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Genomic Organization of Human β3 Subunit Gene of Voltage-Gated Calcium Channel

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= **Abstract** =

Voltage-gated calcium channel (VGCC) is composed of at least four (α1, α2, β, and δ) subunits. Among them β subunit accelerates the kinetics of activation (channel opening) and inactivation (channel closure), and regulates the channel activity by phosphorylation through PKA and PKC that are activated by various signal transduction mechanisms. Until recently four isoforms of beta subunits (β1, β2, β3, β4) have been identified. Our recent data shows that VDCC β3 gene is expressed only in the nervous system and around the perinatal stage at high level. Alternative splicing was also observed at both 5'- and 3'- ends. To elucidate alternative splicing and cis-acting element of gene regulation of the β3 subunit gene we isolated a 12.5kb-sized genomic clone encompassing β3 subunit gene from human genomic library using the whole β3 subunit cDNA from NG108-15 cell line as a probe. The genome was analyzed by Southern hybridization and sequencing. The β3 subunit gene consists at least of 12 exons, and deduced amino acid sequence from the exons showed 98% similarity with that of rat gene. The β3 subunit gene is not alternatively spliced at the middle of the gene, and has many possible phosphorylation sites, which may confer the regulatory role of the β3 subunit gene.

KEY WORDS : Calcium channel · Human genome.

Introduction

Calcium ion in eukaryotes acts as a second messenger molecule like cAMP, inositol triphosphate, and diacylglycerol. Total intracellular concentration is regulated around 2μM in most nonexcitable cells but free calcium concentration is maintained aorund 0.05% of total calcium concentration, 0.1μM, and minute change of free calcium ion mediates various phenomena. Entry of calcium ions into cells through voltage-gated calcium channels(VGCC) mediates a wide variety of physiological fuctions, including linking electrical activity to neurotransmitter release, excitation-contraction coupling, and control of neuronal firing patterns[1].

Traditionally VDCC was grouped into two major categories according to their voltage-dependent properties for activation of channels[1]. Opening of low voltage-activated(LVA) calcium channels is triggered by small depolarizations under membrane holding potentials. Their function in vivo is supposed to be pacemakers, repetative bursting. High-voltage activated (HVA) calcium channel can be open upon depolarization to relatively high potentials, and display divese drug, toxin-binding and kinetic properties.

These channels are classified into L-, N-, and P-type channels based on their electrophysiological and pharmacological properties[2)3)4)5)6)7)8)].

Molecular properties of L-type calcium channel were well known because this type of channel is rich at neuromuscular junctions, and has specific drug binding sites[9)]. Biochemical studies show that skeletal dihydropyridine(DHP)-sensitive L-type VGCC is composed of five distinct subunits(α1, α2, β, γ, and δ)[2)3)10)]. The α1 subunit of the calcium channel complex, which contain the binding sites for DHP, phenylalkylamine, and benzothazepine, can direct expression of functional calcium channels in Xenopus oocyte[11)12)13)]. Coinjection of skeletal muscle α2-δ and β subunit mRNAs with the α1 subunit mRNA, however, drastically changed electrophysiological characteristics of the expressed calcium channels[2)14)]. These findings suggest that α2-δ and/or β subunits of the calcium channel may play a modulatry role in regulating calcium channel function.

Beta subunit is a cytoplasmic protein and accelerates the kinetics of activation(channel opening) and inactivation(channel closure)[14)15)], and regulates the channel activity by phosphorylation through PKA and PKC that are activated by various signal transduction mechanisms[16)17)18)]. Multiple possible phosphorylation sites were shown by cloning of b subuits from various sources. Four isoforms of beta subunits(β1, β2, β3, β4) are known[16)17)18)19)20)21)]. Our recent data shows that VGCC β3 subunit gene is expressed only in the nervous system, and alternative splicing was also observed at both 5'- and 3'- ends. To elucidate the spacial and temporal specificity of the β3 subunit gene we isolated β3 subunit genomic DNA from human genomic library and chracterized.

