Skeletal Muscle and Brain Isoforms of a δ-Subunit of Human Voltage-dependent Calcium Channels Are Encoded by a Single Gene*

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Clones of the β1-subunit of the voltage-dependent calcium channel (VDCC) from human skeletal muscle and hippocampus cDNA libraries, and from human genomic libraries, were isolated using a human skeletal muscle β1 cDNA probe generated by polymerase chain reaction. The skeletal muscle β1 cDNA (β1M) encodes a protein of 523 amino acids that is 97% identical to the rabbit skeletal muscle β-subunit. Two different cDNAs, β1B1 and β1B2, were obtained from the human hippocampus library. The β1B1 transcript encodes a protein of 478 amino acids that is identical to the skeletal muscle β-subunit (β1M), except for an internal region of 52 amino acids. The β1B2 transcript encodes a protein of 596 amino acids. The β1B2 polypeptide is identical to the β1B1 polypeptide at amino acids 1–444; however, it has a unique 152 amino acid carboxyl terminus. Like β1B1, it differs from β1M at the internal 52 amino acids. Analysis of the β1 gene structure demonstrates that these three cDNAs represent transcripts encoded by a single β1 gene. Transcripts from the β1 gene were detected in RNA from skeletal muscle, heart, spleen, and brain, but not in RNA from liver, stomach, or kidney.

Voltage-dependent calcium channels (VDCCs) participate in the regulation of calcium-linked cellular functions such as action-potential generation, muscle contraction, and secretion of hormones and neurotransmitters (Hille, 1984; Hess, 1990; Bean, 1989). One class of long-lasting calcium currents, the L-type current, is antagonized by 1,4-dihydropyridine (DHP) derivatives. DHP-sensitive VDCCs are expressed in many tissues but have been most well characterized in skeletal muscle, cardiac muscle, and brain. In skeletal muscle, the DHP-sensitive VDCCs are expressed in skeletal muscle and brain. The γ-subunit of the skeletal muscle DHP-sensitive VDCC consists of five distinct subunits, α1, α2, β, γ, and δ (Catterall, 1988). α1, β, and γ are encoded by separate genes, whereas α2 and δ are produced by proteolytic cleavage of a larger precursor encoded by a single gene (De Jongh et al., 1991). When introduced into Xenopus oocytes (Mikami et al., 1989) or into fibroblasts (Perez-Reyes et al., 1989; Tanabe et al., 1990) in the absence of the other subunits, the α1-subunit forms a channel that is DHP-sensitive and exhibits many of the characteristics of the native calcium channel.

The subunit composition of cardiac, smooth muscle, and neuronal DHP-sensitive VDCCs has not been completely determined. Analysis of purified cardiac and brain VDCC subunits with antibodies to the skeletal muscle VDCC subunits suggests that there is a high degree of homology between the α1- and α1/δ-subunits expressed in different tissues (Cooper et al., 1987; Ahijianian et al., 1990). Northern blot analysis (Ruth et al., 1989) and monoclonal antibody binding (Sakamoto and Campbell, 1991) demonstrate that the β-subunit is expressed exclusively in skeletal muscle and brain. The γ-subunit appears to be expressed exclusively in skeletal muscle (Jay et al., 1990; Bosse et al., 1990).

