The cDNA and deduced amino acid sequence of the γ subunit of the L-type calcium channel from rabbit skeletal muscle

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Complementary DNAs for the γ subunit of the calcium channel of rabbit skeletal muscle were isolated on the basis of peptide sequences derived from the purified protein. The deduced primary structure is without homology to other known protein sequences and is consistent with the γ subunit being an integral membrane protein.

Ca^{2+} channel; cDNA cloning; Membrane protein; Skeletal muscle

1. INTRODUCTION

L-type calcium channels comprise a group of very similar yet distinct proteins or protein complexes that differ in kinetics, voltage dependence and their relative sensitivity to organic calcium channel blockers (CaCB). When purified from rabbit skeletal muscle the calcium channel consists of three main subunits with molecular masses of 165000 (α_1 subunit or CaCB receptor), 55000 (β) and 32000 Da (γ) [1–5]. A further polypeptide consisting of a disulfide-linked dimer of a 130000 (α_2) and a 28000 (δ) protein is present in this preparation at a variable concentration [2]. The subunits have been reconstituted to functional calcium channels which were modulated by phosphorylation [6] and by monospecific antibodies for the α_1, the β and the γ subunit [7,8]. The cDNAs of the CaCB receptor [9–11], the α_2 polypeptide [10,12] and the β subunit [13] have been cloned. The CaCB receptor is the principal transmembrane subunit, which forms the ion conducting pore. This protein binds organic calcium channel blockers [2–5,9] and is phosphorylated in vitro at Ser-687 by cAMP-dependent protein kinase [14]. The β subunit is a peripheral membrane protein [13]. Heterologous expression of the skeletal muscle CaCB receptor leads to the appearance of functional calcium channels [10,11,15–17] and restores excitation contraction coupling [15,16]. It is not known whether these functions require the presence of the smaller channel subunits. We now report the primary structure of the γ subunit as deduced from the cloned cDNA and the tissue distribution of its mRNA.

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2. MATERIALS AND METHODS

2.1. *Purification of the γ subunit and generation of partial amino acid sequences*

The calcium channel was purified from rabbit white skeletal muscle as in [1]. The γ subunit (200 μg) was separated from the purified channel complex by size exclusion chromatography as described [2]. 5 μg were used to sequence the NH_2-terminus (peptide 1). The remaining 195 μg were dialyzed at 4°C overnight against 50 mM sodium phosphate buffer, pH 7.2, containing 6 M urea and Dowex AG1-X2 resin [13,14]. Dialysis was continued for 80 h against 10 mM $(NH_4)HCO_3$. The dialyzed material was lyophilized and dissolved in 250 μl of 25 mM Tris-HCl, pH 8.0. Trypsin was added at a γ subunit/trypsin ratio of 40:1, and the proteins were incubated at 37°C for 18 h. The dried tryptic digest was suspended in 0.5 ml 0.1% trifluoroacetic acid, loaded onto a Macherey & Nagel 300-5C4 column (30 × 4 mm) and eluted with a gradient of 1% acetonitrile per minute at a flow rate of 0.5 ml/min. One fraction was collected (Fig. 1) and purified further on a Macherey & Nagel 300-7C4 column (microbore 125 × 2 mm) (Fig. 1, inset) and sequenced (peptide 2). Amino acid sequences were determined with a gas phase sequencer as in [13,14].

2.2. *Construction and screening of cDNA libraries*

Single-stranded cDNA was obtained by extension of a synthetic primer (primer A) with poly(A)$^+$ RNA from rabbit skeletal muscle. PCR [18,19] was carried out using 6.5 ng of the single stranded cDNA and 3 4 μg of primer B and primer C, respectively. A random primed cDNA library from rabbit skeletal muscle was constructed [11,13] in pUC9 and 5×10^4 independent recombinant clones were screened with the amplified cDNA fragment. Sequencing of the cDNA was performed on both strands.

2.3. *RNA blot analysis (Northern)*

Poly(A)$^+$ RNA was denatured with glyoxal (1 M) and 50% dimethylsulfoxide, electrophoresed on 1.2% agarose gels and transferred to Biodyne membranes. Hybridization was carried out with the amplified PCR cDNA fragment, labeled by the random priming method [20]. If not stated otherwise, all other cloning procedures were carried out according to [21].

