ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 Apr; 23 (4): 349 - 354
http://www.chinaphar.com

Localization of CCTβ in rat brain and overexpression in insect cells[1]

R96 A

XU Kan-Yan, XIONG Ying, XIE Zhi-Qin, BIAN Wei, JING Nai-He, DU Yu-Cang[2] (*Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China*)

KEY WORDS CTP: phosphocholine cytidylyltransferase; argipressin; *in situ* hybridization; hippocampus; hexadecylphosphocholine; baculovirus

ABSTRACT

AIM: To study the localization of CTP: phosphocholine cytidylyltransferase β isoform (CCTβ) in rat brain, its expression in insect cells and enzymatic properties. **METHODS**: Using digoxigenin-labeled CCTβ probes, *in situ* hybridization was carried out in rat brain wax sections. CCTβ was overexpressed in Trichoplusia Ni (Tn) cells using baculovirus expression system. CTP: phosphocholine cytidylyltransferase assay (CT assay) and [^{3}H] metabolic labeling experiment were used to study its activity, properties, and the effect on phosphatidylcholine (PC) synthesis. **RESULTS**: (1) CCTβ was abundant in CA1, CA2, CA4, and dentate gyrus (DG) region of hippocampus. (2) The content of CCTβ in transfected Tn cells was over 1×10^4 times of that in rat brain, and CCTβ increased the PC synthesis of Tn cells. (3) Hexadecylphosphocholine as well as some ions like Zn^{2+} and PO_4^{3-} could inhibit the activity of CCTβ, dCTP was another adaptive substrate of CCTβ besides CTP. **CONCLUSION**: CCTβ showed a similar localization in rat brain with the memory enhancing peptide argipressin (4 - 8).

INTRODUCTION

CTP: phosphocholine cytidylyltransferase (CCT), which catalyzes the formation of CDP-choline, is a rate-limiting enzyme for phosphatidylcholine (PC) biosynthesis in mammalian cells[1]. PC is the major membrane phospholipid in eukaryotes, its *de novo* synthesis is essential to normal cell growth and death. In our previous research, we reported a new rat gene encoding CCTβ isoform[2]. Tissue distribution analysis showed that it was more abundant in brain than other tissues, and different with the CCTα isoform which had even distribution in all tissues[3]. It was also reported that in rat hippocampus CCTβ mRNA could be upregulated by argipressin(4 - 8), a metabolite of argipressin, which was much potent in facilitating the acquisition and maintenance of learning and memory[4], and also showed neuroprotective functions as facilitation of neurites elongation and prolongation of cell aging[5]. Therefore, it was possible that CCTβ played an important role in AVP_{4-8} affecting memory maintenance, brain development, and aging. In the present work, CCTβ's localization in rat brain, overexpression in Tn cells, and its properties were researched to evaluate CCTβ as a rational drug target in the brain disorders therapy.

MATERIALS AND METHODS

Materials Sprague-Dawley rats (Grade Ⅱ, Certificate № 003) were from Shanghai Experimental Animal Center, Chinese Academy of Sciences. TNM-FH insect medium, CTP, phosphocholine, and oleic acid were from Sigma, USA. Lipofectamine, fetal bovine serum (FBS) from Gibco BRL, USA. Bac to Bac baculovirus expression system (Gibco BRL), and Trichoplusia Ni cells were gifts from Prof WU Xiang-Fu. Anti-His antibody from Qiagen, Germany. [*methyl*-^{14}C] phosphocholine, [*methyl*-^{14}C] CDP-choline, and [*methyl*-^{3}H]choline from Amersham Pharmacia Biotech, England. TLC plate from Whatman, England. Enhancer sprayer from NEN, USA. High performance film from Kodak, USA. Digoxigenin-labeled *in vitro* transcript kit from Boehringer Mannheim, Germany. Pbluescript SK (PBSK) containing CCTβ cDNA was

[1] Project supported by the National Program of Basic Research (№ 1999054000) sponsored by the Ministry of Science and Technology and National Key (973) Program.
[2] Correspondence to Prof DU Yu-Cang. Phn 86-21-6437-4430. Fax 86-21-6433-8357. E-mail duyc@ sunm.shnc.ac.cn
Received 2001-08-29 Accepted 2002-01-14

prepared by our lab. the other chemicals were of analytical grade.