In order to determine the specific contribution of each of the subunits to channel function, α1-subunits from different tissues have been coexpressed with α2/δ-, β-, and γ-subunits from skeletal muscle in Xenopus oocytes and L-cells (Singer et al., 1991; Mori et al., 1991; Varadi et al., 1991; Lacerda et al., 1991). Although the resulting channel properties depend both on the α1 subtype and the recipient cell type, the general conclusion is that the production of calcium channels having normal physiological properties is greatly enhanced by the coexpression of the α1/δ-, β-, and γ-subunits (for review, see Catterall (1991) and Miller (1992)). The skeletal muscle β-subunit has the largest effect on functional expression of the skeletal and cardiac muscle and several forms of the brain α1-subunits. Coexpression of these α1-subunits and the skeletal muscle β-subunit increases the calcium current and accelerates activation and inactivation more than 10-fold (Singer et al., 1991; Mori et al., 1991; Varadi et al., 1991; Lacerda et al., 1991). Skeletal muscle β-subunits have a molecular mass of approximately 58 kDa. They are phosphorylated by cAMP-dependent protein kinases, protein kinase C, and cGMP-dependent protein kinase (Curtis and Catterall, 1985; Takahashi et al., 1987; Jahn et al., 1988) but are not glycosylated nor do they copurify with membranes (Takahashi et al., 1987). Features of the primary structure of the rabbit skeletal β polypeptide (Ruth et al., 1989) and biochemical data suggest that it is a peripheral membrane protein that interacts with an intracellular domain of the α1-subunit and that it may modulate α1 function through a phosphorylation pathway.

The skeletal muscle β1 cDNA detects transcripts of 1.6 and

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†The abbreviations used are: VDCC, voltage-dependent calcium channel; DHP, 1,4-dihydropyridine; kb, kilobase pair(s); bp, base pair(s); PCRam, polymerase chain reaction.

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1.9 kbp in skeletal muscle RNA and a 3-kbp transcript in rabbit brain RNA (Ruth et al., 1989). It is not known whether these transcripts are products of the same gene or of two or more closely related genes. In order to determine the relationship between the skeletal muscle and brain β1 transcripts, we compared the nucleotide sequence of β1 cDNAs from human skeletal muscle and hippocampus cDNA libraries to portions of the β1 gene(s). Our results demonstrate that at least one skeletal muscle and two brain transcripts are the product of the same β1 gene.

**EXPERIMENTAL PROCEDURES**

**Methodology**—Except where noted, recombinant DNA methods were adapted from protocols described by Sambrook et al. (1989) and Ausubel et al. (1987).

**Libraries**—The human fetal skeletal muscle cDNA library was kindly provided by Dr. L. Kunkel (Koenig et al., 1987). The human hippocampus cDNA library and the human placental cDNA library were purchased from Stratagene Inc.

**Screening the Libraries**—Degenerate oligonucleotide primers, B1, 5'-CGCCGGCAATTCACNNCCNNCCNYG-3', corresponding to amino acids 248-253, and B3, 5'-CGCCGGCAATTGCGNGTNGTNGCCYTTCCA-3', corresponding to amino acids 454-460 of the rabbit skeletal muscle β1-subunit (Ruth et al., 1988), were synthesized by the University of Wisconsin Biotechnology Center. Each primer contained an EcoRI restriction site in addition to the 17-20 nucleotides of sequence corresponding to the β1 peptide sequence. These primers were used in a polymerase chain reaction (PCR) using human skeletal muscle cDNA as a template (see below for conditions). The PCR product was cloned into the EcoRI site of pBluescript KS+ (Stratagene Inc.). DNA sequence analysis confirmed the identity of the insert in one clone, PCR3, which was subsequently used as a probe to screen the human cDNA and genomic libraries.

**Sequence—**DNA sequence was determined by the method of Sanger et al. (1977) using the Sequenase kit (United States Biochemical Corp.) and double-stranded plasmid as a template. Nucleotide and polypeptide sequence analyses were performed using GCC software (Devereaux et al., 1984) and a VAX computer.

PCR—All PCRs contained a DNA template (see below), 200 μM of each deoxynucleotide triphosphate, 1 μM of each primer, 50 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl, pH 8.3, 0.001% gelatin, and 1.25 units of Taq DNA polymerase (Perkin-Elmer Cetus) in a total volume of 50 μl. The DNA was denatured for 2 min at 94 °C followed by 25-35 cycles of amplification with each cycle consisting of 45 s at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. After the final extension for 10 min at 72 °C was performed. The template DNA was either 250-500 ng of genomic DNA, first strand cDNA made from 0.5 pg of polyadenylated RNA, or 1 ng of cloned DNA. The number of cycles and the annealing temperature were empirically determined for each primer pair. A 10-μl aliquot of each PCP product was electrophoresed on a 1.5% agarose gel in 0.045 M Tris borate, pH 8.3, stained with ethidium bromide, and visualized under UV light. Products of several closely related genes. We examined the β1 gene structure in order to distinguish between these two possibilities. Several cosmids that contained portions of the β1 gene(s) were isolated and used to determine the gene structure in the regions where the three cDNAs differ.

