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Structure of human voltage-dependent calcium channel (VDCC) β3 subunit gene

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Abbreviation: VDCC, voltage-dependent calcium channel

Abstract

In excitable and endocrine organs, calcium influxes through the voltage-dependent calcium channel (VDCC), composed of four (α1, α2, β, and δ) subunits. Four isoforms of beta subunits (β1, β2, β3, β4) are known to exist. The cytoplasmic β subunits regulate the channel activity by accelerating the kinetics of activation and inactivation through phosphorylation. Regulation of calcium channel activities are also provided by alternative splicing of the β subunits. To elucidate the genomic organization of the VDCC β3 subunit gene, two genomic clones were isolated from human genomic liabrary using the whole rat cDNA for β3 subunit as a probe. The β3 subunit gene in lamda phage DNA was analyzed by Southern hybridization and sequencing. A 19.1 kb clone (2BHG13) contained the whole β3 cDNA sequence, consisting at least 14 exons. The deduced amino acid sequence from the exons shows 97% similarity with that of rat gene. Two alternatively spliced forms of β3 subunit at 5'-end were found. The β3 subunit had many possible phosphorylation sites. Alternative splicing of β3 subunit mRNA at 5'-end and phosphorylation of the β3 subunit protein may play a regulatory role in calcium influxes.

Keywords: voltage-dependent clacium channel, β3 subunit, alternatively splicng, human genome, exon

Introduction

Since calcium ion was known as a necessary element in contraction of myocardial cells, several lines of experiment disclosed that calcium ion has an important physiological role and mediates many intracellular events; linking electrical activity to neurotransmitter release, excitation-contraction coupling in muscle, control of neuronal firing, and hormone release in endocrine cells (Hess, 1990). The concentration of calcium ion is finely tuned through a variety of calcium channels. Cytoplasmic calcium is raised by voltage-dependent calcium channel (VDCC) and N-methyl-D-aspartate (NMDA) receptor through the plasma membrane, and 1,4,5-Inositol triphosphate (IP3) receptor or ryanodine receptor through the endoplasmic reticulum membrane. After its physiological action has been accomplished, the calcium level returns to the basal by Na^+/Ca^{2+} exchanger and Ca^{2+} ATPase.

The calcium channels are classified into L-, N-, P- and T-type based on their electrophysiological and pharmacological properties. L-type calcium channel mediates the influx of calcium ion by strong depolarization under moderately depolarized condition and has binding sites for a member of calcium antagonists. N-type calcium channel exists in nervous system (Dubel *et al*., 1992) and P-type channel is abundant in Purkinje cells of cerebellum (Mori *et al*., 1991; Mintz *et al*., 1992). T-type channel can be opened transiently by small depolarization. Molecular properties of L-type calcium channel was well known because it is abundant at neuromuscular junction, and has specific drug binding sites (Tanabe *et al*., 1987). Biochemical studies show that skeletal dihydropyridine-sensitive L-type VDCC is composed of five distinct subunits (α1, α2, β, γ, and δ) (Catterall, 1991 ; Singer *et al*., 1991 ; Kim *et al*., 1992). The α1 subunit is a transmembrane glycoprotein of 170 kD which can be phosphorylated by protein kinase A. It has calcium pore and contains the binding sites for dihydropyridine, phenylalkylamine, and benzothiazepine. The α2 subunit is about 180 kD in non-reducing condition, but can be divided to α2 subunit of 150 kD and δ subunit of 25 kD under reducing condition. The α2 and δ subunits come from a single transcript, that is processed into two pieces by posttrans-lational protease. The β subunit, protein in cytosol, was shown to comprise of four (β1, β2, β3 and β4) (Ruth *et al*., 1989; Pragnell *et al*., 1991; Hullin *et al*., 1992; Perez-Reyes *et al*., 1992; Castellano *et al*., 1993).

