REGULATION OF CART mRNA EXPRESSION

Ph.D THESIS SUMMARY

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INTRODUCTION

Discovery of CART

Cocaine-induced molecular alterations are believed to be responsible for many of the drug's behavioral consequences. Attention has therefore been focused on determining the changes in gene expression after cocaine treatment. Changes in expression of a number of genes have been observed in response to acute and chronic cocaine administration. CART (i.e., Cocaine- and Amphetamine-Regulated Transcript) gene which was discovered in 1995 by Douglass et al. as an mRNA transcript by differential display method is included in this gene group. CART mRNA was increased in rat striatum and nucleus accumbens after acute cocaine and amphetamine treatment. The profile of CART mRNA within the cerebellum, striatum and hippocampus was determined by Northern blot analysis and surprisingly showed transcriptionally silent brain regions in the hippocampus after acute cocaine and amphetamine treatment. Douglass et al. (1995) already pointed out that CART was a new, previously uncharacterized rat mRNA that was not significantly related to any known mRNA.

Characteristic features of CART DNA and mRNA

CART mRNA expression patterns in the human brain were qualitatively similar to the rat brain CART mRNA distribution indicating that the protein product of the CART mRNA plays a functional role or roles across the mammalian species. Since, CART mRNA has been described in many vertebrate species including rodents (mouse, rat), goldfish and human. Comparing human CART cDNA to rat CART cDNA, the human CART cDNA sequence was about 80 % identical to the rat cDNA with about 92 % homology in the protein - coding region. Subsequently, the CART mouse gene was mapped to chromosome 13 encoding a 2.5 kb segment, and the human gene was mapped to chromosome 5 containing approximately 2 kb segment, while the rat CART gene has not been identified yet. The chromosomal mapping made possible to analyze the genetic variations of CART gene. Several polymorphisms have been described in the 3' UTR region and 5' flanking region. To confirm this association between the CART 5' flanking region polymorphic site and the inherited obesity, further investigations are necessary. Additional rat CART cDNA sequence analysis characterized the rat CART mRNA in helping to understand the appearance of an RNA doublet following Northern blot analysis. The characterization revealed that in the termination of synthesis of CART mRNA, alternate poly A site utilization plays a role in the 3' non-coding region which resulted in the appearance of an RNA doublet, either approximately 700 or 900 bases in length. Moreover, alternative splicing produced further diversity within the coding region of the transcripts in the rat, and resulted in two mRNA species, one with the absence of an in frame 39 base insert within the protein coding region, called short form of CART mRNA apart from the 700 or 900 bases in length. In all probability, the observed CART mRNA doublet following Northern blot analysis corresponds to four mRNAs instead of two mRNAs. The shorter species was about twice as abundant as the longer one in rat. Interestingly, in human, the short form was the only one found.

Characteristic features of CART peptides

Rat CART cDNA sequence analysis also helped to predict the translation product of CART gene. The synthesized protein: either 129 or 116 amino acids in length depending

on the alternative splicing of 39 bases in the coding region. Examination of the deduced amino acid sequence suggested that the CART product would be a neuropeptide that was secreted and processed. This suggestion was based on the identification of a common hydrophobic leader sequence and the amino terminus indicating involvement in a secretion pathway, and several pairs of basic amino acids, which are commonly found in propeptides that are processed before subsequent utilization.

Thim et al. (1999) proved this hypothesis by purifying CART peptides from adrenal gland, hypothalamus, nucleus accumbens and pituitary gland of the rat and determined the peptide structures by using microsequencing and mass spectrometry. It demonstrated a tissue-specific processing of CART that may point to different biological functions of CART peptides in the peripheral and central nervous system. From the adrenal gland, the CART (1-89) and CART (10-89) peptides were isolated. In contrast, from the hypothalamus and nucleus accumbens, shorter form peptides CART (42-89) and CART (49-89) were purified. Surprisingly, the long splice variant was found in all of the tissues examined. Kuhar et al. (1999) confirmed this processing by Western blot analysis. In order to examine the processing and localization of CART putative peptides in the brain, polyclonal antibodies were prepared against several CART peptide fragments. Using these antibodies, several different fragments of the CART protein were identified in the brain. It seemed that the two fragments were likely to be produced from the pre-proCART protein and presumably they had different processing modifications. These antibodies mentioned above were also used for immunohistochemical staining to determine the localization of CART peptides. The distribution of CART peptides corresponded to the expression pattern of CART mRNA.