***In situ* hybridization** Pbluescript SK containing CCTβ cDNA was digested with *Bam*H I and *Hind* III, and subcloned to pSPT18 vector. The recombinant pSPT18 vector was digested with *Hinc* III and *Nde* I or with *Bam*H I and *Nhe* I. The obtained fragments were treated as the template of antisense or sense RNA probes respectively. Using an *in vitro* transcription system, the digoxigenin-labeled RNA were synthesized and stored at −20 ℃ until use. Ten micrometer thick wax section of rat brain was prepared and *in situ* hybridization was performed as described previously[6].

Expression in Tn cells The expression was performed according to the protocal of Bac to Bac baculovirus expression system. Briefly, CCTβ cDNA was cut from PBSK by *Bam*H I and linked to pFastBac HTc transfer vector which was digested with *Bam*H I. The correct linked plasmid was transfected into DH10Bac. The recombinant Bacmid DNA which contained the CCTβ cDNA was extracted. Tn cells were incubated in TNM-FH medium containing 10 % FBS at 27 ℃ until 70 % − 80 % confluent, then the recombinant Bacmid DNA was transfected into the cells with the method of lipofectamine product protocol. After 72 h, the supernatant containing the virus was used for virus collection and retransfection.

Western blot Tn cell lysates (40 μg of protein) were seperated by SDS-GEL electrophoresis on 10 % polyacrylamide gels and transferred onto the polyvinylidene difluoride (PVDF) membranes by electrobloting. Immunoblotting was preformed by incubation of the membranes with anti-His antibody (1:1500 dilution). The enhanced chemiluminescence (ECL) Western blotting reagents and protocol were used to identify the immunoreaction protein.

CCT assay Tn cells were transfected with blank or recombinant virus and incubated for indicated time, washed with phosphate-buffered saline (PBS), and collected in lysis buffer (NaCl 0.1 mol/L, Tris-HCl 10 mmol/L, pH 7.4). The cell lysate were centrifugated at 10 000 × g, the supernatant was used for CCT assay, the protein concentration was assayed by the method of Bradford.

CCT activity was determined by measuring the rate of incorporation of [^{14}C]-phosphocholine into CDP-choline. The reaction mixure contained CTP 4 mmol/L, $MgCl_2$ 10 mmol/L, *bis-tris*-HCl 150 mmol/L (pH 6.5), phosphocholine 1 mmol/L, lipid activator (Ptdcho: oleic acid, 1 : 1) 64 μmol/L, [^{14}C]-phosphocholine (specific activity, 2.0 TBq/mol) 7.4 kBq and certain amount of proteins in a total assay volumn of 50 μL. The reaction was performed at 37 ℃ for 10 min and terminated by addition of 5 μL of edetic acid 0.5 mol/L. Each sample 3 μL was spotted on preabsorbent silica gel G 60 thin layer plate, which was developed in 2 % ammonium hydroxide/95 % ethanol (1 : 1, v : v). The plate was sprayed with enhancer sprayer and exposed to film at −70 ℃, [^{14}C]CDP-choline was identified by co-migration with standard, scraped from the plate, and quantitated by lipid scintillation counting.

Metabolic Labeling Tn cells were transfected with blank or recombinant virus for 36 h, then incubated for 3 h with 74 MBq/L of [^{3}H]choline chloride in fresh FH medium. After that, the medium was removed and cells were washed and collected in PBS. The cell pellets were extracted using chloroform/methanol/concentrated HCl (1:2:0.02, v:v:v), and then, 1/3 volume of chloroform and 1/3 volume of water were added. After mixing violently, the phases were seperated by centrifugation and concentrated by drying under vacuum. The samples of the soluble phase were seperated on silica gel 60 thin layers developed with 2 % ammonium hydroxide/95 % ethanol (1 : 1, v : v), the organic phase was analyzed on silica gel 60 thin layers developed with chloroform/methanol/acetic acid/water (50 : 25 : 2 : 1, v:v:v:v), PC and CDP-choline were identified by comigration with standards. The layer plate was sprayed with enhancer sprayer and exposed to film at −70 ℃, then the film was analysed densitometrically.

