

Cocaine- and amphetamine-regulated transcript (CART) peptide specific binding in pheochromocytoma cells PC12

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Abstract

CART (cocaine- and amphetamine-regulated transcript) peptides have been studied for ten years. We report specific binding of ^{125}I -CART(61–102) to the rat adrenal pheochromocytoma PC12 cell line, both intact cells and cell membranes. Saturation binding to intact plated cells resulted in K_d of 0.48 ± 0.16 nM and B_{\max} of 2228 ± 529 binding sites/cell. ^{125}I -CART(61–102) was also bound to PC12 cells differentiated using nerve growth factor to the neuronal phenotype with non-specific binding below 20%, and K_d of 1.90 ± 0.27 nM and B_{\max} of 11194 ± 261 binding sites/cell. In competitive binding experiments, CART(61–102), CART(55–102) and di-iodinated CART(61–102) were bound to PC12 cell membranes with K_i in low nM range; their affinity to intact non-differentiated and differentiated cells was in low 10^{-8} M range. In order to prove that iodination did not eliminate the pharmacological properties of CART, we tested the biological activity of di-iodinated CART(61–102). It decreased food intake in *in vivo* feeding experiment on fasted mice in a dose of 1 $\mu\text{g}/\text{mouse}$ to the same extent as CART(61–102) in a dose of 0.5 $\mu\text{g}/\text{mouse}$. © 2007 Elsevier B.V. All rights reserved.

Keywords: Radioligand binding; CART; PC12 cells; Food intake

1. Introduction

CART (cocaine- and amphetamine-regulated transcript) peptides were described ten years ago (Douglass et al., 1995) and linked to a previously isolated hypothalamic peptide with unknown function (Spiess et al., 1981). The entire issue of Peptides (Kuhar, 2006) is dedicated to the ten-year anniversary of CART discovery.

CART peptides are involved in reward and reinforcement, feeding and satiety, stress, endocrine regulation, and sensory processing (for reviews, see Broberger, 2000; Hunter et al., 2004, and the above-mentioned issue of Peptides).

CART is evolutionarily conserved across species. ProCART of rat and mouse is identical, consists of 102 amino acids and is

processed to two main active CART peptides, *i.e.* to CART(55–102) and CART(61–102), which are mostly used in CART research. Human proCART lacks sequence 27–39 of the mouse and rat proCART and has valine instead of isoleucine in the position 55 (Douglass et al., 1995; Douglass and Daoud, 1996).

CART is one of the most abundant hypothalamic transcripts (Gautvik et al., 1996) and both CART mRNA and CART immunoreactivity were found in specific nuclei throughout brain, pituitary, adrenals, islets of Langerhans and gut. However, no receptors for CART have been cloned to date. Specific binding of $[^{125}\text{I}]$ -CART(61–102) to the AtT20 mouse pituitary tumor cell line (Vicentic et al., 2005, 2006) has been reported recently. Keller et al. (2006) described binding of a fusion protein consisting of CART(55–102) and green fluorescent protein to dissociated hypothalamic cells. A possible role of CART in the hypothalamo–pituitary–adrenal axis was suggested (Iliff et al., 2005).

In this study, we report specific binding of $[^{125}\text{I}]$ -CART(61–102) to pheochromocytoma cells PC12, both non-differentiated

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and differentiated into a neuronal phenotype. Biological activity of the non-radioactive di-iodinated CART(61-102) was confirmed in *in vivo* feeding test.