It has been shown that CART peptides are neurotransmitters. Electronmicroscopic analysis showed that the peptides were found exclusively in large dense vesicles in neurons in the brain, processed to smaller peptides and released by calcium-dependent mechanisms into synapses. CART peptides are co-localized with numerous well-known neurotransmitters like GABA, galanin, MCH, oxytocin, POMC, somatostatin, as well as nitric oxide synthase.
These neuroanatomical findings suggested various possible physiological functions. CART peptides could play a role in the stress response, feeding behavior, immune function, autonomic regulation, fluid balance, metabolic processes, sexual function and endocrine control. The putative functions are listed in *Table 1*. In order to prove presumed physiological functions, CART peptides were made by using molecular biology. The preparation of CART peptides by peptide synthesis was very difficult. A yeast expression system was developed for the production of relatively large amounts of CART peptides including the naturally occurring CART (55-102) as well.

Injection of the peptides into the brain resulted in behavioral effects. Strong evidence implicates CART in the control of feeding behavior. As mentioned above, CART is highly expressed in the arcuate and paraventricular nuclei of the hypothalamus. These areas are known to be involved in the control of appetite. CART neurons show c-Fos-immunoreactivity after intravenous injections of leptin in rats. Hypothalamic CART mRNA levels are decreased in hypoleptinemic states, such as in the *ob/ob* mouse, and after prolonged fasting. In these conditions, CART expression is restored by leptin administration. Intracerebrovascular injection of recombinant CART peptide into rats and mice inhibits normal and starvation-induced feeding and blocks the feeding response induced by neuropeptide Y. Conversely, after central administration of anti-serum against CART in rats, feeding response was increased. These data strongly implicate CART as an endogenous satiety factor. However, a new finding suggests that CART peptides may stimulate feeding at least in the hypothalamus.The injection of multifunctional CART peptides into the VTA

produces psychostimulant-like behavioral effects including increased locomotor activation and establishment of conditioned place preference. There is also evidence that the CART protein has neurotrophic action *in vitro*. The short form of CART protein, which is found in humans, has been shown to have neurotrophic properties in several primary-cell-culture neurons. These include dopaminergic, hippocampal and retinal neurons, and motoneurons. The effects produced include increased dopamine uptake, increased cell survival and increased neurite length. In a recent study, Balkan et al. suggested the involvement of the CART peptide in the stress response and its modulation by glucocorticoids. CART studies predict its role in development and neurotrophic activity, too.

Even with all of the information available, the CART receptor has not been found yet. Apart from the receptor missing, substantial evidence has been gathered that CART peptides are involved in psychostimulant - like effects, feeding, and stress. To understand and confirm the multifunctional effects of CART protein, the CART receptor needs to be identified. Furthermore, elucidating the detailed molecular mechanism involved in the regulation of CART gene may serve as a model to address the multifunctional behavior of CART peptides.

First, the CART was identified as an upregulated mRNA transcript after acute cocaine and amphetamine treatment. Secondly, the cyclic adenosine 3', 5'-monophosphate (cAMP)- protein kinase A (PKA) signal tranduction pathway is known to be induced by cocaine administration in rat nucleus accumbens. Thirdly, it has been reported that after cAMP activation CART mRNA was increased in GH3 cells. Therefore, the possibility of PKA-mediated transcriptional regulation in the CART gene expression is more than likely.

In this study, based on the aforementioned evidence, the attention was focused on determining regulatory mechanisms driving the changes in the CART gene expression.

Table 1.The possible physiological functions of CART peptide

Feeding behavior
Paraventricular nucleus of the hypothalamus
Arcuate nucleus
Parabrachial nucleus
Perifornical cells in the hypothalamus
Stress
Hypothalamic nuclei
Pituitary (anterior or posterior)
Adrenal medulla
Intermediolateral cell columns of the spinal cord
Sensory processing
Mitral and tufted cells of the olfactory bulb
Some retinal ganglion cells
Layer IV of the cerebral cortex in barrels
Thalamic relay nuclei
Nucleus of the solitary tract
Lamina 1 of the spinal cord
Afferent vagal fibres
Reward and reinforcement
Nucleus accumbens
Ventral tegmental area of the midbrain
Basolateral amygdala
Central autonomic network
Nucleus of solitary tract
Nucleus ambigous
Central nucleus of amygdale
Paraventricular nucleus of hypothalamus

OBJECTIVES OF THE RESEARCH PROJECT

1. Identifying transcription binding sites for the CART promoter region using the Trasfact computer program.

2. To determine whether the putative transcription binding sites on the CART promoter participate in the activation of CART gene expression in GH3 cells.

3. Characterizing the relevant transcription factors and their functions which can be recruited into the CART promoter during CART gene activation.

4. To identify transcriptional regulatory networks that can play substantial role in the regulation of CART mRNA expression.

