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Genomic structure of the regulatory region of the voltage-gated calcium channel α1D

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Abbreviations: CAT, chlorampenicol acyltransferase; DHP, dihydropyridine; NT, nucleotide; PCR, polymerase chain reaction; VGCC, voltage-gated calcium channel

Abstract

In excitable and endocrine organs, calcium influxes through the L-type voltage-gated calcium channel (VGCC) which is composed of four (α1, α2, β, and γ) subunits. Temporal and spatial expression of calcium channel activity is regulated by the transcription of α1 subunit. To elucidate the genomic organization of the VGCC α1D subunit gene, a genomic clone was isolated from the human genomic library and its sequence was analyzed. A 12 kb genomic clone contained the 5'-flanking regulatory region and first two exons was selected and the initiation site for α1D mRNA synthesis was examined by primer extension analysis. The major initiation site was found at the -523 NT position in the translation initiation site. The TATA box could not be found above the transcription initiation site. The CAT vector construct containing the 2.5 kb upstream region had high CAT activity on transfection to NG108-15 and PC12 cells, which confers the neuronal expression of the α1D gene.

Keywords: calcium channel, gene expression, regulation, molecular cloning

Introduction

Intracellular calcium levels can be elevated through extracellular and intracellular storage. The influx from the extracellular source is mediated by the voltage-gated calcium channel (VGCC), the glutamate receptor, and CICR (calcium induced calcium release channel). Intracellular calcium concentrations can also be elevated through the IP3 receptor and ryanodine receptor of intracellular storage. Calcium influxes through the glutamate receptor is rapid and is an initial regulatory step in neuronal intracellular calcium signaling (Barritt, 1992). Calcium influx through the VGCC mediates a wide variety of physiological functions, including linking electrical activity to neurotransmitter release, excitation-contraction coupling, and control of neuronal firing patterns (Hess, 1990). Traditionally, the VGCC was seperated into two major calcium channels (low voltage-activated and high voltage-activated) according to voltage-dependent properties for activation of the channels (Catteral, 1991). High voltage-activated calcium channels can be opened by relatively large depolarizing potentials, and are classified into L-, N-, and P-type channels based on electrophysiological and pharmacological properties.

Molecular properties of the L-type calcium channel are well known because it is concentrated at neuromuscular junctions, and has specific drug binding sites. Biochemical studies show that the skeletal dihydropyridine (DHP)-sensitive L-type VGCC is composed of five distinct subunits (α1, α2, β, γ, and σ) (Isom *et al.*, 1994; Hofman *et al.*, 1994). The α1 subunit of the calcium channel complex, which has binding sites for DHP, phenylalkylamine, and benzothiazepine, has a pore for calcium entry that can form the functional calcium channels when expressed in Xenopus oocyte. Six isoforms of α1 subunit are known; α1A, α1B, α1C, α1D, α1E, α1S (Tanabe *et al.*, 1987; Mikami *et al.*, 1989; Starr *et al.*, 1991; Hullin *et al.*, 1992; Snutch *et al.*, 1992; Soong *et al.*, 1993).

The cDNA of α1D was cloned from rat brain and human brain, and human pancreatic cell (Hui *et al.*, 1991; Seino *et al.*, 1992; Williams *et al.*, 1992). Analysis revealed that its expression is limited to neuronal and endocrine tissues (Hui *et al.*, 1991; Chin *et al.*, 1992; Williams *et al.*, 1992). Immunohistochemical study has shown that α1D L-type VGCCs are evenly distributed in neuronal cell bodies and proximal dendrites (Hell *et al.*, 1993). NG108-15 cells, which express the VGCC α1 gene, was differentiated into neuron-like cells by PGE1 via elevation of cAMP. Elevation of cAMP levels resulted in the expression of VGCC with the dihydropyridine binding site (Nierenberg *et al.*, 1983). The transcriptional regulation of 5'-end flanking region of VGCC subunit gene has not been reported except α1D in rat brain (Kamp *et al.*, 1995). To understand the regulation of expression the 5'-flanking region of the L-type VGCC α1 subunit gene was cloned from the human genomic library, and its genomic

structure analyzed.