Statistical analysis Data were expressed as $\bar{x} \pm s$, and compared by t-test.

RESULTS

Localization of CCTβ in rat brain and hippocampus The distribution of CCTβ in adult rat brain and hippocampus was detected by *in situ* hybridization with digoxigenin-labeled antisense RNA probes. The cells hybridizing to CCTβ probes were located in CA1, CA2, CA4, and dentate gyrus of hippocampus (Fig 1B), no hybridization could be observed in the control section that hybridized with the sense RNA probes (Fig 1A). CCTβ could not be detected in other part of brain (not shown).

Expression of the CCTβ The recombinant

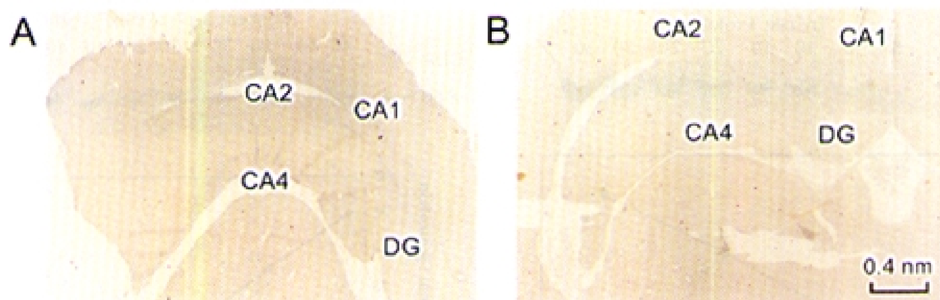

Fig 1. *In situ* hybridization of CCT mRNA. A: rat hippocampus with sense probe; B: rat hippocampus with antisense probe. ×32. DG: dentate gyrus.

Bacmid DNA which contained the CCTβ cDNA was transfected into Tn cells. The supernatant was collected for retransfection, this process was repeated several times to exclude the contamination caused by the DNA added during the transfection. The virus DNA in Tn cells and supernatant were extracted as the template. PCR with CCT primers (5′ primer: 5′-ACG TTT ATA AGC ATA TCA AG-3′, 3′ primer: 5′-GAT AAG GCC TGT AGC ATC CG-3′) showed that CCTβ was successfully reconstructed into the virus genome (Fig 2A).

The protein expressed through pFastBac HTc transfer vector contained a 6 His-tag at the N-terminal for immunological detection. Western blot showed a band of an approximate size of M_r 43 000 using 6-His antibody (Fig 2B). The size was similar to the human CCTβ2 expressed in COS-7 cells which was about M_r 40 000[7].

Both Western blot and CCT assay (not shown) revealed that the expression of CCTβ was induced after 36 h and increased with time of incubation, but did not increase after 72 h, because most of the transfected cells were dead at that time.

Properties of expressed CCTβ The enzymatic activity of expressed CCTβ was determined by measuring the incorporation of [^{14}C]-phosphocholine into CDP-choline. The introduction of the CCTβ into the Tn cells led to a significant increase in the CCT specific activity in cell lysates (Fig 3), according to the slope of the line segment between 0 and 10 mg, the activity of CCTβ was (35.7±1.6) $\mu mol \cdot min^{-1} \cdot g^{-1}$ protein, which was 1×10^4 times of the reported activity of CCT in normal rat brain which only showed several $nmol \cdot min^{-1} \cdot g^{-1}$ protein[8].

Alternative substrates for CCTβ (ATP, GTP, and dCTP) were screened. Although ATP and GTP yielded little activity, the activity in the presence of 2 mmol/L dCTP was even a little higher than that at CTP 2 mmol/L, suggesting dCTP as an adaptive substrate of expressed CCTβ (Tab 1).

The effect of ions on CCTβ activity was investigated. Mg^{2+} (10–40 mmol/L) could largely raise the

Tab 1. Effect of different substrates on expressed CCTβ activity. $n=3$. $\bar{x} \pm s$. $^bP<0.05$ *vs* control.

