Douglass et al., 1995). Subsequently, tissue specific processing of CART into smaller peptides has been demonstrated in rat hypothalamus, pituitary and adrenal gland, suggesting different biological functions in the nervous and endocrine systems (Koylu et al., 1997; Thim et al., 1999; Kuhar and Yoho, 1999). It has been shown that CART peptide-(55–102) plays an anorectic role in the hypothalamus, where intracerebroventricular administration inhibits, while antibody neutralization of the endogenous peptide stimulates food intake (Kristensen et al., 1998; Lambert et al., 1998).

In the hypothalamus, thyrotropin releasing hormone (TRH), besides its key role in stimulating the pituitary–thyroid axis, is also involved in the suppression of feeding activities, as either intraventricular (Vijayan and McCann, 1977) or intrahypothalamic (Suzuki et al., 1982) injection suppresses food intake. Interestingly, TRH and CART mRNAs are co-expressed in hypothalamic paraventricular nucleus neurons (Broberger, 1999), suggesting their common involvement in the modulation of anorectic signaling.

Catecholamines are also involved in food-intake control (Shiraishi, 1991; Wellman et al., 1993; Yang and Meguid, 1995) and we have previously found that leptin, an adipocyte-derived hormone, which has been shown to play a pivotal role in inhibiting food intake and restraining body fat stores (Zhang et al., 1994), inhibits norepinephrine and dopamine release in the hypothalamus (Brunetti et al., 1999), which could explain part of the anorectic effects of leptin.

In the present study, we have investigated the effect of CART peptide-(55–102) and TRH on norepinephrine and dopamine release in the hypothalamus.

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2. Materials and methods

2.1. Animals

Male adult Wistar rats (200–220 g) were housed in a thermoregulated environment (23 ± 1°C), with automatic control of light/darkness cycle. Food and water were available ad libitum.

2.2. Drugs

Synthetic rat CART peptide-(55–102), 0.5 mg, was purchased from American Peptide (CA, USA); TRH acetate salt, 50 mg, and the selective serotonin reuptake inhibitor fluoxetine, 10 mg, from Sigma-Aldrich (Italy). The specific activity of CART was also evaluated after reduction of its disulfide bonds with 2-mercaptoethanol and subsequent alkylation of the resulting thiol groups with 4-vinylpyridine (Friedman et al., 1970). The specific activity of TRH on hypothalamic catecholamine release was compared to the activity of a TRH analog where pyroglutamic acid was replaced by its sulphonamido analog (*S*)-isothiazolidine-1,1-dioxide-3-carboxylic acid (synthesized by Prof. F. Pinnen, G. D'Annunzio University, Chieti, Italy).

2.3. Hypothalamic synaptosomes

Hypothalamic synaptosomes were prepared according to Gray and Whittaker (1962). Briefly, male Wistar rats (200–250 g) were sacrificed by decapitation, the hypothalami quickly dissected, homogenized in 0.32 M saccharose and centrifuged, first at $1000 \times g$ for 5 min, and then at $12{,}000 \times g$ for 20 min, to isolate neuronal endings from cell nuclei and glia. Then, the synaptosome suspension was incubated, at 37°C, under O_2/CO_2 95/5%, pH 7.2–7.4, in Krebs–Ringer buffer (mM/l: NaCl 125, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, NaH_2PO_4 5, Tris–HCl 10, glucose 10, ascorbic acid 1), supplemented with 0.05 μM fluoxetine, a selective serotonin reuptake inhibitor, with either 0.05 μM [^{3}H]norepinephrine, or 0.05 μM [^{3}H]dopamine, for 15 min, to make synaptosomes uptake [^{3}H]norepinephrine or [^{3}H]dopamine, substituting for the endogenous norepinephrine or dopamine pool. Then, synaptosomes were layered onto 0.8 μM Millipore filters, placed into 37°C water-jacketed superfusion chambers (18 different chambers for each experiment), and perfused with the above buffer (0.6 ml/min). After 30 min, to allow stable release (equilibration period), perfusate was collected in 2-min fractions, and after the first 3–4 fractions (basal release) testing drugs were added to the perfusion buffer for 10 min (stimulus), followed by 10 min with Krebs buffer alone (return to basal). To evaluate the effect of CART peptide or TRH on neurotransmitter release during depolarization [3 min perfusion with K^+ (15 mM), after removal of equimolar concentrations of Na^+ in perfusion buffer], these peptides were added, after the equilibration period, both 20 min prior (pre-stimulus) and 3 min during K^+ (15 mM) perfusion (stimulus). Finally, beta-emission from perfusate fractions, corresponding to [^{3}H]norepinephrine or [^{3}H]dopamine release, was detected by liquid scintillation scanning. Ca^{2+}-mediated catecholamine release was investigated by perfusing the synaptosomes with Ca^{2+}-free Krebs–Ringer buffer, supplemented with 1 mM ethylene glycol-bis(β-aminoetil ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA).