Materials and Methods

Cloning of voltage-gated calcium channel α1 subunit gene (α1D) from the human genomic library

The VGCC α1 subunit gene was isolated from the human genomic DNA library using 5'-end 0.73 kb sized Bst XI fragment of α1D, ranging from -314 to 420 NT of the translation initiation. About 1.2×10^6 plaques were screened with a random primed cDNA probe. The plaques were transferred and immobilized onto nitrocelluose paper, and hybridized with hybridization solution containing the ^{32}P-dCTP labeled cDNA probe(10^6 cpm/ml) in 5x SSC, 50% formamide, 0.1% SDS. The membrane was washed three times with 0.2x SSC/ 0.1% SDS for 10 min at room temperature, followed by incubation at 65˚C for 10 min. One positive plaque was isolated. The phage DNA was purified and digested with *Eco*RI, and then Southern blot analysis was performed. To find the fragment containing the 5'-end regulatory sequences, two end-labeled oligonucleotides were used as probes. One was from the 5'-end of the rat α1D cDNA, from -239 to -262 NT, and the other from the 5'-end of the translation region, 204 -225 NT. Their sequences were 5'- TAC AGG TAT ATA TTA CTA AGA TAA TAT –3' and 5'- ATG AGC ACG TCT GCA CCC CCA C –3' respectively. The fragment positive for the probe was subcloned and its sequence analyzed.

Sequencing and data analysis

Sequencing was carried out by the directional deletion method. Deletion mutants were prepared by Erase-a-base deletion kits according to the manufacturer's manual (Promega). The 5'-/3'-overhang DNA were made and digested with Exo III nuclease followed by S1 nuclease. The unidirectional deletion DNA was ligated and trans-formation was carried out. Plasmid DNA from the deletion clones were prepared by Wizard miniprep kit(Promega). The sequencing template DNA was prepared by alkali denaturation-neutralization of the double-stranded plasmid. Sequencing was carried out by the dideoxy-termination method using the Sequenase v.2.0(Amersham). The se-quencing data from the deletion mutants were assembled by using the Asseblign program from IBI Co. Exon sequences were analyzed by comparing the genomic DNA sequences with cDNA sequence from the human α1D subunit gene. The exon sequence was translated into the amino acid sequence, and possible transcription factor-binding sites were analyzed using the MacVector program (IBI Co.).

Primer extension

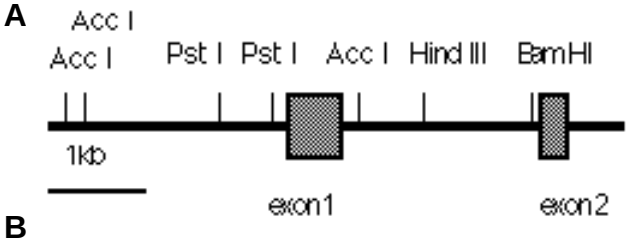
Primer extension analysis was performed as previously described (Sambrook *et al.*, 1989). The total RNA was isolated by the acid phenol GITC method from human brain biopsy tissue. The primer was constructed from the -305 to -326 NT region (5'-CAA AGG AGA ACT GGC TTG TCC C-3') of the human genomic DNA. [^{32}P]-labeled (about 1×10^5 cpm) primer was mixed with 150 µg brain RNA. The mixed DNA and RNA were recovered by ethanol precipitation. The precipitated nucleic acids were redis-solved in 30 µl of hybridization buffer (40 mM PIPES, pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% formamide). The hybridization mixture was denatured at 85˚C for 10 min, and quickly transferred to a water bath at 30˚C. The mixture was incubated overnight. The hybridized nucleic acids were recovered, and cDNA was synthesized in reverse transcriptase buffer (50 mM Tris-HCl, pH 7.6, 60 mM KCl, 10 mM $MgCl_2$, 1 mM of each dNTP, 1 mM dithiothreitol, 1 unit/µl placental RNAase inhibitor) using MMLV reverse transcriptase at 37˚C for 2 h. one µl of 0.5 M EDTA (pH 8.0) and 1 µl of DNase-free pancreatic RNase (5 µg/ml) were added and incubated at 37˚C for 30 min. The synthesized cDNA was recovered by ethanol precipitation after phenol: chloroform (1:1) extraction. The dissolved pellet in 4 µl of TE, pH 7.4 was analyzed by sequencing gel along side α1D 5'-flanking genomic DNA.