EXPERIMENTAL PROCEDURES

CELL LINES

List of cell lines used:

GH3	rat pituitary adenoma cell line
AtT20	mouse pituitary adenoma cell line
PC12	rat phaeochromocytoma cell line

CELL CULTURE

PC12 cells were grown in Dulbecco's modified eagle medium (DMEM), supplemented with 5% calf serum and 10% horse serum. GH3 cells were maintained in F-12K Ham's (Kaighn's modification) media supplemented with 12% horse and 2.5 % fetal bovine serum.Mouse AtT20 cells were grown in Dulbecco's Minimal Essential Medium supplemented with 10 % horse serum. All tissue cultures were maintained in a humidified incubator at 37 ^{0}C under 5 % CO_2.

SEQUENCING AND ANALYSIS OF THE MOUSE CART GENE PROMOTER REGION

A *Bam*HI subclone, BAM5'C (approximately 9.5 kb in length), containing the entire CART gene was generated. The CART 5'-flanking region in BAM5'C (*BglII*/*Sac*I segment) was sequenced. All sequences were determined by cycle sequencing reactions.
The sequenced region was analyzed for putative transcription factor binding sites using the Transcription Factor Database (TRANSFAC) and MatInspector

GENERATION OF PLASMID CONSTRUCTS

pGL3-Luciferase Reporter (Promega) and pSV-Galactosidase Control Vectors (Promega) provide a basis for the quantitative analysis of factors that potentially regulate mammalian gene expression. These factors may be *cis*-acting (promoters and enhancers) or *trans*-acting (various DNA-binding factors).

pGL3-Basic Luciferase Reporter Vector: This vector lacks eukaryotic promoter and enhancer sequences. Expression of luciferase activity in cells transfected with this plasmid depends on the promoter activity of the insertion in certain cells. Without insertions, this vector serves as a control for the background activity.

pGL3-Control Luciferase Reporter Vector: This vector contains SV40 promoter and enhancer sequences, resulting in strong expression of luciferase gene in many types of mammalian cells. The control vector serves as positive control to monitoring transfection efficiency.

pSV-Galactosidase C Control Vector: This vector is co-transfected with the DNA of interest and acts as an internal control for transient expression assays.

Cloning: Various lengths of 5' upstream sequences were cloned into the promoter-less vector; pGL3-Basic itself and generated clones were tested for promoter activity. Constructs were generated by digesting the genomic clone, Bam5'C with *BglII/NcoI*, or *KpnI/SacI*, or *SmaI/NcoI*. pGL3-Basic was digested and dephosphorylated followed by ligation with the digested genomic fragment using T4 DNA ligase. The clones were transformed into *E. coli* TOP10 cells and plasmid DNA was isolated. Constructs were confirmed by dideoxy nucleotide sequencing.
The pGL3-luciferase expressing constructs made -3451 CART-LUC, spanning -3451 to +23, -641 CART-LUC, spanning -641 to +30, and -102 CART-LUC, spanning -102 to + 23 to +30 where +1 is the predicted site of transcription initiation. Furthermore, mutated 641CART-LUC with abrogated CRE site and CREB dominant negative mutant, (pCMV500-A-CREB) in which basic residues within the bZIP domain have been mutated to acidic residues, referred to as A-CREB were generated.

TRANSFECTION

For each transfection, 2 μg of one of the pGL3-luciferase expressing constructs, 2μg of pSV-β-Galactosidase Vector and 6 μl of FuGENE 6 Transfection Reagent (Roche) were mixed in the appropriate serum-free media and incubated at 25 ^{0}C for 45 minutes following the manufacturer's instruction. 100 μl of the complexed DNA/FuGENE 6 mixture was added to each well and incubated for 18 hours at 37 ^{0}C under 5 % CO_2. In some cases, 2 ml of supplemented media containing 20 μM forskolin was added and incubated for additional amounts of time. Cells were lysed and luciferase expression measured using the Luciferase Assay System (Promega).Luciferase activity was quantified using a luminometer. β-Galactosidase activity was measured using the β-Galactosidase Enzyme assay system (Promega) and the absorbance read at 420 nm. Protein concentration was determined using the Bradford method (Bio-Rad Protein Assay). In all experiments, luciferase expression was normalized to β-Galactosidase activity using equal protein concentration. Statistical significance was determined using one-way ANOVA followed by a Tukey Test or a Student t-test (SigmaStat 2.0.).

RNase PROTECTION ASSAY

10 μg of RNA from AtT20, GH3 and PC12 cells was dissolved in High-Speed Hybridization buffer (Ambion) and ^{32}P-CART RNA antisense probe was added to each sample. After 20 minutes hybridizing at 68 ^{0}C, the probe and RNA were digested with an RNase mixture at

37 ^{0}C for 30 minutes. After inactivation and precipitation of samples, the protected fragments were subjected to 8% denaturing polyacrylamide gel electrophoresis. Then, the gel was dried and exposed to X-ray film at minus 80 ^{0}C for 15 hours.