Although the α1 subunit is neccessary for the transport of calcium ions, it is not sufficient for the full activity. When the α1 subunit only was expressed in Xenopus oocyte,

the channel activity was only 1/10 of native one, and did not reveal the typical channel kinetics. Coinjection of skeletal muscle $\alpha 2$-δ and β subunit mRNAs with the $\alpha 1$ subunit mRNA, however, drastically changed electrophysiological characteristics of the expressed calcium channels. Beta subunit is a cytoplasmic protein and accelerates the kinetics of activation (channel opening) and inactivation (channel closure) (Lacerda *et al*., 1991; Varadi *et al*.,1991). It regulates the channel activity by phosphorylation through PKA and protein kinase C that are activated by various signal transduction mechanisms. These findings suggest that β subunits of the calcium channel may play a modulatory role in regulating calcium channel function (Ruth *et al*., 1989 ; Pragnell *et al*., 1991; Hullin *et al*., 1992).

According to recent data (Kim *et al*., unpublished observation), VDCC $\beta 3$ subunit gene is expressed only in the nervous system, and alternative splicing at 5'-end was also observed. To elucidate the structure of the VDCC $\beta 3$ subunit gene the genomic DNA was isolated from human genomic liabrary and chracterized.

Materials and Methods

Cloning of $\beta 3$ subunit gene of VDCC from human genomic library

VDCC $\beta 3$ subunit gene was screened from human genomic DNA library (Stratagene 944201, W138 cell line from human lung fibroblast) using NG51 cDNA which has full coding region of VDCC $\beta 3$ subunit. About 7.0×10^5 plaques were screened with the cDNA probe which was labelled with [α-^{32}P]dCTP using random-priming. The plaques were transfered onto nitrocellulose paper, immobilized, and hybridized with hybridization solution containing [α-^{32}P]dCTP labeled cDNA probe. The membrane was incubated in $6\times$ SSC, 0.1% SDS, $5\times$ Denhardt's reagent, 50% formamide, and 1×10^6 cpm/ml labeled probe at 42˚C overnight. The membrane was washed with $0.2\times$ SSC/ 0.1% SDS for 10 min three times at room temperature, followed by at 65˚C for 10 min three times. After the autoradiography, the second screening was performed as above, and finally seven plaques were isolated.

The phage DNAs were purified and digested with *Sac*I, and Southern blot analysis was carried out. The hybridization condition was the same as described above. The probes used were a random-primed NG51 cDNA for confirming the clones and an end-labelled oligonucleotide corresponding to the 5'-end of NG51 for the selection of clones containing this sequence. The sequence of the oligonucleotide probe was 5'-TCC TCA AAC CCG GGC ACG TAG GAG TCG TCA TAC ATG-3'. The fragments positive for the probe were subcloned and their sequences were analyzed.

Sequencing and data analysis

The *Sac*I fragments of the genomic DNA were subcloned into pGEM 7zf(+) vector. The deletion mutants were prepared by Erase-a-base deletion kits according to the manufacturer's manual (Promega). To make deletion mutants the restriction maps were made. The 5'-/3'-overhang DNA were made and digested with *Exo*III nuclease followed by S1 nuclease. The unidirectionally deleted DNA was ligated and transformation was carried out. Resulting plasmid DNAs from the deletion clones were prepared by Wizard miniprep kit (Promega). The sequencing template DNA was prepared by alkali denaturation of the double-stranded plasmid. The sequencing was carried out by the dideoxy-termination method using the Sequenase v. 2.0 (Amersham).

Cyclic sequencing was carried out using the SequiTherm cyclic sequencing kits (Epicentre Technologies). The oligonucleotide primer was end-labelled with [γ-^{32}P] dATP using polynucleotide kinase. The sequencing reaction mixture is consisted of 50 ng phage DNA, 1.5 pmol of ^{32}P-labelled primer, 5 units of thermostable DNA polymerase, and dideoxynucleotides. The reaction mixtures were overlayed with mineral oil, and cycled 33 times as follows; 30 sec at 95˚C, 30 sec at 50˚C, and 1 min at 70˚C. The reaction product was seperated on a 6% denaturing acrylamide gel and visualized by autoradiography.