Construction of CAT vector and analysis

PHGRI was generated by cloning the 6.0 kb *Eco*RI frag-ment from α1D genomic clone into pGEM 3zf(-). The 2.5-kb *Acc* I fragment, which contains the 5'-regulatory sequences, first exon, and 131-bp of first intron, was cloned into pCATBasic vector. NG108-15, PC12, and NIH3T3 cells were maintained in DMEM (Gibco) with 10% fetal bovine serum. Transfection of the clone into the cells was performed by the calcium phosphate precipitation method (Wigler *et al.*, 1978). In each 100-mm petri dish, 10^6 cell were seeded 1 day prior to transfection. Seventeen micrograms of plasmid DNA to be tested and 3 µg of pSV-β gal DNA were added to each petri dish. The medium was changed 12 h after transfection, and cells were harvested after incubation for an additional 24 h. Lysates of transfected cells were prepared and suspended in a total volume of 200 ml of phosphate buffered saline. Five to 40 µl of the extract were used for both CAT and β-galactosidase assays as previously described (Sambrook *et al.*, 1989).

Results and discussion

The genomic DNA of α1 subunit gene of VGCC was isolated by high stringency screening of the genomic library and probed with the rat VGCC α1D subunit cDNA, which encompasses the 730 NT of the 5'-end of cDNA including the 5'-end untranslation region. A phage clone, HG5, with 12 kb-sized insert was isolated. The insert consisted of



B

Figure 1. Restriction map and nucleotide sequence of the 5'- upstream region of the human VGCC α1D subunit gene. **A.** The clone includes part of the 5' upstream region of the gene and exon 1 to the initial part of intron 2. **B.** The nucleotide sequence of part of the 5' upstream region, exon 1, intron 1, exon 2, and the initial part of intron 2 of the 5688-bp portion of genomic DNA clone, pHGRI. The lower case shows the 5'-flanking regulatory region and introns, and the upper case shows the exons. The deduced amino acid sequences encoded by exon 1 and 2 were shown below the corresponding nucleotides using the single-letter amino acid code. The nucleotide +1 shown as an arrow corresponds to the transcription initiation site by primer extension analysis. The arrow head represents RNA splice sites that were identified by comparison of nucleotide sequences of CACNN1A2 from human genomic DNA and human cDNA of Williams (1992) and Seino (1992). The positions of putative transcription factor binding sites for Sp-1 are underlined. The broken arrows shows the Acc I fragment for the CAT vector construct. The above sequence was deposited in the GenBank database (access number, AF055575).

3 *Eco*RI fragments, 6.0, 4.5 and 1.5 kb. Among them the 6.0 kb fragment was strongly positive for the cDNA probe and oligonucleotide probes, while the 4.5 kb fragment was positive only for cDNA probe on Southern hybridization. The result suggested that the 6.0 kb fragment contains the transcription initiation site and 5'-flanking regulatory region. Sequencing was carried out for the 6.0 kb fragment, pHGRI clone. Analysis of its sequence revealed that it contained the first two exons and 5'-flanking regulatory sequences (Figure 1). The nucleotide sequences of two exons were well matched with that of human α1D VGCC cDNA (Williams *et al*., 1992), and the sequences of the 5'-regulatory region and introns were 95% similar with that of the rat gene (Kamp *et al*., 1995).

To investigate the transcriptional start site, a primer extension analysis was carried out using RNA isolated from human fetal brain. A 22-mer primer (5'-CAA AGG AGA ACT GGC TTG TCC C-3') was designed from -326 to -305 NT from the translation initiation of human α1D cDNA. A strong signal was shown at -523 nucleotide position and minor bands were detected at the -350 NT (Figure 2). This suggests that the multiple

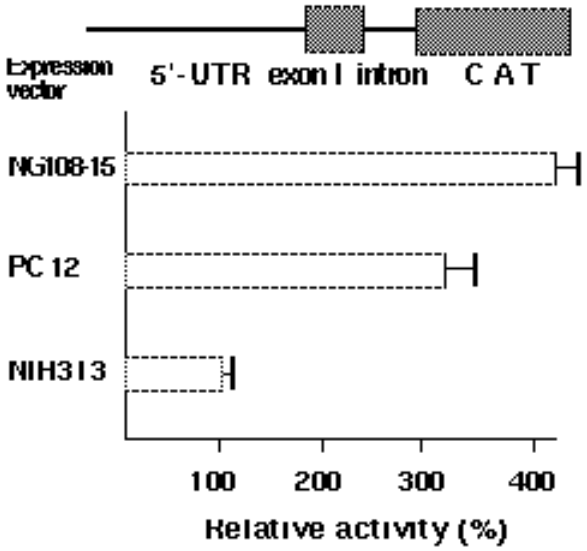


Figure 3. Relative CAT activities of cells transfected by the promotor-CAT fusion plasmid vector. The CAT activities of the cells transfected by the plasmids containing CAT vector (open bar) and promotorless pCATBasic plasmid (solid bar) are shown. Their activities are compared to the CAT activity in NIH3T3 cells transfected with the construct. The transfection efficiency was normalized by cotransfection with pSV-β gal plasmid. The standard deviation is indicated by error bars.