NORTHERN BLOT ASSAY

Total RNA was isolated from frozen rat cerebellum and hypothalamus or GH3 cells. Tissues (50-100 mg) were homogenized in 1 ml of Trizol Reagent (Life Tech.) using a power homogenizer. 10^7 GH3 cells were lysed in 1 ml of Trizol Reagent and RNA was isolated according to the manufacturer's recommendation. Total RNA was run in a 1.2 % formaldehyde agarose gel. RNA was transferred by capillary action in the presence of 10x SSC and fixed by UV cross-linking. Hybridization buffer was changed and 2x 10^6 cpm of a ^{32}P-labeled cocktail consisting of 2 or 3 distinct oligonucleotides was incubated at 50 ^{0}C for 24 hours. Oligonucleotides were 5' end labeled by using γ-^{32}P-ATP (6000 Ci/mmol) and polynucleotide kinase (Stratagene). The CART cocktail consisted of three oligonucleotides with the following sequence: 5'TGAAAACAAGCACTTCAAGAGGAAAG3', 5'TGCAACGCTTCGATCTGCAACATAG3', 5'CTCATGCGCACTCTCTCCAGCG3'. Blots were stripped and re-probed with the GAPDH probe. CART mRNA levels were normalized to GAPDH mRNA.

WESTERN BLOT ASSAY

Total protein was extracted in 100 µl of lysis buffer containing the following protease and phosphatase inhibitors: 5 nM ocadaic acid, 200 µM sodium othovanadate, 1x protease mixture (Maniatis) and 0.2 mM PMSF.Equal amounts of 1x sample buffer were added to 25µg of protein lysate. The gel was run at 120V for 2 hours and then electrically transferred overnight (50V at 4 ^{0}C) onto Immobilon -P membrane (Millipore) using a transfer buffer. The blot was incubated in PBS-T blocking buffe for 1 hour. After the initial blocking step, the blot was incubated in blocking buffer that contained anti-CREB antibody at a 1:500 dilution (Santa-Cruz) for 1 hour at room temperature. Then the blot was washed at room temperature and then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody, anti-rabbit IgG (Santa Cruz). CREB binding was detected using a chemiluminescent detection kit (ECL+Plus). The blot was washed with 0,2 M NaOH to remove previous protein complexes and re-incubated overnight at 4 °C with anti-phosphorylated - CREB antibody (P- CREB) (Cell Signaling).

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

DNA-protein interactions were studied by EMSA. Nuclear protein extracts were prepared from forskolin-treated GH3 cells as described by Szeberényi et al. (1998). Total nuclear protein (15 µg) was incubated with 2 ng of ^{32}P-5'end-labeled oligonucleotide, containing the CART-CRE site (5'AGCATTGACGTCA3') Total nuclear protein extract and labeled oligonucleotide were incubated for 10 minutes at room temperature. In some cases, a 20- or 100-fold molar excess of a specific competitor (nonlabeled CART oligo), or 100-fold molar excess of a nonspecific competitor Oct 1 (5' TGTTGAGGGGACTTTCCCAGC 3') was added to the mixture, to assess the specificity of the DNA-protein binding complex before the addition of the labeled oligonucleotide. „Incubation with unlabeled oligonucleotide, the ^{32}P-labeled CART oligonucleotide was added and incubation was continued for 20 minutes. For the supershift assay, 2 µg of CREB antibody (Santa Cruz) was incubated with the nuclear extract and the ^{32}P-labeled CART oligonucleotide for 45 minutes. Supershift analysis was also done using 4 µg of P-CREB antibody (Santa Cruz). P-CREB antibody was incubated with the nuclear protein extract in binding buffer for 30 minutes at room temperature, followed by addition of the ^{32}P-labeled CART oligonucleotide and incubated for another 45

minutes. The DNA -protein complexes were separated by electrophoresis on a 6% non-denaturing polyacrylamide gel. Gels were run (120 V) in the presence of 0.5x TBE buffer for 1.5 hour at 4 ^{0}C. Dried gels were exposed for 24 hours and analyzed by using a PhosphoImager.