2. Materials and methods

2.1. Iodination of CART(61-102)

CART(61-102) (Bachem, Bubendorf, Switzerland) was iodinated at Tyr⁶² either with Na[¹²⁵I] (MP Biomedicals, Illkirch, France) or non-radioactive NaI (Sigma, St. Luis, MO, USA) using Iodo-Gen (Pierce Chemical Co., Rockford, IL, USA) (Fraker and Speck, 1978) in 0.1 M Na-phosphate buffer, pH 7.2, containing 0.15 M NaCl for 15 min at room temperature according to manufacturer's instructions. For mono-iodination, 3 equivalents of peptide were used for 1 equivalent of non-radioactive NaI or Na[¹²⁵I]. For di-iodination, 10-fold excess of non-radioactive NaI to the peptide was used. Non-, mono- and di-iodinated CART(61-102) were separated by RP-HPLC using an Agilent Prep-C18, 5 µm, 250×4.6 mm column (Agilent Technologies, Palo Alto, CA, USA) applying 20–28% gradient of acetonitrile in H₂O with 0.1% trifluoroacetic acid in 45 min (flow rate 1 ml/min, UV detection at 215 and 275 nm and gamma detection of radioactive peptide). The molecular weights of the iodinated peptides were confirmed by MALDI-TOF, Reflex IV mass spectrometry (Bruker Daltonics, Billerica, MA, USA). Specific activity of [¹²⁵I]-CART(61-102) was about 2000 Ci/mmol. [¹²⁵I]-CART(61-102) was kept in aliquots at -20 °C and used for saturation and competition binding studies within 1 month.

2.2. Food intake experiments

Male C57Bl/6 mice from the Institute of Molecular Genetics (Prague, Czech Republic) were housed at a temperature of 23 °C and a daily cycle of 12 h light and dark (light from 6:00). They were given *ad libitum* water and standard chow diet (St-1, Velaz, Kolec, Czech Republic). All experiments followed the ethical guidelines for animal experiments and the Act of the Czech Republic Nr. 246/1992.

At the age of three months, mice were implanted with intracerebroventricular (i.c.v.) cannulas. Briefly, mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine/xylazine (100 mg/kg ketamine, 16 mg/kg xylazine, Spofa, Prague, Czech Republic). Once anesthesia had been established, the head of the mouse was fixed in a stereotaxic frame and a single guide cannula (26 gauge, Plastics One Inc., Roanoke, VA, USA) was implanted just above the third ventricle (AP 2 mm, V 3 mm). A stainless steel screw was attached to the skull and the cannula was fixed in place using Duracrol resin (Spofa-Dental, Prague, Czech Republic) applied around the screw and cannula. An appropriate dummy cannula (Plastics One Inc.) was inserted into the cannula to prevent blockage. Animals were placed into separate cages and allowed at least seven days to recover from surgery before being used in the experiment.

For three days before the food intake experiment, mice were fasted overnight with access to food from 8:00 to 15:00 h and

free access to water for 24 h. The amount of food consumed was monitored. On the day of the experiment at 8:00, fasted mice were injected i.c.v. with 5 µl of saline or CART(61-102) at doses 0.5 and 1 µg/5 µl or I₂-CART(61-102) at a dose 1 µg/5 µl (all dissolved in saline), using an infusion pump. All solutions were infused in 20 s and the infusion cannula was left in place for a further 20 s to prevent reflux. Fifteen min after injection, mice were given weighed food pellets. The pellets were replaced with fresh ones every 30 min and weighed. Animals had free access to water during the experiment. The results are expressed in grams of food consumed. The placement of cannula was verified histologically after the experiment.

2.3. Cell culture

Rat pheochromocytoma cell line PC12 was from ATCC (Manassas, VA, USA). The cells were grown in RPMI 1640 medium (Sigma, St. Luis, MO, USA) supplemented with 10% horse serum, 5% fetal bovine serum, 4.5 g/l glucose, 1 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, pH 7.4, and passaged once a week to maintain the cells in exponential growth. For binding studies, cells were seeded on polyethylene imine-coated 24-well plates (Corning, NY, USA), diameter 15 mm/well. To reach the density of 5×10⁵ cells/well that was found optimal for binding experiments, non-differentiated cells were allowed to grow for 2 days. Differentiation of cells was performed by the addition of the nerve growth factor (NGF, 50 ng/ml of medium) to the fresh medium on days 1, 3, and 5. On day 7, the cells at a density of 5×10⁵ cells/well were used in the experiment. The differentiation was checked microscopically and the number of cells/well was counted before and after the experiment.