The sequencing data from the deletion mutants and subclones were assembled by using the Assemblign program from IBI (New Haven, CT). Exon sequences were analyzed by comparing the genomic DNA sequences with cDNA sequence from NG51 cells. The exon sequence was translated into amino acid sequence, and possible phosphorylation and other motifs were searched using MacVector program (IBI).

Results and Discussion

Initially seven plaques were obtained by high stringency screening of the genomic library using NG51 cDNA. Out of seven clones only two clones, 2BHG13 and 2BHG14, were positive for Southern hybridization with NG51 cDNA probes. Their sizes were 19.1 and 18.0 kb, respectively (Figure 1). 2BHG 13 had nine *Sac*I fragments, and 2BHG14 has five *Sac*I fragments.Two *Sac*I fragment from two clones were the same size, which suggested that the two genomic clones are overlapping. Another round of Southern blot analysis was carried out using the oligonucleotide probe corresponding to 5'-end to identify whether 5'-end of the $\beta 3$ subunit gene is contained. 2BHG13 clones was positive for 5'-end of NG51, where first exon exists. 2BHG14 does not have the first exon, which could not be found in 2.85 kb upstream from the second exon. That is relevant to the negative result of

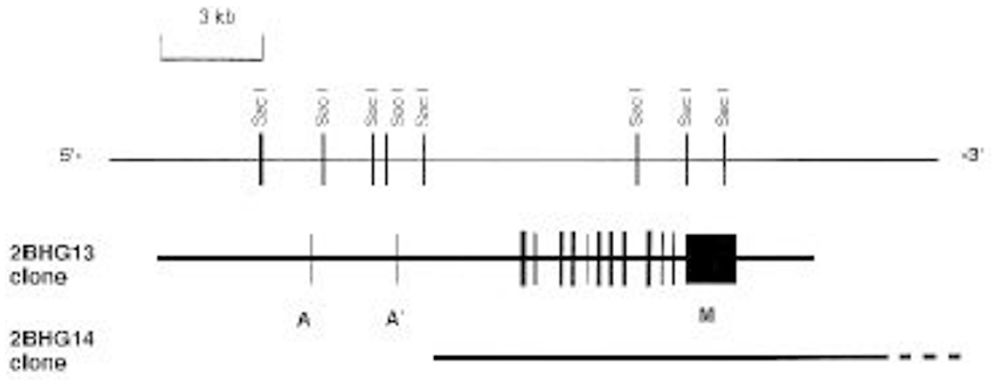

Figure 1. The genomic structure of human β3 subunit gene of VDCC. Two genomic clones were shown as thick lines and restriction sites were shown as thin lines. The BHG13 contains 14 exons from A and A', and M vertical bars indicates the exons. Each exons of A and A' contain translation initiation codon and the exon M contains termination codon.

Figure 2. Nucleotide sequence of VDCC spliced from exons. Nucleotide sequence of VDCC β3 subunit gene was obtained by splicing of the 14 exons from human genomic DNA. The nucleotide sequence shown as italic is the alternatively spliced variant of subunit gene. Splicing sites were marked with (◆). The translation initiation codon at the first two exons and the termination codon at the 14th exon were underlined.

Table 1. Exon/intron junction of 2BHG13 phage genomic clone.