EXPERIMENTAL RESULTS

Computer search for enhancer elements in the CART promoter region

To understand the molecular mechanism involved in the transcriptional regulation of CART gene expression, a mouse BAC genomic library was screened using rat CART cDNA probe. One hybridization clone was identified (Adams et al.1999). A *Bam*HI subclone, BAM5'C, containing the entire CART gene was generated. The CART 5' flanking region in BAM5'C (*Bgl*II / *Sac*I segment) was sequenced. The sequenced region was analyzed for putative transcription factor binding sites. This analysis revealed a region in the mouse CART gene 5' upstream sequence containing a cluster of transcription factor binding sites, including a canonical cyclic AMP response element (CRE), two AP-1 sites, one SP1 and several AP2 sites, along with a TATA-like sequence and an E-box .There is also a STAT-response element, TT(N)$_6$AA, in an overlapping STAT/CRE/AP1 site. Further upstream, there is a putative binding site for the pituitary-specific transcription factor Pit-1, AWWWTATNCAT, where W is either an A or T. The CART gene upstream sequence was further analyzed using a neural network promoter predicting algorithm in order to predict transcription initiation sites for the mouse CART gene. The transcription initiation for the mouse CART gene was predicted at the A located 19 nucleotides upstream of the initiating AUG codon in agreement with the previously published rat and human 5' ends. The alignment of the mouse proximal promoter region with the corresponding human region (Accession Number NT019389) identified a 320 bp region immediately upstream from the CART coding sequence that shares 83.4% nucleotide identity. The region contains the binding site for the clustered set of transcription factors, including the overlapping CRE/AP1/STAT site.

Assessing the transcription factor binding sites for promoter activity

To examine the regulation of CART gene promoter, GH3 mouse pituitary adenoma cell line served as an *in vitro* model. Furthermore, to compare the activities, predict tissue specificity, and determine the importance of certain CART promoter regions in the gene regulation, AtT20 and PC12 cell lines were used as well as an *in vitro* model.
The activity of various 5'-proximal regions of the CART gene was tested for the ability to drive gene expression when cloned into pGL3-Basic vector upstream of the luciferase gene. Three luciferase expressing constructs were made and tested, -3451CART-LUC, -641CART-LUC, and -102CART-LUC that contain 3451, 641, and 102 bp mouse CART 5' upstream sequence (+1 is the predicted site of transcription initiation), respectively. The -102CART-LUC construct contains the TATA-like box and an SP1 site. The -641CART-LUC construct contains the STAT/CRE/AP1 composite site and the -3451CART-LUC contains a putative Pit-1 binding site. Pit-1 is a transcription factor belonging to the POU domain proteins that is expressed exclusively in the central nervous system and in the pituitary.
Cells were transiently co-transfected with pSV-β-Galactosidase and one of the CART promoter containing constructs. Luciferase activity of each construct was compared to that produced by pGL3-BASIC, the parent vector that lacks a promoter for background activity.

pGL3-CONTROL, which has SV40 promoter to drive luciferase expression, was used as a positive control. In GH3 cells, pGL3-CONTROL had a 6-fold increase in luciferase activity above pGL3-BASIC. This is similar to the 5.4-fold activation by -102CART-LUC, indicating that this segment of CART upstream sequence can function as a promoter and drive luciferase expression. Moreover, the -641CART-LUC construct containing a larger upstream segment of CART promoter produced much greater, 29-fold activity. A dramatic 59-fold increase in luciferase activity was observed after the -3451CART-LUC construct transfection, suggesting the importance of this region in mediating the promoter activity. Because the promoter analysis revealed a CRE element in the CART promoter, this element should respond to an elevated cAMP level in the cells.

Then, the endogenous CART gene was measured for responsiveness to forskolin treatment. GH3 cells were treated with 20 μM forskolin for 0, 1, 3, 6, 12 and 24 hours and 40 μg total RNA was analyzed by Northern blot analysis. Forskolin treatment increased CART mRNA levels compared to 0 hour, control exposure. The involvement of protein kinase A (PKA) was also tested. In the same experiment, some GH3 cells were treated with 30 μM H89, a selective inhibitor of PKA for 2 hours prior to and during forskolin treatment. The time course showed that the maximal increase in CART mRNA levels occurred after 6 hours of forskolin treatment. Treatment with H89 prior and during forskolin treatment reduced CART mRNA level up to 50%. This result confirms that cAMP is a second messenger for positive regulation of CART gene expression and PKA as protein kinase is also involved in the hypothetical transcription pathways of CART gene.

To determine which cis-elements present in the CART promoter were responsible for the responsiveness to cyclic AMP induction by forskolin, two luciferase expressing constructs were tested. The -641CART-LUC and -102CART-LUC constructs were transfected into GH3 cells and 18 hours after transfection media was changed and cells were treated with 20μM forskolin for 7 hours. A 2-fold increase in luciferase activity after forskolin treatment was produced with -641CART-LUC, while no statistically significant increase in luciferase activity was observed with -102CART-LUC and pGL3-CONTROL. This suggests that the region between -102 and -641 containing overlapping STAT/CRE/AP1 transcription factor binding sites that are responsive to cyclic AMP.