2.4. Binding to intact plated cells

Saturation and competition binding experiments were performed according to Motulsky and Neubig (1997). Plated cells were incubated with 0.25–16 nM [¹²⁵I]-CART(61-102) in saturation experiments or with 0.1 nM [¹²⁵I]-CART(61-102) and 10⁻¹¹–10⁻⁶ M non-radioactive ligands in competitive binding experiments, in a total volume of 0.25 ml of binding buffer (20 mM HEPES buffer pH 7.4, 118 mM NaCl, 4.7 mM KCl and 5 mM MgCl₂, 5.5 mM glucose, 1 mg/ml BSA, and 0.1 mg/ml basic pancreatic trypsin inhibitor) for 30 min at 37 °C. Non-specific binding was determined using CART(61-102) in concentrations 10⁻⁶ M (competitive experiments) or 2×10⁻⁶ M (saturation experiments). After incubation, cells were washed and then solubilized in 0.1 N NaOH. Bound radioactivity was determined by γ -counting (Wizard 1470 Automatic Gamma Counter, Perkin Elmer, Wellesley, MA, USA). The total binding was 2–5% of the radioactivity added. Non-specific binding in competitive binding experiments amounted to less than 15% of the total binding unless otherwise stated. Experiments were carried out in triplicates at least three times. For competition experiments, CART(55-102) and CART(61-102) from Bachem (Bubendorf, Switzerland), and di-

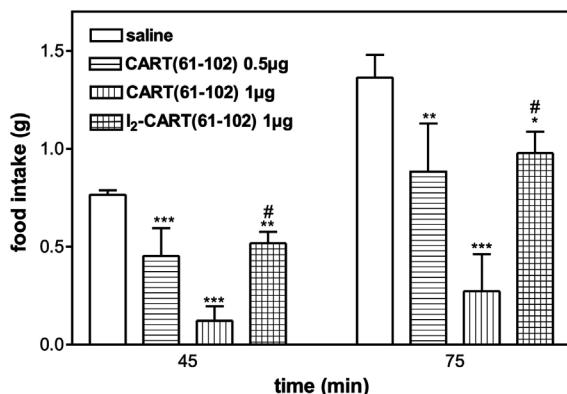


Fig. 1. Effect of CART(61-102) and I₂-CART(61-102) on food intake of overnight fasted mice. CART(61-102) was administered i.c.v. in doses of 0.5 and 1 µg/mouse and I₂-CART(61-102) in a dose of 1 µg/mouse. Food intake was monitored 45 and 75 min after injection and is expressed in grams of food consumed. Significance is *P<0.05, **P<0.01 and ***P<0.001 versus the respective saline-treated group ($n=6$ –8 mice per group). #P<0.01 versus group treated with CART(61-102), 1 µg/mouse.

iodinated CART(61-102) (I₂-CART(61-102), see the second paragraph above) were used.

2.5. Binding to cell membranes

Cell membranes from non-differentiated PC12 cells were isolated from cells stored frozen at –70 °C. The cells were suspended in 20 mM HEPES, pH 7.4, with 2 mM EDTA, homogenized with a Teflon-glass homogenizer and centrifuged at 500 ×g for 10 min. The supernatant was centrifuged at 100 000 ×g for 45 min, the sediment was re-suspended and its aliquots stored at –70 °C.

Binding experiments were performed with 12.5–100 µg of membrane protein in a total volume of 0.25 ml under conditions described in the previous paragraph for the intact cells, only glucose was omitted in the binding buffer. Binding was terminated by quick filtration in a Brandel cell harvester (Biochemical and Development Laboratories, Gaithersburg, MD, USA). The total binding was 3–10% of the radioactivity added. Non-specific binding in competitive binding experiments amounted to less than 15% of the total binding unless otherwise stated. The assays were performed in duplicates at least three times. Competitive binding of [¹²⁵I]-CART(61-102) with cholecystokinin octapeptide (CCK-8, Neosystem, Strasbourg, France), α-melanocyte stimulating hormone (α-MSH), thyroxin releasing hormone (TRH), angiotensin II (ATII), neurotensin (NT) and leptin (Sigma, St. Luis, MO, USA) was also tested.

This procedure was also used in the competitive binding experiment to floating intact cells, only the binding buffer contained glucose. Floating cells were cells that were detached from the support before passaging.