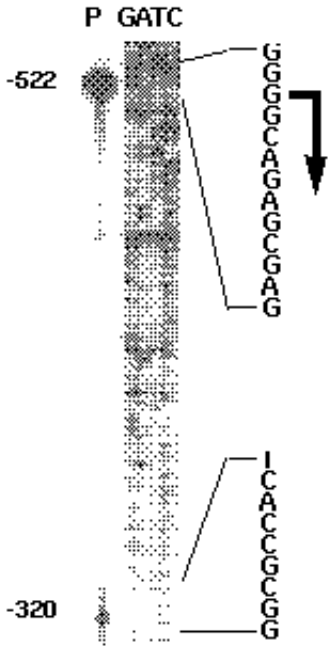


Figure 2. Determination of the transcription initiation site of human voltage dependent calcium channel α1 subunit gene. A ^{32}P-labelled 22-mer oligonucleotide primer(5'-CAA AGG AGA ACT GGC TTG TCC C -3',NT -305 - -326) was annealed with 50 mg of yeast tRNA or 50 mg of total RNA derived from human biopsied brain, and extended using MMLV reverse transcriptase. The extended cDNA products (lane P) were analyzed by sequencing gel along with the human VGCC α1D genomic DNA sequencing ladder (lane G, A, T, C). The sites of initiation are indicated to the right of the panel by arrows.

α1D transcript can be made from the gene. The NT with the upper strong band was designated as the +1 position of transcription initiation. This starting point is 32 NT upper region higher than that of Kamp(1995). This is because he did not determine the transcription starting point by primer extension and species differentiation of the genomic DNA of humans and rats.

To identify putative transcription factor binding sites in the 5'-upstream region of the VGCC gene, a computer search was carried out comparing the upstream sequence with known transcription factor binding sequences. The putative transcription factor binding sites are indicated below the nucleotide sequence of the 5'-flanking region of VDCC in Figure 1. The TATA box could not be found in the upstream regions from the putative transcription initiation site as rat α1D gene (Kamp *et al*., 1995). Several lines of other genes, such as house keeping genes, were also devoid of a TATA box. In the absence of the TATA box, binding sites for transcription factors other than TATA binding proteins are known to initiate transcription (Azizkhan *et al*., 1993; Latchman, 1995). Several putative transcription factor binding sites were detected for C/EBP (Johnson *et al*., 1987) Sp-1 (Jones *et al*., 1988), AP-1 (Angel *et al*., 1987), and AP-2 (Imagawa *et al*., 1987). Sp-1 binding sequences were found thirteen times between -750 and 130 regions, but were clustered at the -85 to -10 region, just above the transcriptional initiation site.

To determine that the clone contained neuronal specific

elements, expressional regulation of the 5'-regulatory region through CAT activities were analyzed. The CAT expression vector was constructed into CATbasic vector (Promega) for the 2.5 kb sized *Acc* I fragment, ranging from -1840 to 710, which contained part of the first exon and second intron. The DNA construct was cotransfected into various cell types, NIH3T3, PC12, NG108-15 cells with plasmid DNA containing a β-galactosidase gene. The β-galactosidase activities were then used to normalize CAT activities. The construct transfected into NG108-15 and PC12 cells resulted in relative CAT activity 4.2-fold and 3.2-fold, respectively, greater than that in NIH3T3 cells (Figure 3). The CAT activity in the NG108-15 cells was similar to that of Kamp (1995). The difference in CAT activities between the NG108-15 and PC12 cell reflects the neuronal character of each cell line. NG108-15 cell binds DHP (Nierenberg *et al*., 1983), which means that the cell line expressed the α1D gene, and has transacting neuronal elements. A moderate high level of CAT activity might reflect the undifferentiated state of neuronal characters. These results suggest that the 5'-upstream sequence of the α1D gene contains multiple transcriptional regulatory elements and neuronal specific elements. For the elucidation of essential transcription factors in neurons and upper regulatory elements of the gene, further characterization including deletion analysis and DNA foot printing would be needed.

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