Materials and Methods

1. Cloning of voltage-gated calcium channel β3 subunit gene from human genomic library

Voltage-gatedt calcium channel β3 subunit gene was isolated from human genomic DNA library (Stratagene 944201, W138 cell line from human lung fibroblast) using NG51 cDNA which has full coding region of VDCC 3 subunit. About 7.0×10^5 plaques were screened with the cDNA probe which was labelled with ^{32}P-dCTP using random priming. The plaques were transfered onto nitrocellulose paper, immobilized, and hybridized with hybridization solution containing ^{32}P-dCTP labeled cDNA probe. The membrane was washed with 0.2×SSC/0.1% SDS for 10 min three times at RT, followed by at 65℃ for 10 min three times. The signal was visualized by exposure onto X-OMAT film overnight. The positive plaques was picked, and second screening performed as above. The resulting four plaques were cultured and their phage DNAs were isolated. Among them only one plaque, BHGx was positive for oligonucleotide from 5'-end of NG51 cDNA. The BHG was digested with Sac I, and transferrred onto Nylon membrane and Southern blot analysis was carried out with NG51 cDNA as a probe. The fragments poitive for the prove was subcloned into pGEM 7zf(+). and their sequences were analyzed.

2. Sequencing

The Sac I fragments of the genomic DNA were subcloned into pGEM 7zf(+) vector. Basically the deletion mutants were preparaed by manufacturer's manual(Promega). To get deletion mutants the restriction enzymes maps were made. The 5'-/3'-overhang DNA were made and digested with Exo III nuclease followed by Sl nuclease. The unidirectional deletion DNA was liagated and transformation was carried out. Resuting plasmids DNA from the deletion clones were prepared by Wizard miniprep kit(Promega). The sequening template DNA was prepared by alkali denaturation-neutralization of the double-stranded plasmid. The sequencing was carried out by using the dideoxy-termination method of Sanger, the Sequenase v.2.0 (Amersham).

3. Data analysis

The sequencing 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 NG51 cell lines. The exon sequence was translated into amino acid sequence, and the possible phos-

phorylation and other motifs were analyzed using MacVector program(IBI)

Results

Calcium channel genomic DNA was isolated from genomic library of W138 cell line from human lung fibroblast(Stratagene 944201) which was cloned into λFix bacteriophage vector. The genomic DNA of β3 subunit gene of VGCC was isolated by high stringency screening of the genomic library with NG51 cDNA , which encompass whole coding region of β3 subunit of VGCC. A phage clone, BHG, with 12.5kb-sized insert was isolated. The insert consists of 4 Sac I fragments, 5.8, 4.5, 1.3, and 0.8kb. Among them 5.8kb fragment was the most strong positive and 1.1kb fragment was positive for Southern hybridization using NG51 cDNA as a probe. All of above fragments were sequenced using the Exo III deletion kit from Progega. The sequencing data from deletion mutants were analyzed by using MacVector and Assemblign sequencing program.

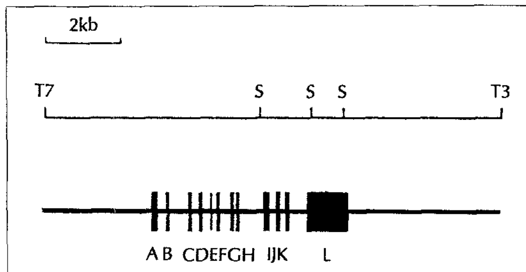

Fig. 1. Organization of the voltage-gated calcium channel β3 subunit gene.
As indicated, the VGCC β3 subunit gene consists of at leasr 12 exons from A to L in 12.5 kilobases. S denotesSac I enzymes and T7/T3 indicates the prmotor site in the λFix cloning vector. Closed rectangualr in lower figure represents exons. The exon-intron junction sequences are dipicted in Table 1.

The analyzed result disclosed that the genomic clone, BHG, has 12 exons from A to L as shown on Fig. 1. The order of Sac I fragment from 5'- to 3'-direction is 5.8kb, 1.3kb, 0.8kb, and 4.5kb. 5.8kb Sac I fragment has 8 exons, and 1.3kb Sac I fragment has 4 exons. The genomic clones dose not have full cDNA sequences. The first exon observed started at 2864th bp which encodes the 16th amino acid of β3 subunit gene. The 12th exon has termination codon.