2.4. Analysis of data

Catecholamine release has been calculated as either the means ± S.E.M. of the percentage of [^{3}H]norepinephrine or [^{3}H]dopamine recovered in each fraction with respect to total (fractions + filter) or the means ± S.E.M. of the area under the time–response curve (AUC); each group represents the mean ± S.E.M. of 3–5 experiments performed in triplicate. Treatment and control group data from experiments run on a single pool of hypothalamic tissue have been compared by the analysis of variance (ANOVA).

3. Results

We have found that both CART peptide-(55–102) and TRH, in the dose range (0.1–10 nM), do not affect basal norepinephrine and dopamine release (means ± S.E.M. of the percentage of [^{3}H]norepinephrine recovered in each fraction with respect to total: CART: control 1.15 ± 0.15; 0.1 nM, 1.07 ± 0.02; 1 nM, 1.01 ± 0.02; 10 nM, 0.87 ± 0.01. TRH: control, 1.00 ± 0.05; 0.1 nM, 0.91 ± 0.04; 1 nM, 1.07 ± 0.01, 10 nM, 1.10 ± 0.05. Means ± S.E.M. of the percentage of [^{3}H]dopamine recovered in each fraction with respect to total: CART: control, 1.36 ± 0.03; 0.1 nM 1.38 ± 0.02; 1 nM, 1.27 ± 0.03; 10 nM, 1.36 ± 0.03. TRH: control, 1.46 ± 0.03; 0.1 nM, 1.54 ± 0.06; 1 nM, 1.43 ± 0.06; 10 nM, 1.38 ± 0.05). We have further investigated the possible role of these peptides in potentiating or inhibiting the stimulated release of both catecholamines, by perfusing the synaptosomes with graded concentrations of either CART peptide-(55–102) or TRH, 20 min before and during a 3-min depolarizing stimulus, which was induced by raising the K^+ concentration in the Krebs–Ringer perfusion buffer to 15 mM. CART peptide-(55–102) (0.01–1 nM) was able to inhibit, in a concentration-dependent manner, depolarization-induced dopamine release (Fig. 1), while it did not modify the stimulated release of norepinephrine (Fig. 2). The effect of CART-(55–102) was lost after reduction of its disulfide bonds with 2-mercaptoethanol and subsequent alkylation of the resulting thiol groups with 4-vinylpyridine (Friedman et al., 1970) [means ± S.E.M. of the area under the time–response curve of the percentage of [^{3}H]dopamine recovered, with respect to total (fractions + filters): control (K^+ (15 mM)), 6.81 ±