Exon	Exon size	Sequence at exon/intron junction			Intron size	Amino acid
Name	(bp)	5' splice donor		3' splice acceptor	(kb)	interrupted
A	>40	AAC GAG	gtgagcttcc		1.5	
A'	>60	GAG GCG	gtgagtgccc------cccaacccag	GGT TCA GCA	4.0	Ala- 15
B	123	AGG GCC AAG	gtatactttc------aattctccag	CAC AAA CCT	0.2	Lys- 56
C	123	ATT AAA GAG	gtgatcgacc------ctgcccccag	AAG TAC AGC	0.5	Glu- 97
D	116	AAG GCC AG	gtgagagttg------ttcttctcag	G AGA TCT	0.3	Arg-136
E	65	TCT CTA G	gtagcctccc------ttccaaaaag	CC AAG CAG	0.2	Ala-158
F	20	CAA AAG CAG	gtgagtcaag------cattctgcag	GCG GAA CAT	0.2	Gln-164
G	81	GGT TAT CAG	gtgagaggat------cttcccccag	GTC ACA GAC	0.2	Glu-191
H	59	GAT GGC AG	gtaagctgcc------cccaccccag	G ATC TCC	0.2	Arg-211
I	110	AGC ATT G	gtgagaagtc------ccctccatag	CG GAA GTG	0.6	Ala-248
J	152	TCA CCA AAG	gtaagtcagc------ccccgctcag	GTA CTC CAG	0.2	Lys-298
K	96	TGC CCA CCG	gtgagtgcct------ccctccccag	GAG TCA TTT	0.1	Pro-330
L	150	GGA CTT CAG	gtgaaccatt------tggcccccag	AAC CAG CAG	0.5	Gln-380
M	>835	exon/intron junction not determined				

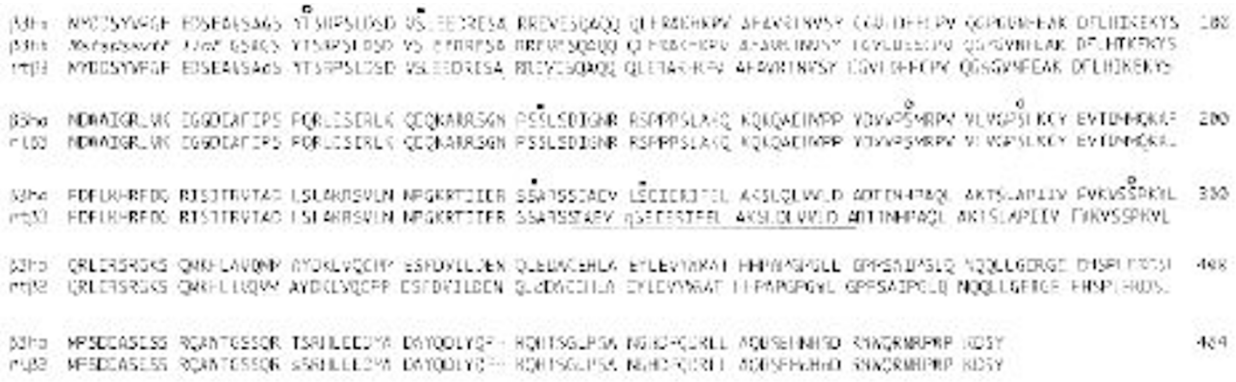

Figure 3. Alignment of deduced amino acid sequences of human VDCC β3 subunit gene with that of rat. The human β3 sequences (β3ha and β3hb) are obtained by splicing of the exons and translation of the exon sequences. The nucleotide sequence shown in italic is the alternatively spliced variant of subunit gene. Identical sequences were shown as capital letter and nonidentical sequences were marked as lower cases. The conserved potential sites for phosphorylation are indicated with the following symbols: protein kinase C (○), casein kinase II (■). Underline shows the predicted helices deduced from protein analysis program.

Southern analysis of 2BHG14 using the oligonucleotide probe. The analyzed result disclosed that the genomic clone, 2BHG13 encompasses the whole coding region of subunit gene, containing 14 exons from A and A' to M as shown on Figure1.