Substrate/mmol·L^{-1}	Relative activity
Control (CTP 2)	1.00±0.01
ATP 2	–
GTP 2	–
dCTP 2	1.15±0.03^b

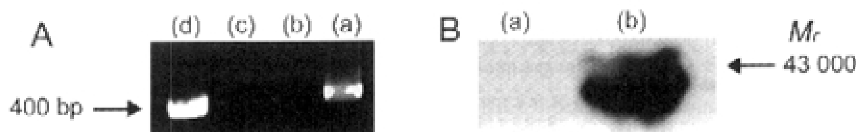

Fig 2. Identification of rat CCTβ recombinant baculovirus and expression. A: PCR results. Template DNA: (a) PBSK vector including rat CCTβ gene; (b) Tn cells after transfected by blank baculovirus for 60 h; (c) supernatant after transfected by recombinant baculovirus for 60 h; (d) Tn cells after transfected by recombinant baculovirus for 60 h. B: Western blot with anti-His antibody. (a) Tn cells after transfected by blank baculovirus for 60 h; (b) Tn cells after transfected by recombinant baculovirus for 60 h.

ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 Apr; 23 (4)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

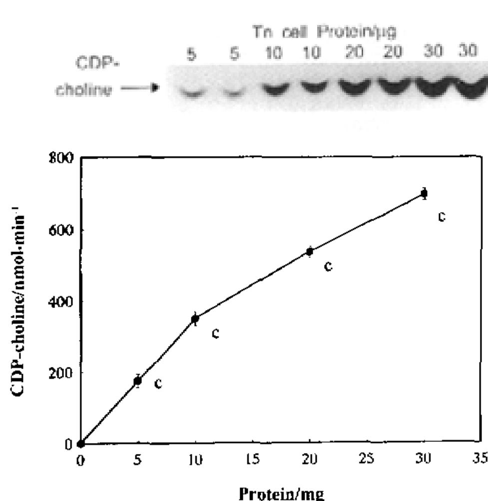

Fig 3. Reaction of [^{14}C]-phosphocholine with lysate of Tn insect cells transfected with recombinant baculovirus for 10 min. Reaction with protein 50 μg of Tn cells transfected with blank virus was treated as negative control and no band was detected. $n = 4$. $\bar{x} \pm s$. $^cP<0.01$ *vs* negative control.

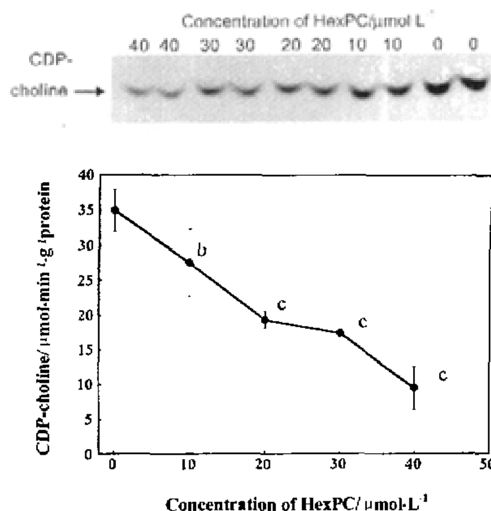

Fig 4. The inhibition of CCTβ activity by hexadecyl-phospho-choline. All incubations were performed with [^{14}C]-phosphocholine 7.4 kBq and protein 10 μg for 10 min at 37 ℃ in 50 μL of reaction volumn with different concentrations of hexadecylphosphocholine and without lipid activator. $n=4$. $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ *vs* without hexadecylphosphocholine.

activity of CCTβ, but high concentration of Mg^{2+} had a negative effect. A strong inhibition of the enzyme activity was caused by Zn^{2+} and PO_4^{3-}. Yet Na^+ only caused slight inhibition (Tab 2). These results were similar to the previous reports[9].

Tab 2. Effect of different ions on expressed CCTβ activity. $n=3$. $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ *vs* control.