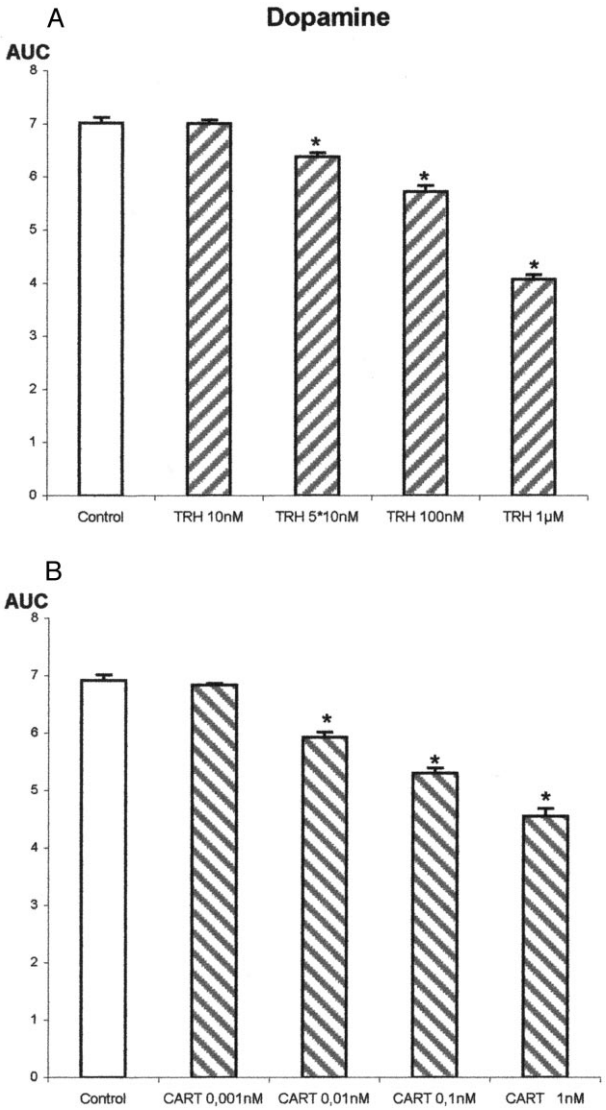

Fig. 1. Effect of CART peptide-(55–102) (0.001–1 nM) (A), and of TRH (10 nM–1 μM) (B), on depolarization-induced dopamine release. The control group (white column) was perfused with K^+ (15 mM) in Krebs–Ringer buffer for 3 min; the CART peptide and TRH groups (hatched columns) were perfused with graded concentrations of the respective peptides in K^+ (15 mM) Krebs–Ringer buffer for 3 min, after a 20-min pre-incubation with the peptide in Krebs–Ringer buffer. The columns represent the area under the time–response curve (AUC) of the percentage of [^{3}H]dopamine recovered, with respect to total (fractions + filters); each column represents the mean ± S.E.M. of 3–5 experiments performed in triplicate; $^* P < 0.001$.

0.13; CART (0.01 nM), 6.64 ± 0.32; CART (0.1 nM), 7.04 ± 0.20; CART (1 nM), 6.92 ± 0.18].

On the other hand, TRH, in the dose range 0.1–10 nM, did not affect depolarization-induced catecholamine release, but, at higher concentrations (50 nM–1 μM) with respect to CART peptide, it inhibited depolarization-induced dopamine release (Fig. 1), while norepinephrine release was not affected (Fig. 2). The specific activity of TRH on hypothalamic dopamine release was lost when pyroglutamic acid in the tripeptide sequence was replaced by its sulphonamido analog (*S*)-isothiazolidine-1,1-dioxide-3-carboxylic acid [means ± S.E.M. of the area under the time–response curve of the percentage of [^{3}H]dopamine recovered, with respect to total (fractions + filters): control (K^+(15 mM)), 7.13 ± 0.16; TRH analog (1 nM), 6.84 ± 0.21; 10 nM, 7.20 ± 0.25; 100 nM, 7.30 ± 0.15; 1 μM, 6.90 ± 0.25].

We also tested the additive effect of co-perfusion with subthreshold inhibitory concentrations of CART peptide