The first exon A', which has the nucleotide sequence of known β3 subunit gene, was found in 0.7 kb-size *Sac*I fragment. The 2BHG13 clone seemed to have another exon A located about 1.5 kb upstream of exon A'. The nucleotide sequence of exon A was similar to that of alternatively spliced variant of rat β3 subunit gene at 5'-end (Kim *et al*.,unpublished observation). The second exon B was far from the first exon as shown in Table 1 and Figure 1. Most of the exons were clustered in 6.5- and 1.3-kb *Sac*I fragments. 6.5 kb *Sac*I fragment has eight exons, and 1.3-kb *Sac*I fragment has four exons. The exon M containing termination codon is located on 1.3-kb *Sac*I fragment. The exon-intron junction of β3 subunit gene of VDCC is shown in Table 1. The size of intron in the BHG genome ranges from 118 bp (between exon K and L) to 597 bp (between I and J). The size of the 14 exons varies from 21 bp (exon F) to more than 835 bp (exon M). All introns begin with 'GT' and end with 'AG'. The codon phases were variable, from the first to third base of codon.

The presumed nucleotide sequences spliced from the exons were shown at Figure 2 and the number of the nucleotide is 1525. The open reversed triangle indicates the spliced junctions of the exons. Deduced amino acid sequence of β3 subunit gene from the exons starts with methionine and was terminated at exon M. The amino acid sequence has high degree of similarity with that of rat gene (Perez-Reyes *et al*., 1992) and identical to that of the human β3 cDNA (Collin *et al*., 1994). Alternatively spliced forms of β1, and β2 subunits gene, which contribute to the functional diversity of the VDCC, were reported (Powers *et al*., 1992). Alternative splicing of α1 subunit and β1 subunit confers the diversity of tissue specific regulation of calcium channel in various tissues. The genomic clones that we have cloned do not have the exons which could be alternatively spliced in the middle of the gene as β1 subunit (Powers *et al*., 1992; Williams *et al*., 1992) but at 5'-end (Kim *et al*., unpublished observation). Although the marked differential expression of the spliced variant could not be obseved, the alternatively spiced form might have different kinetic properties and the diversity of β3 subunit gene could confer the regulatory role of calcium channel activity.

The nucleotide sequences from the exons were translated into amino acid sequences (Figure 3). Deduced amino acid sequences of human β3 subunit of VDCC was compared with that of rat, which showed the 98% similarity, but it is less similar to β1 or β2 subunit of VDCC as 75% and 78% respectively (data not shown). The computer analysis of the amino acid sequence of the β3 subunit reveals that β3 subunit does not have typical membrane-spanning region but contains three major α helical domains. The β3 subunit of VDCC has many possible phosphorylation sites. Potential protein kinase C (Ser/Thr-X-Lys/Arg) domains were found at 4 locations and 4 possible casein kinase II domains (Ser/Thr-X-X-Asp/Glu) were observed. No consensus sequence for cAMP-dependent protein kinase was found in β3 subunit gene.

Calcium currents through the cardiac and skeletal muscular VDCC are known to be modulated by phosphorylation mediated through several protein kinases, protein kinase C, cAMP-dependent protein kinase, *etc* (Ruth *et al*., 1989; Hullin *et al*., 1992; Castellano *et al*., 1993). Although it has been demonstrated that preferred substrate for protein kinases are the α1 subunits, β subunits have several potential phosphorylation sites (Catterall, 1991;

Hullin *et al*., 1992; Castellano *et al*., 1993). Among them possible phosphorylation sites for protein kinase C were the most abundant, but possible phosphorylation sites for cAMP-dependent kinase and cGMP-dependent kinase did not appear. These suggests that phosphorylation through the activation of the protein kinase C system might be an important regulatory machinary in calcium signalling.

The β3 subunit protein is supposed to have theoretical 3 major α-helical domains. The β1 subunit protein was known to have 4 α-helical domains, and the first two α-helical domains was proposed to be a binding domain to the α1 subunit of skeletal muscle type VDCC (Waard *et al*., 1994). Missing the second α-helical domain in the β3 subunit might contribute to the different binding patterns with various α1 subunits. It is not characterized that which type of α1 subunits take part in the functional calcium channel with β3 subunits. Our recent result on colocalization of β3 and α1B by *in situ* hybridization suggests that the β3 subunits is the partner subunit of α1 subunit of P-type calcium channel.

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