2.6. Analysis of binding data and statistics

Data are presented as means±S.E.M. of 3–6 independent experiments. Saturation and competitive binding curves were plotted using Graph-Pad Software (San Diego, CA, USA)

comparing the best fit for single or two binding site models (K_d , B_{max} and IC₅₀ values were obtained from non-linear regression analysis). Inhibition constants (K_i) were calculated from IC₅₀ using Cheng–Prusoff equation (Chang and Cheng, 1978).

Food intake data were analyzed by one-way ANOVA (analysis of variance) followed by Tukey *post hoc* test using Graph-Pad Software; $P<0.05$ was considered statistically significant.

3. Results

3.1. Iodination of CART(61-102)

CART(61-102) was mono-iodinated with radioactive and stable isotope. One major peak was found both in radioactive and non-radioactive preparation; it was identified as mono-iodinated peptide by mass spectrometry. By HPLC analysis, [¹²⁵I]-CART(61-102) was proven to stay stable under conditions of the binding experiments (results not shown).

To prove that iodination did not eliminate the biological activity of CART, we needed a rather high amount of non-radioactive iodinated CART(61-102). As it was easier to prepare the di-iodinated derivative, we decided to use non-labeled di-iodinated CART(61-102) in our experiments. Its molecular weight was confirmed by mass spectrometry.

3.2. Biological activity of I₂-CART(61-102)

CART(61-102) administered i.c.v. lowered food intake in fasted mice significantly and dose-dependently (see Fig. 1) until about 120 min after injection, with the highest effect measured 45 and 75 min after injection. I₂-CART(61-102) at the dose of 1 µg/mouse reduced food intake to 67% of food intake of saline-treated group; this value was significantly different from the food intake after administration of CART(61-102), dose 1 µg/mouse (Fig. 1).

3.3. Optimization of binding experiments

In binding experiments to PC12 cell membranes, specific binding of [¹²⁵I]-CART(61-102) at 37 °C was linear from

Table 1
Displacement of [¹²⁵I]-CART(61-102) binding by CART peptides on PC12 cells

Peptide	K_i (nM)		
	Membranes from non-differentiated cells	Non-differentiated cells on plates	Differentiated cells on plates
CART(61-102)	2.99±0.65	13.61±2.62	8.01±2.14
CART(55-102)	2.98±0.33	12.94±1.65	5.40±0.44
I ₂ -CART(61-102)	1.27±0.20	3.00±0.89	4.46±1.05
CCK-8, α-MSH, TRH, AT II, NT, leptin	≥ 10 000		

Mean±S.E.M. of three to six separate experiments.

K_i was calculated using Cheng–Prusoff equation (concentration of the radioligand was 0.1 nM and K_d taken from saturation experiments was 0.48 nM for non-differentiated cells and 1.90 nM for differentiated cells).

12.5 µg to 100 µg of protein. It reached the maximum at 15 min and remained constant at least for the next 100 min. Binding of [¹²⁵I]-CART(61-102) to floating PC12 cells at 37 °C was linear in a range of 1.25×10^5 to 10^6 cells/tube, the binding reached maximum at 15 min and was constant at least for an additional 60 min. Specific binding at 4 °C reached the maximum at 60 min and was constant for further 4 h of incubation; non-specific binding at this temperature, however, amounted to 40% of total binding.

After optimization of binding conditions, 30 min incubation at 37 °C with 50 µg of protein (cell membranes) or 5×10^5 cells (floating or plated intact cells) was routinely used.

3.4. Competitive binding to non-differentiated PC12 cells and cell membranes

CART(61-102), CART(55-102), and I₂-CART(61-102) competed with [¹²⁵I]-CART(61-102) for binding to PC12 cell membranes with K_i in the low nM range (see Table 1). Non-related peptides such as CCK-8, α-MSH, TRH, AT II, neuropeptin, and leptin did not displace [¹²⁵I]-CART(61-102) binding to the cell membranes even at a concentration of 10^{-5} M.

Specific binding of [¹²⁵I]-CART(61-102) to floating cells was displaced by CART(61-102) with $K_i = 3.00 \pm 0.51$ nM.

In competitive binding experiments to plated PC12 cells, non-differentiated cells bound CART(61-102) and CART(55-102) with K_i in low 10^{-8} M range and I₂-CART(61-102) in 10^{-9} M range (see Table 1).