Group/mmol·L^{-1}	Relative activity	Inhibition/%
Control (Mg^{2+} 10)	1.00 ± 0.04	-
Mg^{2+} 0	0.44 ± 0.03^c	66
Mg^{2+} 100	0.79 ± 0.08^b	21.5
Na^+ 500	0.82 ± 0.06^b	17.6
Zn^{2+} 3	0.31 ± 0.02^c	69.5
PO_4^{3-} 150	0.10 ± 0.02^c	90

Hexadecylphosphocholine could inhibit PC synthesis *in vivo* at the CDP-choline step[10]. To find out whether hexadecylphosphocholine could inhibit expressed CCTβ activity, different amount of hexadecylphosphocholine was added and CCTβ activity was inhibited to different extent (Fig 4). Furthermore, hexadecylphosphocholine could also inhibit the PC synthesis of Tn cell transfected with recombinant virus for 36 h (data not shown).

From these results above, we could conclude that the expressed CCTβ shared the same characteristics with CCT *in vivo*.

Effect of CCTβ expression on PC metabolism Tn cells were transfected with blank or recombinant virus for 36 h and labeled with [^{3}H]-choline for 3 h to determine whether the overexpression of CCTβ affected the PC biosynthesis pathway. Although the cellular content of [^{3}H]-labeled PC only slightly increased (26 %) in CCTβ-transfected cells. The relative incorporation ratio was 1.00 ± 0.05 and 1.26 ± 0.10 for Tn cells infected with blank virus and infected with recombinant virus respectively (Fig 5B), the [^{3}H]-labeled CDP-choline greatly increased (Fig 5A). These results proved that overexpression of CCTβ led to an increasing accumulation of its product, CDP-choline in Tn cells.

DISCUSSION

It has been reported that CCTβ is much more abundant in brain than other tissues as liver, kidney, and spleen, *etc*, in rat[2], however the localization of CCTβ in brain is unknown. The result of the present work showed that in brain the distribution of CCTβ was limited

Fig 5. A: incorporation of [^{3}H]-choline into CDP-choline in Tn insect cell after 3 h incubation with [^{3}H]-choline. (a): Tn cells infected with blank virus; (b): Tn cells infected with recombinant virus. B: incorporation of [^{3}H]-choline into PC in Tn insect cell after 3 h incubation with [^{3}H]-choline. (a): Tn cells infected with blank virus; (b): Tn cells infected with recombinant virus.

in special areas of hippocampus which were also the binding sites of AVP_{4-8}[11]. It is well known that AVP_{4-8} can facilitate the learning and memory in rat[4] and have the neuroprotective effect on cell proliferation and aging[5], and PC is considered to be related with memory maintenance in mouse brain in accordance with its important structural roles[12]. Moreover, the product of the CCT reaction, CDP-choline, has been proved to have protective effects on brain aging and neurodegenerative disorders like Alzheimer's disease (AD)[13]. Considering CCT mRNA's upregulation by AVP_{4-8}, we deduced that the gene encoding CCTβ was one of AVP_{4-8} target genes, it also had relationship with learning and memory and might be used as a therapy of the neurodegenerative disorders such as AD.

To get much information about CCTβ's enzymatic properties, CCTβ was overexpressed in Tn cells by baculovirus system. The CCT activity in Tn cells transfected with recombinant virus was 1×10^4 times of the activity in rat brain[8], PC synthesis also increased in the CCTβ-overexpressed Tn cells. These results suggested the expressed CCTβ was functionally effective in Tn cells. The characteristics of CCTβ, such as ion effects, substrate specificity, and activity inhibited by hexadecylphosphocholine were investigated too. The fact that dCTP is also an adaptive substrate is quite different with previous reports[3,9]. As the endogenous lipid activator from the cell lysate could not be excluded, many research works, such as the regulation of CCTβ by different lipids remained to be done. Purification of the expressed CCTβ is necessary for further investigation.

In conclusion, our study indicated that the localization of a CCT isoform was similar to that of the memory enhancing peptide AVP_{4-8}. All the results verified that CCTβ expressed in Tn cells had similar properties with CCT *in vivo*, but had a different substrate specificity.

ACKNOWLEDGEMENT Thanks to Prof WU Xiang-Fu for the expression system and valuable discussions.