**To investigate the difference between β1 and β2 at amino acids 210-261 in βM and 210-216 in βB, primers B7 and B8 that flank this region in the cDNAs were used in a PCR assay. A single 2-kb PCR product was obtained when either cosmid D3-3 or genomic DNA was used as template. This genomic fragment was cloned and sequenced. Exons were identified by comparing the genomic sequence with the βM and βB cDNA sequences. The genomic sequence (shown in Fig. 2) contains two exons, one that is present in β1 and the other that is present in βM. Consensus splice sites flank each of the putative exons. Thus the difference between β1 and β2 arises from the use of tissue-specific exons contained within a single gene. The structure of this region of the β1 gene is represented in Fig. 4A.

**RESULTS**

**Human cDNAs**—cDNAs from the fetal skeletal muscle library and the hippocampus cDNA library were obtained by screening with PCR3D.

**DNA Sequence Comparison**—The DNA sequences of a skeletal muscle cDNA and portions of the hippocampus cDNAs were determined. Some of the hippocampus cDNAs contained a 450-bp insert with no homology to the rabbit skeletal muscle β1 cDNA (Ruth et al., 1989). The sequence of this insert contained stop codons in all three forward frames and was flanked by consensus splice sites, so it most likely represents an unspliced intron present in about half of the hippocampus cDNAs.

The correctly spliced hippocampus cDNAs fall into two classes, β1 and β2, representing two different transcripts. The number of cycles and the annealing temperature were empirically determined for each primer pair. A 10-μl aliquot of each PCR product was electrophoresed on a 1.5% agarose gel in 0.045 M Tris borate, pH 8.3, stained with ethidium bromide, and visualized under UV light. Products of several closely related genes. We examined the β1 gene structure in order to distinguish between these two possibilities. Several cosmids that contained portions of the β1 gene(s) were isolated and used to determine the gene structure in the regions where the three cDNAs differ.

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**To investigate the difference between the 3' ends of β1 and β2, three primers; B5 (present in both β1 and β2) B14 (present only in βB1), and B12 (present only in βB2) were used in PCR assays using cDNAs, cosmids, and genomic DNA as templates. Fig. 3A summarizes the results of PCRs using primers B5 and B14. A 228-bp fragment was produced when β1 cDNA, cosmid D3-2, and genomic DNA were used as templates. As expected, no product was seen when β2 cDNA was used as template. Fig. 3B summarizes the results of PCRs using primer B5 and B12. A 154-bp product was produced when βB2 was used as template. No product was observed when βB1 was used as template. A 1.8-kb fragment was produced when genomic DNA or cosmid D3-2 DNA was used as template.

The data shown in Fig. 3 allow us to deduce the β1 gene structure in this region which is summarized in Fig. 4. First, β1 cDNA and genomic DNA are colinear between primers B5 and B14, indicating that there are no introns in this region. DNA sequence analysis of a portion of cosmid D3-2 confirms that this portion of β1 cDNA is colinear with genomic DNA.
Fig. 1. Nucleotide sequence and conceptual translation of the human skeletal muscle βM and hippocampus β1B and β2B cDNAs. The sequence of β2B (B2) is shown in its entirety, with its conceptual translation shown directly below the sequence. Positions where the βM (M) and/or β1B and β2B (N1) nucleotide sequence and amino acid sequence differ are shown above the P1B2 sequence. Lowercase letters represent untranslated sequences, and uppercase letters represent translated sequences. Gaps, represented by dots, were introduced to maximize alignment. These gaps are included in the coordinates of the nucleotide sequence.