3.5. Saturation binding to non-differentiated PC12 cells and cell membranes

The saturable, specific binding of [¹²⁵I]-CART(61-102) to non-differentiated plated PC12 cells exhibited K_d of 0.48 ± 0.16 nM and B_{max} 2228 ± 529 binding sites/cell. Non-specific

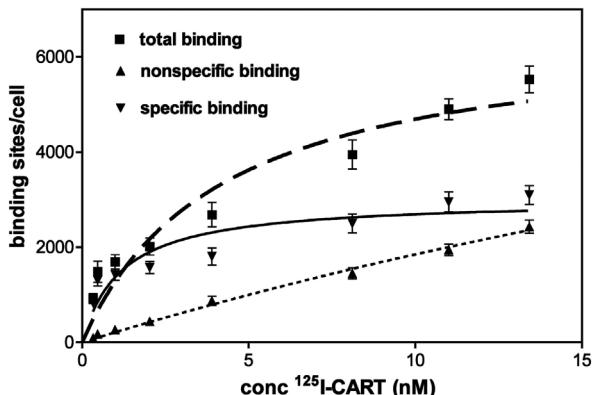


Fig. 2. [¹²⁵I]-CART(61-102) saturation binding to non-differentiated PC12 cells on plates (in all experiments, 5×10^5 cells/well were used). The cells were incubated at 37 °C for 30 min with increasing amount of [¹²⁵I]-CART(61-102) in the absence (total binding) or presence (non-specific binding) of 2×10^{-6} M CART(61-102). Specific binding was calculated by subtracting the non-specific from total binding. Binding curves were plotted using nonlinear regression. The figure is a representative example of five experiments carried out in triplicates.

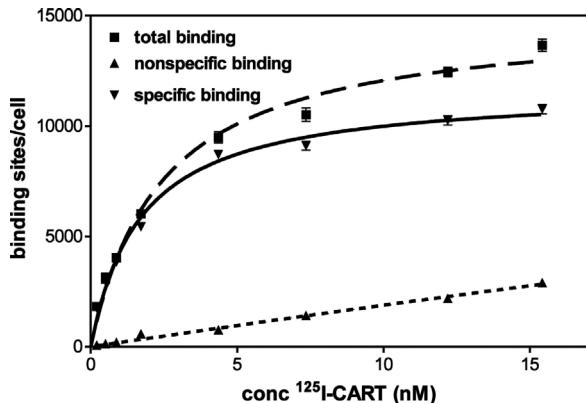


Fig. 3. [¹²⁵I]-CART(61-102) saturation binding to NGF-differentiated PC12 cells on plates (in all experiments, 5×10^5 cells/well were used). The cells were incubated at 37 °C for 30 min with increasing amount of [¹²⁵I]-CART(61-102) in the absence (total binding) or presence (non-specific binding) of 2×10^{-6} M CART(61-102). Specific binding was calculated by subtracting the non-specific from total binding. Binding curves were plotted using nonlinear regression. The figure is a representative example of three experiments carried out in triplicates.

binding was about 40% and never exceeded 50% of the total binding. Non-linear regression analysis showed one binding site (see Fig. 2). The saturation binding to cell membranes gave K_d of 0.60 ± 0.16 nM and B_{max} 155 ± 8 fmol/mg of membrane protein, but the non-specific binding slightly exceeded 50% of the total binding.

3.6. Differentiation of PC12 cells

The PC12 cell line was successfully differentiated using NGF treatment to the neuronal phenotype, as described earlier (Greene and Tischler, 1976). A fully developed net of neurons and axons was clearly visible using a phase contrast microscope. CART(61-102), CART(55-102) and I₂-CART(61-102) were bound to differentiated cells with K_i comparable with that of non-differentiated cells (Table 1).

3.7. Saturation binding to differentiated plated PC12 cells

The specific binding of [¹²⁵I]-CART(61-102) to non-differentiated plated PC12 cells was saturable, with non-specific binding less than 20%, with K_d 1.90 ± 0.27 nM, and B_{max} 11194 ± 261 binding sites/cell (Fig. 3).