A comparison of luciferase activity between -641CART-LUC and pGL3-CONTROL after forskolin treatment showed that -641CART-LUC exhibits a continuous increase in expression beginning at 1 hour and continuing through 48 hours. The promoter activity of -641CART-LUC was examined in AtT20 and PC12 cells as well. -641CART-LUC showed higher activity in PC12 cells than in AtT20 cells. However, promoter activity was the highest in GH3 cell (29-fold above background). Forskolin treatment did not have any significant effect on luciferase expression of -641CART-LUC construct in PC12 and AtT20 cells. The high activity of -641CART-LUC in GH3 cells, and the lower activity in PC12 and AtT20 cells suggest that the STAT/CRE/AP1 overlapping transcription binding sites enhance expression in a cell type specific manner.

Additionally, a mutation that abrogated the CRE site in -641CART-LUC construct resulted in a 4.7-fold drop of luciferase expression in GH3 cells after forskolin treatment. All these results together delineate the relevance of the CRE transcription binding site on the CART promoter in GH3 cells. Also, these findings propose that this overlapping STAT/CRE/AP1 site plays an important role in the determination of tissue specificity of CART gene expression.

Characterizing the putative transcription factors and their involvement in the regulation of the CART gene

Subsequently, electromobility shift assays were carried out to identify in particular the transcription binding sites that are responsive to elevated cAMP level and responsible for the promoter activity in transfected GH3 cells and the induced endogenous CART mRNA expression after forskolin treatment. A 27-bp double-stranded oligonucleotide containing the CRE site, corresponding to nucleotides -153 to -127 of the CART promoter, was synthesized and 5' end labeled with ^{32}P, referred to as CART oligo.

In order to test if this CRE site binds protein factors from GH3 cells, nuclear extracts from cells treated with 20 μM forskolin for varying periods of time (0.5, 1 and 3 hours) were incubated with ^{32}P-labeled CART oligo. Treatment with forskolin under the same conditions has been shown to enhance endogenous CART gene expression in GH3 cells.The ^{32}P-labeled CART promoter oligonucleotide clearly binds to nuclear factors in gels and results in the detection of a shifted DNA/protein complex. The binding increased with forskolin treatment time, and addition of 20x excess unlabeled CART oligo served to assess the specificity of the DNA-protein binding complex. This experiment indicates that the GH3 cells, in response to forskolin treatment, produced increased binding to the CART promoter oligonucleotide.

Western blotting study was conducted to examine whether the treatment with forskolin under the same conditions produced changes in CRE binding protein (CREB) and/or phosphorlylated-CREB (P-CREB) levels. CREB is a well-characterized transcription factor known to be activated by cAMP and binds to the CRE site of promoters. Consequently, CREB became the first candidate for the transcription factor that can bind to the CRE site of CART proximal promoter and be accountable for the increased CART mRNA expression. It revealed an increased P-CREB level with duration of treatment with forskolin, while CREB levels remained unchanged. These results suggested that the nuclear protein binding to the CART promoter oligonucleotide could be P-CREB. Accordingly, mobility supershift assay was used to detect if the protein binding to the CART promoter site would interact with CREB and P-CREB antibodies since an increase of P-CREB was observed by Western blot. It was found that CREB and P-CREB antibodies supershifted the CART oligo/protein complex. In addition, molar excess of unlabeled cold CART oligo competed with radiolabeled CART oligo, whereas an unrelated oligo (Oct1) did not, indicating specificity in the CART oligo/protein complex. The CART oligo/protein/CREB antibody complex was found in both untreated and treated cells as expected. Similar data were found after 0.5, 1 and 6 hours of forskolin treatment as well.

To confirm the role of CREB transcription factor in CART gene regulation, a CREB dominant negative mutant, A-CREB was co-transfected with the -641CART-LUC construct into GH3 cells treated with forskolin. A-CREB functions by heterodimerizing with endogenous CREB transcription factor and preventing its interaction with the CRE element. GH3 cells, co-transfected with the A-CREB mutant had a 3.4-fold reduction in luciferase expression as compared to cells co-transfected with the empty vector.

As a result of the experiments, in GH3 cells, the transcriptional regulation of the CART gene after activation of the cAMP pathway is likely to be mediated by the CREB transcription factor.

Investigating the importance of different signal transduction pathways in the regulation of CART gene

Ionomycin, an antibiotic has been reported to trigger the release of Ca^{2+} ions from the intracellular storage resulting in an increase of P-CREB. GH3 cells were treated with 10 μM ionomycin under the same condition as forskolin treatment. Northern blot analysis demonstrated a significantly changed expression pattern after 1 hour ionomycin treatment. It seems that Ca^{2+} ions are stronger activators for the CART gene than AC activation.