βM sequence:

| M,El.82 | M,El.82 | M,Bl overrides to maximize alignment. These gaps are included in the coordinates of the nucleotide sequence.

β1B sequence:

| M,El.82 |

β2B sequence:

| M,El.82 |

FIG. 2. The nucleotide sequence of the portion of the β-subunit gene containing the skeletal muscle-specific exon used in βM and the exon used in β1B and β2B. Uppercase letters represent exons, and lowercase letters represent introns. The location of exons are highlighted by bars.

![Diagram of exon-intron structure]

FIG. 3. PCR products obtained using primers B5, B12 and B14. A. PCR products obtained using primers B5 and B14 and templates βB1 cDNA, βB2 cDNA, cosmid D3-2, and human genomic DNA. B. PCR products obtained using primers B5 and B12 and templates βB1 cDNA, βB2 cDNA, cosmid D3-2, and human genomic DNA.
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DNA from nucleotide 1354 to its poly(A) tail (data not shown). Second, when primers specific to the 3' region of $\beta$B1 (primers B5 and B14) or $\beta$B2 (primers B5 and B12) are used in PCRs with cosmid D3-2 as the template, products of 228 bp and 1.85 kb are produced, respectively. Thus the B12 primer sequence (from the B2 exon) is located 1.6 kb downstream of the B14 primer sequence. The 1.85-kb B5-B12 PCR product was cloned into the pCR1000 vector (Invitrogen, Inc.) and portions of it were sequenced. Fig. 4B summarizes the structure of the $\beta$ gene in this region and shows the sequence of the exon-intron junctions used in the $\beta$B2 transcript. These data show that the $\beta_1$ primary transcript undergoes a novel splice in $\beta$B2, skipping over both the termination codon and the polyadenylation signal that are used in $\beta$M and $\beta$B1. The completely processed $\beta$B2 transcript is 1.98 kb longer than the $\beta$M transcript and includes one or more 3' exons.

As shown in Fig. 4, all of the differences between the $\beta$M, $\beta_1$, $\beta$B1, and $\beta$B2 cDNAs are the result of alternative splicing of a single $\beta_1$ gene.

**Tissue Specificity**—First strand cDNA was synthesized from polyadenylated RNA isolated from mouse brain, heart, liver, kidney, spleen, and stomach. These cDNAs were used in PCRs with primers B7 and B8 which span amino acids 191–280 in $\beta$M and amino acids 191–235 in $\beta$B1 and $\beta$B2. No product was detected in reactions using cDNA made from liver, kidney, or stomach RNA. A 265-bp product was amplified in PCRs using cDNA made from skeletal muscle RNA or the $\beta$M cDNA clone. A 130-bp product was amplified in PCRs using cDNA made from brain, heart, or spleen RNA or the $\beta$B1 or $\beta$B2 cDNA clones (Fig. 5).

**Peptide Sequence Motifs**—A comparison of peptide sequence motifs was undertaken in an attempt to detect potentially significant differences between the brain and the skeletal muscle polypeptides. The peptide sequence of human $\beta$B1, human $\beta$B2, and human $\beta$M (this study) and the rabbit skeletal muscle $\beta$M (Ruth et al., 1989) were scanned for over 400 protein sequence motifs using the GCG software (Devereaux et al., 1984). Four different motifs were identified: protein kinase C phosphorylation sites, cAMP-dependent protein kinase phosphorylation sites, casein kinase phosphorylation sites, and N-glycosylation sites. The location of these sites is shown in Fig. 6. The majority of these sites were conserved between the rabbit and human sequences and are found in the $\beta$B1, $\beta$B2, and both the human and rabbit $\beta$M isoforms. However, a potential protein kinase C phosphorylation site at serine 238 is located in a region exclusive to the brain.
skeletal muscle isoforms. $\beta_1$B1 and $\beta_2$B2 contain a potential protein kinase C phosphorylation site at serine 209 that is not present in $\beta_3$M. Instead the human and rabbit $\beta_3$M isoforms have a potential casein kinase phosphorylation site at serine 209. $\beta_2$B2 lacks the potential protein kinase C phosphorylation sites at serine 495 and serine 509 present in $\beta_3$M and $\beta_1$B1 but has potential casein kinase phosphorylation sites at serine 509, serine 540, serine 563, and serine 590 and a potential protein kinase C site at threonine 535. A CAMP-dependent protein kinase phosphorylation site, threonine 205, is present in all four sequences and can be phosphorylated in vitro (De Jongh et al., 1989). Serine 182 has also been phosphorylated in vitro by CAMP-dependent protein kinase (Ruth et al., 1989); however, the replacement of this residue with glycine in the human polypeptide suggests that this phosphorylation may not be physiologically relevant.