The exon-intron junction of β3 subunit gene of VGCC was shown on Table 1. All introns began with 'gt' and ended with 'ag'. The codon phases were variable, from the first to third base of codon. The nucleotide sequence spliced from the exons is shown at Fig. 2 and the numer of the nucleotide is 1410. The double aterisks indicates the spliced junctions of the exons.

The nucleotide sequences from the exons were translated into amino acid sequences(Fig. 3). Deduced amino acid sequences of human β3 subunit of VGCC was compared with that of rat, which showed the 98% similarity, but it is less similar to β1 or β2 subunit of VGCC 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

Table 1. Exon-intron junction sequences

A	cccaacccag	GGTTCAGCCG	AGGGCCAAGG	gtatactttc
B	aattctccag	CACAAACCTG	CATTAAAGAG	gtgatcgacc
C	cctggcttgg	GCATCAGTCT	CATGTCTGTC	gaagtgagca
D	ctgcccccag	AAGTACAGCA	AGAAGGCCAG	gtgagagttg
E	ttcttctcag	GAGATCTGGG	CCATCTCTAG	gtagcctccc
F	ttccaaaaag	CCAAGCAGAA	GCAAAAGCAG	gtgagtcaag
G	cattctgcag	GCGGAACATG	AGGTTATGAG	gtgagaggag
H	cttcccccag	GTCACAGACA	TTGATGGCAG	gtaagctgcc
I	cccacccccag	GATCTCCATC	TCCAGCATTG	gtgagaagtc
J	ccctccgtag	CGGAAGTGCA	CTCACCAAAG	gtaagtcagc
K	ccccgctcag	GTACTCCAGC	GTGCCCACCG	gtgagtgcct
L	ccctccccag	GAGTCATTTG	CGGACTTCAG	gtaaccattt
M	tggcccccag	AACCAGCAGC		

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                                                                                                90
GGTTCAGCCGGCTCCTACACCAGCCGCCCA   TCTCTGGACTCAGACGTCTCCCTGGAGGAG   GACCGGGAGAGTGCCCGGCGTGAAGTAGAG
                                                                                               180
AGCCAGGCTCAGCAGCAGCTCGAAAGGGCC   AAGCACAAACCTGTGGCATTTGCGGTGAGG   ACCAATGTCAGCTACTGTGGCGTACTGGAT
                                                                                               270
GAGGAGTGCCCAGTCCAGGGNCCTGGAGTC   AACTTTGAGGCCAAAGATTTTCTGCACATT   AAAGAGAAGTACAGCAATGACTGGTGGATC
                                                                                               360
GGGCGGCTAGTGAAAGAGGGCGGGGACATC   GCCTTCATCCCCAGCCCCCAGCGCCTGGAG   AGCATCCGGCTCAAACAGGAGCAGAAGGCC
                                                                                               450
AGGAGATCTGGGAACCCTTCCAGCCTGAGT   GACATTGGCAACCGACGCTCCCCTCCGCCA   TCTCTAGCCAAGCAGAAGCAAAAGCAGGCG
                                                                                               540
GAACATGTTCCCCCATATGACGTGGTGCCC   TCCATGCGGCCTGTGGTGCTGGTGGGACCC   TCTCTGAAAGGTTATGAGGTCACAGACATG
                                                                                               630
ATGCAGAAGGCTTTCTTCGACTTCCTCAAA   CACAGATTTGATGGCAGGATCTCCATCACC   CGAGTCACAGCCAGCCTCTCCCTGGCAAAG
                                                                                               720
CGATCTGTGCTCAACAATCCGGGCAAGAGG   ACCATCATTGAGCGCTCCTCTGCCCGTCC    AGCATTGCGGAAGTGCTGAGTGAGATCGAG
                                                                                               810
CGCATATTTGAGCTGGCCAAATCCCTGCAG   CTAGTAGTTGGACGCTGACACCATCAAC     CACCCAGCAGCTCGCCAAGACCTCGCTG
                                                                                               900
GCCCCCATCATCGTCTTTGTCAAAGTGTCC   TCACCAAAGGTACTCCAGCGTCTCATTCGC   TCCCGGGGGAAGTCAGAGAAGCACCTG
                                                                                               990
GCCGTACAGATGATGGCATATGATAAGCTG   GTTCAGTGCCCACCGGAGTCATTTGATGTA   ATTCTGGATGAGAACCAGCTGGAGGATGCC
                                                                                              1080
TGTGAGCACCTGGTGAGTACCTGGAGGTT    TACTGGCGGGCCACGCACCACCCAGCCCCT   GGCCCCGGAhCTTCTGGGTCCTCCCAGTGCCC
                                                                                              1170
ATCCCCGGACTTCAGAACCAGCAGCTGCTG   GGGGAGCGTGGCGAGGAGCACTCCCCCCTT   GAGCGGGACAGCTTGATGCCTCTGATGAG
                                                                                              1260
GCCAGCNNGAGCTCCCGCCAAGCCTGGACA   GGATCTTCACAG,CGTACGTCCCGCCACCTG   GAGGAGhGACTATGCAGATGCCTACCAGGAC
                                                                                              1350
CTGTACCAGCCTCACCGCCAACACACCTCG   GGGCTGCCTAGTGCTAACGGGCATGACCCC   CAAGACCGGCTTCTAGCCCAGGACTCAGAA
                                                            1410
CACAACCACAGTGACCGGAACTGGCAGCGC   AACCGGCCTTGGCCCAAGGATAGCTACTGA
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Fig. 2. Nucleotide sequence of VGCC β3 subunit gene spliced from exons.