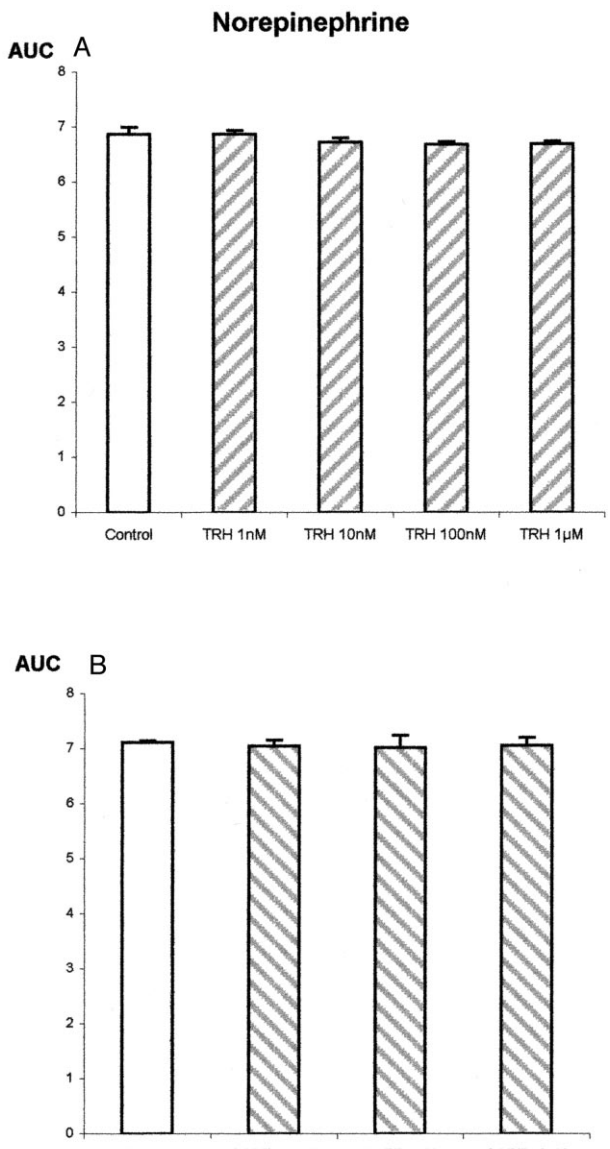

Fig. 2. Effect of CART peptide-(55–102) (0.1–10 nM) (A), and of TRH (1 nM–1 μM) (B), on depolarization-induced norepinephrine release. The control group (white column) was perfused with K^+ (15 mM) in Krebs–Ringer buffer for 3 min; the CART peptide and TRH groups (hatched columns) were perfused with graded concentrations of the respective peptides in K^+ (15 mM) Krebs–Ringer buffer for 3 min, after a 20-min pre-incubation with the peptide in Krebs–Ringer buffer. The columns represent the area under the time–response curve (AUC) of the percentage of [^{3}H]norepinephrine recovered, with respect to total (fractions + filters); each column represents the mean ± S.E.M. of 3–5 experiments performed in triplicate.

and TRH on the stimulated dopamine release, and we found a significant inhibitory activity [means ± S.E.M. of the area under the time–response curve of the percentage of [^{3}H]dopamine recovered, with respect to total (fractions + filters): control (K^+(15 mM)), 8.70 ± 010; CART 0.001 nM, 8.50 ± 0.09; TRH 10 nM, 9.01 ± 0.06; CART 0.001 nM + TRH 10 nM, 5.43 ± 0.17 *; * $P < 0.001$ vs. control].

We have finally investigated the role of extracellular Ca^{2+} on depolarization-induced catecholamine release, by perfusing the synaptosomes in Ca^{2+}-free Krebs–Ringer buffer, supplemented with 1 mM EGTA, a Ca^{2+} chelating agent. We found that, in extracellular Ca^{2+}-free conditions, K^+ (15 mM)-induced depolarization of the hypothalamic synaptosomes did not evoke either dopamine or norepinephrine release, neither CART peptide nor TRH had any effect [means ± S.E.M. of the percentage of [^{3}H]norepinephrine recovered in each fraction with respect to total: control, 1.36 ± 0.03; K^+ (15 mM), 1.44 ± 0.03; K^+ (15 mM) + CART (0.01 nM), 1.40 ± 0.04; K^+ (15 mM) + TRH 50 nM, 1.53 ± 0.05. Means ± S.E.M. of the percentage of [^{3}H]dopamine recovered in each fraction with respect to total: control, 1.50 ± 0.03; K^+ (15 mM), 1.49 ± 0.05; K^+ (15 mM) + CART (0.01 nM), 1.59 ± 0.03; K^+ (15 mM) + TRH 50 nM, 1.43 ± 0.04].

4. Discussion

Food intake is regulated by the interaction of stimulatory and inhibitory signals which integrate each other in the hypothalamus (Kalra et al., 1999). A possible role for CART peptides in restraining feeding behavior has been suggested by the marked decrease in the expression of arcuate nucleus CART mRNA in food-deprived as well as in genetically obese rats (Kristensen et al., 1998). Moreover, either intraventricular administration of recombinant CART peptide-(55–102) or antibody neutralization of endogenous CART peptides, inhibits or increases food intake, respectively (Kristensen et al., 1998; Lambert et al., 1998).