Ruth et al. (1989) identified a block of 8 similar amino acids (marked as shaded boxes in Fig. 8) repeated within four $\alpha$-helical domains. The second of these $\alpha$-helical domains is present in all four sequences and can be phosphorylated in vitro by CAMP-dependent protein kinase (Ruth et al., 1989); however, the replacement of this residue with glycine in the human polypeptide suggests that this phosphorylation may not be physiologically relevant.

**DISCUSSION**

A single gene encodes three different $\beta$-subunits, including the $\beta_1$-subunit of the skeletal muscle dihydropyridine-sensitive calcium channel and at least two other $\beta$-subunits that are expressed in the brain, heart, and spleen. A polypeptide of 523 amino acids ($\beta_3$M) is produced in skeletal muscle, whereas two polypeptides of 478 ($\beta_1$B1) and 596 amino acids ($\beta_2$B2) are produced in the brain. The sequences of $\beta_3$M and $\beta_1$B1 are identical with the exception of a region of 52 amino acids in $\beta_3$M that is replaced by 7 different amino acids in $\beta_1$B1. DNA sequence analysis of the corresponding portion of the $\beta_1$ gene demonstrates that this difference is the result of use of one exon in skeletal muscle transcripts and another exon in brain transcripts. The two brain polypeptides, $\beta_1$B1 and $\beta_2$B2, are identical from amino acids 1–444, but differ at their carboxyl termini. $\beta_1$B1 shares the same 34-amino acid carboxyl terminus as $\beta_3$M, $\beta_2$B2 has a unique 152-amino acid carboxyl terminus. Analysis of the gene structure in this region demonstrated that the primary transcript undergoes a novel splice in $\beta_2$B2, skipping over both the termination codon and the polyadenylation signal used in $\beta_3$M and $\beta_1$B1. The $\beta_2$B2 transcript is 1.87 kb longer than the $\beta_1$B1 and $\beta_3$M transcripts and differs by the use of one or more 3′ exons which we have not yet characterized.

The $\beta_2$B2 cDNA probably corresponds to the 3.0-kb transcript seen using rabbit brain RNA on Northern blots (Ruth et al., 1989). The $\beta_1$B1 cDNA is 1.9 kb in length and has the same 5′ end and uses the same polyadenylation site as the skeletal muscle $\beta_3$M cDNA. Since the $\beta_1$B1 cDNA contains a complete open reading frame and a poly(A) tail, it probably represents a minor class of transcripts not detected on the Northern blots. Alternatively, it is possible that the $\beta_1$B1 transcript is actually 3.0 kb in length but utilizes a large 5′ untranslated region not included in the $\beta_1$B1 cDNAs described here.

While the manuscript was in preparation, Pragnell et al. (1991) reported the sequence of a rat brain $\beta_3$ cDNA, and Williams et al. (1992) reported the sequence of a human brain $\beta$ cDNA that they called $\beta_5$. These cDNAs correspond to our $\beta_2$B2 and $\beta_1$B1 cDNAs, respectively. In addition, cDNAs from two other $\beta$ genes have recently been identified. Perez-Reyes et al. (1992) reported a rat brain $\beta$-subunit cDNA that represents the transcript of the $\beta_3$ gene that is expressed in heart, lung, and brain. Hullin et al. (1992) described a collection of cDNAs from heart, aorta, and brain. Several of these cDNAs represent transcripts from the $\beta_3$ gene, whereas others represent transcripts from a third gene, $\beta_5$.