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       1                                                   51                                                 100
b3h                GSAGS       YTSRPSLDSD  VSLEEDRESA  RREVESQAQQ  QLERAKHKPV  AFAVRTNVSY  CGVLDEECPV  QGPGVFEAK   DFLHIKEKYS
beta3  MYDDSYVPGF  EDSEAGSAdS  YTSRPSLDSD  VSLEEDRESA  RREVESQAQQ  QLERAKHKPV  AFAVRTNVSY  CGVLDEECPV  QGPGVFEAK   DFLHIKEKYS

       101                                                 151                                                200
b3h    NDWWIGRLVK  EGGDIAFIPS  PQRLESIRLK  QEQKARRSGN  PSSLSDIGNR  RSPPPSLAKQ  KQKQAEHVPP  YDVVPSMRPV  VLVGPSLKGY  EVTDMMQKAF
beta3  NDWWIGRLVK  EGGDIAFIPS  PQRLESIRLK  QEQKARRSGN  PSSLSDIGNR  RSPPPSLAKQ  KQKQAEHVPP  YDVVPSMRPV  VLVGPSLKGY  EVTDMMQKAF

       202                                                 251                                                300
b3h    FDFLKHRFDG  RISITRVTAD  LSLAKRSVLN  NPGKRTIIER  SSARSSIAEV  LSEIERIFEL  AKSLQLVVLD  ADTTNHPAQL  AKTSLAPIIV  FVKVSSPKVL
beta3  FDFLKHRFDG  RISITRVTAD  LSLAKRSVLN  NPGKRTIIER  SSARSSIAEV  LSEIERIFEL  AKSLQLVVLD  ADTTNHPAQL  AKTSLAPIIV  FVKVSSPKVL

       301                                                 351                                                400
b3h    QRLIRSRGKS  QMKHLAVQMM  AYDKLVQCPP  ESFDVILDEN  QLEDACEHLA  EYLEVYWRAT  HHPAPGPGLL  GPPSAIPGLQ  NQQLLGERGE  EHSPLERDSL
beta3  QRLIRSRGKS  QMKHLAVQMM  AYDKLVQCPP  ESFDVILDEN  QLEDACEHLA  EYLEVYWRAT  HHPAPGPGLL  GPPSAIPGLQ  NQQLLGERGE  EHSPLERDSL