In order to test if the CRE site binds protein factors from ionomycin-treated extracts, GH3 cells were treated with 10 μM ionomycin for 0, 0.5,1 and 3 hours and were incubated with ^{32}P-labeled CART oligo. Nuclear proteins bound to ^{32}P-labeled CART oligo and these DNA/protein complexes were supershifted with CREB and P-CREB antibody. In the same gel, the 100x excess of unlabeled AP1 oligo (Promega) competed for the labeled CART oligo indicating that the same nuclear factor or factors have binding affinity to the CART and AP-1 oligo. The binding activity of ^{32}P-CART after 1 h ionomycin treatment demonstrated increased binding to the probes, respectively.

CONCLUSIONS

CART mRNA expression has been reported to be altered in response to various stimuli including fasting, leptin, and psychostimulant drugs but the signal transduction pathways involved in the CART mRNA regulation have not been studied yet.

The aim of this work was to characterize the CART promoter regulation by identifying putative transcription factor binding sites in order to describe signal transduction pathways involved in the regulation of CART gene expression. Sequence analysis of a 3.4 kb mouse genomic DNA fragment revealed a 320 bp region located upstream from the transcription start site, which is highly conserved between human and mouse CART gene. This region contains several transcription factor binding sites including an overlapping STAT/CRE/AP1 site located 148 nucleotides upstream from the predicted transcriptional initiation site. This binding may play an important role in the regulation of the CART gene.

The transient transfection system is the primary tool for identifying and characterizing the interaction of *cis*-acting elements with their corresponding *trans*-acting factors and associated cofactors in mammalian cells. In order to test the presumed promoter activity of the individual DNA fragment, different lengths of the mouse CART promoter were transfected for transcriptional activity into the GH3 rat adenoma line. This comparative transfection analysis revealed diverse transcriptional expression patterns after forskolin treatment in accordance with the different lengths of the CART promoter. These studies indicate that this individual DNA fragment displays significant promoter activity, moreover a major role for the overlapping STAT/CRE/AP1 transcription binding site in the regulation of the CART gene. This suggestion is supported by the mutation of the CRE site in the -641CART-LUC construct that showed significant decrease in the promoter activity. In addition, -3451CART-LUC displayed the highest promoter activity suggesting the importance of other transcription factor binding sites in CART gene expression. Additionally, the transfection studies on AtT20 and PC12 cells showed promoter activity considerably reduced indicating a highly tissue – specific regulation of this promoter. Likewise, the Pit-1 binding site at position -818 on the mouse CART promoter suggest the Pit-1 transcription factor involvement in a cell-type specific transcriptional activation of the CART gene. Pit-1 is

a POU-homeobox transcription factor that is responsible for either the commitment or maintenance of somatotrop, lactotrop, and thyreotrop cell lineages in the pituitary.

To identify the possible regulation pathway/pathways, endogenous CART gene responsiveness to forskolin treatment was tested. Transcription activation of endogenous CART gene in GH3 cells showed a continuous increase after cyclic AMP activation. In accordance with the recent scientific viewpoint this elevation was probably due to PKA activation since forskolin turns on the cAMP-PKA pathway. PKA stimulates different transcriptions factors including CREB. CREB is known to bind to CRE elements in the promoter of number of genes (e.g. somatostatin and tyrosine aminotransferas). Consequently, treatment with H89, an inhibitor of PKA, with simultaneous forskolin treatment, reduced the increase in the expression of endogenous CART mRNA. In summary, these studies together suggest that the CRE site in the CART proximal promoter could mediate the increased expression in CART mRNA levels in response to forskolin treatment via the PKA pathway.

Following this train of thoughts, it has been well described that the binding of cAMP to two PKA regulatory subunits, releases the catalytic subunits and enables them to phosphorylate target proteins. ATF/CREB family is one of the target proteins including several members, of which the CREB, CREM and ATF1 gene products are directly phosphorylated by the cAMP-dependent PKA. ATF/CREB family belongs to a larger leucine zipper family. This domain allows CREB, ATF1 and CREM to be able to bind to CRE sites as a dimer. The presence of highly conserved and related dimerization domains suggests that the different members of the CREB/ATF family might be able to form heterodimers and homodimers as well. Furthermore, composite CRE/AP1 sites have been found in a number of genes in playing very important roles in neurotransmitter synthesis, including dopamine hydroxylase, prodynorphin, proenkephalin, and cholecystokinin.