Polymerase chain reactions that distinguish the skeletal muscle ($\beta_3$M) and brain ($\beta_1$B1 and $\beta_2$B2) transcripts from the $\beta_3$ gene in RNA isolated from various tissues suggest that the brain contains only the brain isoforms ($\beta_1$B1 and $\beta_2$B2), and skeletal muscle contains only the skeletal muscle isoform...
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(β,M). Brain isoforms (β,B1 and/or β,B2 or some closely related gene) are also expressed in the heart and spleen. No β-subunit RNA was detected in liver, kidney, or stomach.

Detection of β transcripts in the heart and spleen is in contrast to the results of Ruth et al. (1989) who observed β-subunit transcripts of 3.0 kb in rabbit brain RNA and 1.6 and 1.9 kb in rabbit skeletal muscle RNA and no hybridization with polyadenylated RNA from smooth muscle or heart. This discrepancy may be due to the increased sensitivity of PCR as compared with Northern analysis, or it could result from the detection of a closely related gene that is expressed in heart and spleen. The observed PCR products in heart and spleen RNA are not likely to be derived from either the β2 or β gene, since within the region containing the B7 and B8 primers, both genes are less than 70% identical to the β gene. Therefore, the cardiac and spleen PCR products are produced from transcripts from the β gene or another more closely related gene or genes.

The identification of two β isoforms expressed in the brain raises questions about their association with specific channels. A recent study showed that a monoclonal antibody to β,M immunoprecipitates both a DHPR receptor and a ω-conotoxin GVIA receptor in rabbit brain membrane preparations (Sakamoto and Campbell, 1991). Two polypeptides of 68 and 78 kDa were detected with this monoclonal antibody in partially purified ω-conotoxin receptors from rabbit brain membranes. It seems likely that both the 478-amino acid β,B2-subunit (approximately 52 kDa) and the 596-amino acid β,B1-subunit (approximately 64 kDa) were isolated in this preparation.

Several different peptide sequence motifs were identified in an analysis of the conceptual translation products of the rabbit β,M cDNA (Ruth et al., 1989), the human β,M cDNA, and the human β,B1 and β,B2 cDNAs. The β-subunit from the skeletal muscle DHSP-sensitive calcium channel has been phosphorylated by a protein kinase intrinsic to isolated triads (Imagawa et al., 1987) and by cAMP-dependent protein kinases, protein kinase C, and a cGMP-dependent protein kinase (Curtis and Catterall, 1985; Takahashi et al., 1987; Jahn et al., 1988; De Jongh et al., 1989). Our analysis shows that the majority of the phosphorylation sites are conserved between the rabbit and human sequences and are present in all three (β,M, β,B1, and β,B2) isoforms. However, some potential phosphorylation sites are found exclusively in the skeletal muscle (β,M) isoforms or the brain (β,B1 and β,B2) isoforms or only in the β,B2 isoform. The observation that a subset of the phosphorylation sites are conserved in all four peptide sequences while others are exclusive to the brain or skeletal muscle isoforms implies specific functional correlates. However, the significance of the differences between the brain and skeletal muscle isoforms cannot be assessed without first characterizing the functional differences between the brain and skeletal muscle β polypeptides.

The tissue- and cell-specific functions of different calcium channels may depend not only on the α1-subunit expressed but also on the specific α2/δ-, β-, and γ-subunits expressed (see Catterall, 1991). In order to dissect the contribution of each of these subunits, it is essential to understand the genetic relationship and pattern of expression of tissue-specific isoforms of each subunit. Our identification of three isoforms of the human β-subunit encoded by a single gene provides a key tool in the dissection of the role of the β-subunit in the tissue- and cell-specific regulation of voltage-gated calcium channels.

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