       401                                                 451                    484
b3h    MPSDEASESS  RQAWIGSSQR  TSRHLEEDAY  DAYQDLYQPH  RQHTSGLPSA  AQDSEHNHSD  RNWQRNRPWP  KDSY
beta3  MPSDEASESS  RQAWIGSSQR  TSRHLEEDAY  DAYQDLYQPH  RQHTSGLPSA  AQDSEHNHSD  RNWQRNRPWP  KDSY
```

Fig. 3. Deuced amino acid sequences of huamn VGCC β3 subunit gene.
Alignment of deduced amino acid sequences of huamn VGCC β3 subunit gene and rat β3 gene. The human β3 sequence is obtained by translation of sequence spliced together from exons. Identical sequences were shown as capital letter and nonidentical sequences were shown 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 a helices deduced from hydropathy profile(not shown).

typical membrane-spanning region and contains three major a helical domains. The β3 subunit of VGCC has many possible phosphorylation site. Potential protein kinase C(Ser/Thr-X-Lys/Arg) sites were found at 4 locations and 4 possible casein kinase II sites(Ser/Thr-X-X-Asp/Glu) were observed. No consensus site for cAMP-dependent protein kinase was found in β3 subunit gene.

Discussion

The first exon of the genomic DNA clone, BHG, as shown at Table 1. is GGTTCA---GCCAAG, from which other exon could not found reaching 2.85kb upstream. The size of intron in the BHG genome ranges from 118 bp(between exon J and K) to 597 bp(between H and I). Deduced amino acid sequence of β3 subunit gene from the exons starts with GSAD--, 16th amino acid of β3 subunit protein[18)19)20)]. The other effort should be done to find the exons encoding 5'-end of the β3 subunit gene, where 3 alternatively spliced forms of β3 subunit gene from rat were observed(unpublished data).

The deduced amino acid sequence from the exons has high degree of similarity with that of rat gene[20)] and identical to that of the human β3 cDNA[22)] Alternatively spliced forms of β1, β2 subunit gene, which contribute to the functional diversity of the VGCC, were reported[23)]. The genomic clones so far we have cloned doesnot have the exons which could be alternatively spliced in the middle of the gene as β1 subunit[23)24)].

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 first two α-domains was proposed to be a binding domain to the α1 subunit of skeletal muscle type VGCC[25)]. Missing the second α-helical domain in the β3 subunit might contribute to the different binding patterns with various α1 subunits.

Calcium currents through the cardiac and skeletal muscular VGCC are modulated by phosphorylation mediated by several protein kinases, pretin kinase C, cAMP-dependent protein kinase, etc[16)18)19)]. Although it has been demonstrated that preferred substrate for protein kinases are the α1 subunits, β subunits have several potential phosphorylation sites[2)18)19)]. Among them possible phosphorylation sites for protein kinase C were the most abundant, but possible phosphorylation sites for cAMP-dependent kinase and cGMP-dependent kianse could not found. These suggests that phosphorylation through the activation of the protein kinase C system might be important regulatory machinary in calcium signalling.

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Genomic Organization of Human β3 Subunit Gene of Voltage-Gated Calcium Channel

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전압의존성 칼슘이온통로는 α1, α2, β, 및 δ 등 적어도 네 개 이상의 아단위로 구성되어 있다. 이중 β아단위는 칼슘채널의 활성화(Activation ; 이온통로의 열림) 및 비활성화(Inactivation ; 이온통로의 닫힘)를 크게 강화시키며, 또한 여러 신호전달 체계를 통해 활성화된 PKA 혹은 PKC 등에 의해 인산화됨으로서 칼슘채널의 활성도를 조절한다. β 아단위는 여지껏 β1, β2, β3, β4의 네가지 아형이 알려져 있는데 이중 β3형이 신경계에 특이적으로 발현되며 칼슘채널을 조절하는 β3형 칼슘채널의 유전자조절을 알아보고자 게놈수준에서 조사하였다. β3형 게놈 유전자는 사람 폐에서 유래한 섬유아세포의 게놈으로부터 만든 유전자은행으로부터 NG108-15세포주에서 분리한 β3형 cDNA를 탐색자로 이용하여 분리하였다. 이 게놈 DNA의 크기는 12.5kb였으며 12개의 exon을 포함하며, 이 유전자 단백질의 16번째 아미노산으로부터 끝 아미노산까지를 포함하고 있으며 흰쥐 cDNA 유전자에서 밝힌 아미노산 서열과 비교하면 98% 동일하였다. Exon들로부터 유추한 아미노산서열에는 인산화될 수 있는 많은 아미노산들이 발견되어 이 전압의존성 칼슘이온통로의 β3 아단위 유전자가 칼슘이온의 흐름조절에 중요함을 알 수 있다.