CREB is one of the main transcription factors involved in many genes' regulation in the brain; therefore, CREB became the first candidate, which may moderate the expression of CART gene via the PKA pathway by binding to the CRE site on the CART promoter. The results of mobility shift assays pointed out that treatment of GH3 cells with forskolin produced an increased level of P-CREB via the PKA pathway that binds to the CART promoter oligonucleotide containing CRE/AP1 site. These findings are very important because phosphorylation of CREB affects the ability of dimerization with different bZIP partners or the phosphorylation at Ser133 might trigger CREB binding to CRE. Transfection experiments with the dominant negative mutant CREB-A also supported a crucial role for CREB in the regulation of CART gene expression. On the other hand, it is a well-known fact, that increase in the intracellular Ca^{2+} level causes an elevation of P-CREB. Many kinases, some of whose activities are enhanced by Ca^{2} are capable of phosphorylating CREB *in vitro*, including Ca^{2+}/calmodulin-dependent kinases I, II, and IV (CaMKI,II, and IV) and PKC. In this study, it was also demonstrated that the increase of intracellular Ca^{2+} level by ionomycin treatment induced the gene expression of CART through CREB recruitment to the CRE site of the promoter in GH3 cells. These observations indicate that the CART gene may be regulated by other signal transduction pathways activating CREB, not exclusively by cAMP-PKA. Additional studies are required to determine the precise role of intracellular Ca^{2+} in the regulation of the CART gene. Moreover, the mobility shift assay of ionomycin-treated cells showed a competition between labeled CART probe and unlabeled AP-1 oligonucleotide indicating the possible role of other transcription factors (e.g. AP-1) in the regulation of CART gene expression.

The fact that other transcription factors can form complex with CRE can explain this phenomenon. After cAMP and Ca^{2+} stimuli, CREB makes different complexes involving

several transcription factors. The molecular mechanism that accounts for the CRE/AP1 specificities of bZIP proteins has been a focus of research since this DNA recognition motif was first identified in 1987. The CRE (TGACGTCA) and AP1 (TGACTCA) target sites share the same consensus half-site and differ by only a single, central G-C base pair. It has been reported that the CRE/AP1 specificity of CREB is controlled by charged residues has interesting implications about how transcription factors seek and selectively bind to precise sequences within genomic DNA (Montclare et al. 2001) emphasizing the ease with which specificity can be altered through the formation of different heterodimers or through combinational interactions with cellular factor.

Recently, *in vitro* binding studies have indicated that phosphorylation of CREB on Ser 133 does not alter the affinity of CREB or a palindromic CRE site (TGACGTCA; CART promoter has the same palindromic sequence), but it can be crucial at different levels of the transcription activation pathway for promoting target gene activation through recruitment of the 265-kDa, 2442 amino acid co-activator protein, CREB binding protein (CBP). CBP co-activator is a member of acetyltransferase family (AT) including p300/CBP-associated factor (P/CAF), TAF_{II} 250, steroid receptor coactivator-1 (NCo-1/SRC-1), p300/CBP-interacting protein (P/CIF), and p160. These proteins are able to acetylate transcription factors (e.g. p53), histone proteins and other nuclear proteins.

Based on studies, in accordance with our results, the Ser-133 phosphorylation of CREB is both necessary and sufficient for complex formation promoting the interaction between KIX domains. This interaction domain of CBP is required for binding to the P-CREB. CBP can serve as a molecular bridge that allows CREB to recruit and stabilize the RNA polymerase II (Pol II) transcription complex at the TATA box. Some studies have shown that the AT containing domain of CBP can only stimulate transcription from certain promoters and that the PKA activation of CREB required CBP, P/CAF and P/CIF. The diversity amongst the AT domains in different co-activators may account for their observed promoter selectivity.

As mentioned above, CREB forms heterodimers with its family members (ATF1, CREM, ICER) and seems to make complexes with other factors binding to the CRE site, for example c- Jun and NF-κB through CBP. In this regard, our findings postulate a hypothesis in which the direct and indirect interaction of different transcription factors, co-activators with CREB involving the alternation of AT activity are also involved in the CART promoter regulation. Additional mutational analysis and mobility shift assay of the STAT/CRE/AP1 composite site with different transcription factors including CBP will be important in identifying which transcriptional binding sites and factors beside CREB are involved in the Ca^{2+}/cAMP responsiveness of CART promoter.

The present results may be of physiological significance since cocaine administration increases P-CREB levels in the brain and increases CART mRNA levels in the striatum as well. Since the CART peptide appears to play a role in the action of diverse neurotransmitters and has itself multiple functions in the central and peripheral nervous system (Table 1), the further investigation of the regulation of the CART gene can elucidate the background of its multiple functions. On the other hand, using CART gene promoter studies as a model for investigating gene regulation, can provide a new approach helping to understand the complex regulation of genes and their expression in central nervous system.

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ABSTRACT, POSTERS AND PRESENTATIONS FOR THE PRESENT THESIS

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