4. Discussion

The CART receptor molecule(s) has not been characterized yet. Binding studies using radiolabeled CART were mostly unsuccessful due to the high background attributed to disruption of the binding site of the active ligand during the iodination reaction (Keller et al., 2006; Vicent et al., 2006). Just recently, specific binding of ¹²⁵I-CART (61-102) to AtT20 mouse pituitary tumor cells has been reported (Vicent et al., 2005, 2006) and linked to CART-induced activation of the extracellular signal-regulated kinase (ERK) pathway via a putative G-protein-coupled receptor (Lakatos et al., 2005).

Lakatos et al. (2005) registered a negligible response to CART(55-102) in pheochromocytoma PC12 in terms of ERK phosphorylation. However, dense CART immunostaining was reported in chromaffin cells of adrenal medulla (Dun et al., 2006) and thus could also be expected in PC12 cells because the line is derived from chromaffin cells and keeps the original character.

In our competition binding experiments with cell membranes and intact floating PC12 cells, the equilibrium binding was reached already after 15 min and was stable at least for the next 60 min. CART(61-102), CART(55-102) and I₂-CART(61-102) were bound to cell membranes with a similar affinity in a low nM range and to plated non-differentiated and differentiated cells with a lower affinity than to cell membranes (Table 1).

The di-iodinated derivative of CART had the highest binding affinity of all tested ligands both to cell membranes and plated non-differentiated and differentiated PC12 cells. It is not a rare phenomenon that iodinated compounds have higher affinity to the receptors than non-iodinated ones. Enhancement of ligand affinity after iodination has already been described in the literature, e.g. for the specific oxytocin receptor antagonist (Elands et al., 1988).

In this study, all K_d and B_{max} determined were in usual common range (Motulsky and Neubig, 1997). [¹²⁵I]-CART(61-102) was specifically bound to intact plated non-differentiated PC12 cells, with K_d 0.48 ± 0.16 nM (Fig. 2). The results of the saturation experiment with cell membranes confirmed the K_d value. Under identical experimental conditions [¹²⁵I]-CART(61-102) was specifically bound to differentiated PC12 cells with K_d 1.90 ± 0.27 nM (Fig. 3). Differentiated cells displayed lower non-specific binding than non-differentiated cells (20% versus 40%). We calculated that if non-specific binding to non-differentiated cells were also 20%, K_d for non-differentiated would have been similar to the value for differentiated cells. In AtT20 pituitary tumor cells, specific binding of [¹²⁵I]-CART(61-102) with K_d only of 21.9 ± 8.0 pM was reported (Vicentic et al., 2005). It could be explained by the fact that binding experiments with AtT20 cells were done at 4 °C while ours with PC12 cells at 37 °C.

Interestingly, B_{max} for differentiated cells, $11,194 \pm 261$ binding sites/cell (Fig. 3), was 5 times higher than B_{max} for non-differentiated cells, 2228 ± 529 binding sites/cell (Fig. 2).

Detection of CART peptide binding sites in both non-differentiated and differentiated PC12 cells pointed to a possible role of CART in the sympatho-adreno-medullar system (Dun et al., 2006) and in response to stress (Koylu et al., 2006).

We have not succeeded in detecting any specific [¹²⁵I]-CART(61-102) binding to dissociated cells and cell membranes of hypothalamus and gastrointestinal tract or to stable neuroglial cell line NG 108-15 (not shown). The reason might be the presence of different CART receptor(s) in normal and tumor cells or much lower density of the binding sites in tissues than in cultured cells.

Using feeding test with mice, we confirmed partial preservation of the biological activity of CART(61-102) after its iodination (Fig. 1). I₂-CART(61-102) in a dose of 1 µg/mouse lowered food intake to the same extent as CART(61-102) in a

dose of 0.5 µg/mouse. Together with the specific binding of [¹²⁵I]-CART(61-102) to PC12 cells, it proved that [¹²⁵I]-radiolabeling did not change to a great extent the pharmacological properties of CART.

The finding of binding sites for [¹²⁵I]-CART(61-102) on PC12 cells, both non-differentiated and differentiated, and the fact that di-iodinated CART(61-102) retained biological activity could be used in further studies aiming at characterization of potential CART receptor molecule(s).

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