TRH also plays a role of central anorectic mediator, as either intraventricular (Vijayan and McCann, 1977) or intrahypothalamic (Suzuki et al., 1982) administration inhibits food intake.

CART and TRH mRNAs are co-expressed in paraventricular nucleus neurons of the hypothalamus (Broberger, 1999). The paraventricular nucleus is one of the possible sites of interaction of neurotransmitters/neuromodulators that affect feeding (Kalra et al., 1999). This nucleus also contains noradrenergic and dopaminergic fibers arising from the brain stem (Swanson and Sawchenko, 1983). Norepinephrine release from presinaptic terminals in the paraventricular nucleus plays a dual role on food intake, inhibiting via α_1-, and stimulating via α_2-adrenoceptors (Wellman et al., 1993). The role played by dopamine in the hypothalamic control of food intake is also still unsettled: on one side, the classical anorectic agents, such as amphetamine, are supposed to produce their effects through dopamine reuptake inhibition, and consequent increase in synaptic dopamine transmission in the lateral hypothalamus (Samanin and Garattini, 1993), and dopamine itself injected into the hypothalamus inhibits food intake (Gillard et al., 1993); on the other hand, dopamine administration into the lateral hypothalamus increases food intake (Shiraishi, 1991) and raised dopamine levels are found in obese animals (Yang and Meguid, 1995). More recently, dopamine has been shown to be required for the increased food intake that follows leptin deficiency (Szczypka et al., 2000), and we have previously suggested that the anorectic effects of leptin could be partially mediated by inhibition of dopamine release in the hypothalamus (Brunetti et al., 1999). Since dopamine release is associated with rewarding behaviours (Robbins and Everitt, 1996), our results, showing similar effects of CART peptide-(55–102) and TRH in inhibiting depolarization-induced dopamine release in the hypothalamus (Fig. 1), although with a greater sensitivity for the former peptide (minimal effective concentrations: CART peptide, 0.01 nM; TRH, 50 nM), suggest that the anorectic activity of these peptides could involve a blunting of the rewarding effects of dopamine. Moreover, CART-(55–102) and TRH show a synergic effect, as co-perfusion with both peptides at subthreshold concentrations significantly inhibit dopamine release, suggesting a common pathway downstream their membrane receptors. On the contrary, other authors have found that TRH does stimulate dopamine release from hypothalamic slices, but actually at strikingly higher concentrations (10 μM–1 mM) (Sharp et al., 1982).

The inhibitory effects of CART peptide-(55–102) and TRH seem to be specific for dopaminergic terminals, as hypothalamic norepinephrine is not affected, both basally and after depolarization (Fig. 2), a quite different result compared with the anorectic hormone leptin, which we found able to inhibit both dopamine and norepinephrine release (Brunetti et al., 1999): this could reflect a weaker anorectic effect of CART peptide and TRH with respect to leptin.

Our experiments also confirm that CART peptide-(55–102) effects are strictly dependant on the secondary structure of the peptide, as disruption of the intrachain disulfide bonds, by reduction and pyridylethylation, results in the loss of both the anorectic (Kristensen et al., 1998) and dopamine release inhibitory activities.

The dopamine release inhibitory effect of TRH seems to be specific for the pyroglutamyl–histidyl–prolinamide sequence of TRH, as a sulphonamido [(*S*)-isothiazolidine-1,1-dioxide-3-carboxylic acid] substitution for pyroglutamic acid generates a TRH analog lacking such an activity.

Furthermore, the experiments in Ca^{2+}-free medium have shown that depolarization-induced catecholamine release is strictly dependant on extracellular Ca^{2+} availability, since

neither norepinephrine nor dopamine release is stimulated by depolarization in the absence of extracellular calcium.

In conclusion, CART peptide-(55–102) and TRH are able to specifically inhibit dopamine release in the hypothalamus, while norepinephrine release is not affected; considering the role played by dopamine in the central mechanisms of reward, this effect could underlie the decreased appetitive behaviour induced by these peptides.

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