REFERENCES

1 Pelech SL, Vance DE. Regulation of phosphatidylcholine biosynthesis. Biochim Biophys Acta 1984; 779: 217–51.
2 Xiong Y, Liu XL, Wang Y, Du YC. Cloning of cytidine triphosphate: phosphocholine cytidylyltransferase mRNA upregulated by a neuropeptide arginine-vasopressin (4–8) in rat hippocampus. Neurosci Lett 2000; 283: 129–32.
3 Lykidis A, Murti KG, Jackowski S. Cloning and characterization of a second human CTP: phosphocholine cytidylyltransferase. J Biol Chem 1998; 273: 14022–9.
4 Liu RY, Lin C, Du YC. Facilitation of arginine-vasopressin analogs on learning and memory in rats. Acta Pharmacol Sin 1990; 11: 97–100.
5 He M, Miao HW, Chen XF, Du YC. Effect of arginine-vasopressin short analogs on the growth of C_6 cells. Chin J Cell Biol 1995; 17: 176–80.
6 Bain W, Yang J, Ding YY, Jing NH. *In situ* hibridization with digoxigenin labeled RNA probe: localization of nestin mRNA in mouse embryos. J East China Normal Univ 1996; 12: 28–33.
7 Lykidis A, Baburina I, Jackowski S. Distribution of CTP: phosphocholine cytidylyltransferase (CCT) isoforms. J Biol Chem 1999; 274: 26992–7001.
8 Mages F, Roy C, Fonlupt P, Pacheco H. Kinetic and biochemical properties of CTP: choline-phosphate cytidylyltransferase from the rat brain. Eur J Biochem 1988; 178: 367–72.
9 Yeo HJ, Larvor MP, Ancelin ML, Vial HJ. Plasmodium falciparum CTP: phosphocholine cytidylyltransferase expressed in *Escherichia coli*: purification characterization and lipid regulation. Biochem J 1997; 324: 903–10.
10 Rudiger H, Thomas W, Christoph CG, Werner R. The phospholipid analogue hexadecylphosphocholine inhibits phosphatidylcholine biosynthesis in Madin-Darby canine kidney cells. FEBS Lett 1991; 288: 129–32.
11 Du YC, Guo NN, Cheng ZF. Autoradiographic approach to the developmental study on the binding sites of AVP_{4-8} in rat hippocampus. Acta Physiol Sin 1994; 46: 435–40.

12 Chung SY, Moriyama T, Uezu K, Hirata R, Yohena N, Masuda Y, *et al*. Administration of phosphatidylcholine increases brain acetylcholine concentration and improves memory in mice with dementia. J Nutr 1995; 125: 1484-9.

13 Cacabelos R, Camano J, Gomez MJ, Fernandez-Novoa L, Franco-Maside A, Alvarez XA. Therapeutic effects of CDP-choline in Alzheimer's disease. Cognition, brain mapping, cerebrovascular dynamics, and immune factors. Ann NY Acad Sci 1996; 777: 399-403.

CCTβ 在大鼠脑内的分布及在昆虫细胞中的表达[1]

徐侃彦，熊 缨，谢值勤，边 玮，景乃禾，杜雨苍[2]（中国科学院上海生命科学研究院生物化学与细胞生物学研究所，上海 200031，中国）

关键词 CTP：磷酸胆碱胞苷转移酶；精氨酸加压素；原位杂交；海马；软脂酰磷酸胆碱；杆状病毒

目的：考察大鼠 CTP：磷酸胆碱胞苷转移酶 β (CCTβ)在脑内的分布及在昆虫细胞中的表达以研究其活性和特征．**方法**：用地高辛标记 CCTβ 探针对大鼠脑切片进行原位杂交；利用杆状病毒表达系统在粉纹夜蛾细胞中表达 CCTβ；利用酶测活实验和代谢标记实验，研究表达蛋白的活性和特征，及对细胞磷脂酰胆碱(PC)合成的影响．**结果**：(1) CCTβ 在海马的齿状回、CA1、CA2 和 CA4 区有明显分布．(2) Tn 细胞中表达的 CCTβ 含量是天然脑内的 1×10^4 倍，它的表达加快了细胞的 PC 合成．(3) 软脂酰磷酸胆碱以及一些离子会抑制 CCTβ 活性；dCTP 是 CCTβ 另一最适底物．**结论**：CCTβ 在大鼠脑内有与精氨酸加压素片段(4-8)相似的分布．

（责任编辑 韩向晖）