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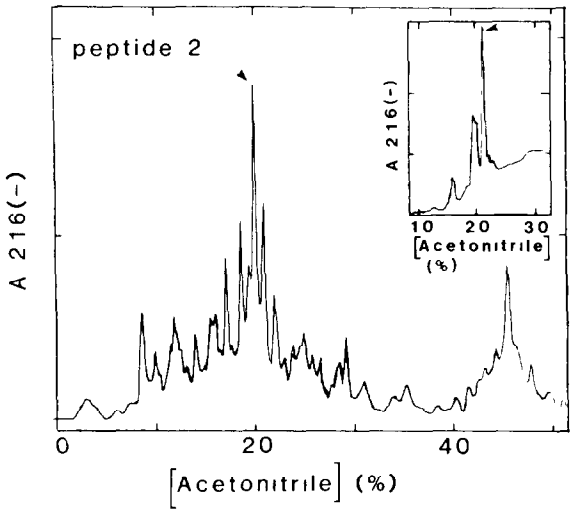

Fig. 1. Isolation of tryptic peptide 2 from the γ subunit. Elution profiles of the reverse-phase HPLC of tryptic peptides and rechromatography of peptide 2 (inset). Solid line and left-hand axis A216.

3. RESULTS

3.1. *Cloning strategy and isolation of the cDNA for the γ subunit*

Fig. 2 summarizes the amino acid sequences of peptide 1 (NH_2-terminus) and peptide 2 and the strategy for cloning the γ subunit. 48 oligodeoxyribonucleotides each containing 17 nucleotides (nt) which are complementary to all possible cDNA sequences encoding the amino acid sequence Glu-Ile-Phe-Glu-Phe-Thr (excluding the third nucleotide residue of the Thr codon) in peptide 2 were synthesized (primer A in Fig. 2a). These oligomers were used as specific primers for reverse transcription of the γ subunit mRNA. The resulting single-stranded cDNA served as target sequence for polymerase chain reactions, which were primed by an equimolar mixture of 192 (primer C) and 128 synthetic 17 nt oligodeoxyribonucleotides (primer B). Primer C corresponds to all possible cDNA sequences encoding the NH_2-terminus of peptide 1 (Met-Ser-Pro-Thr-Glu-Ala, excluding the third nucleotide of the Ala codon). Primer B is complementary to all possible cDNA sequences encoding the amino acid sequence His-Phe-Asn-Pro-Gly-Glu (excluding the third nucleotide of the Glu codon) of peptide 2, respectively. The gel resolved amplified cDNA was eluted, subcloned and sequenced. It consists of 266 nt and includes the coding region for peptide 1 (excluding the Met codon) and part of peptide 2. This cDNA fragment was used as a probe for cloning larger cDNA sequences from a randomly primed cDNA library.

The 1268 nt cDNA obtained contains an open reading frame encoding a sequence of 222 amino acids (Fig. 3A). It includes the two independently determined

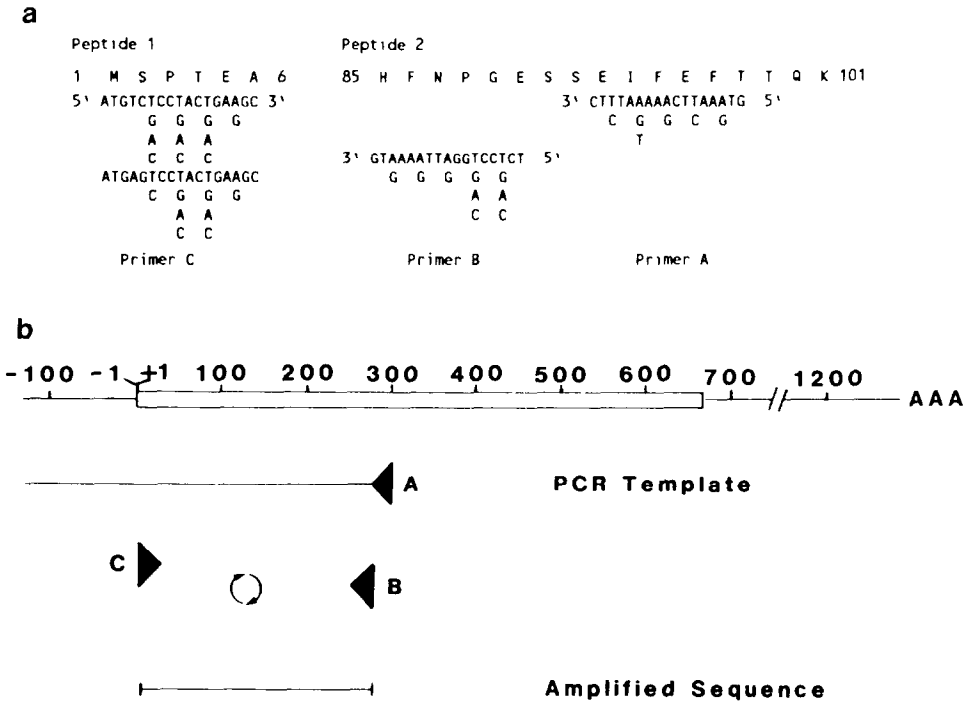

Fig. 2. Partial amino acid sequences (a) and cloning strategy for the γ subunit (b). (a) Peptide sequences derived from gas phase microsequencing with deduced oligodeoxynucleotides used as primers A, B, C. The primers represent all possible nucleotide combinations which are complementary to (A, B) or identical with (C) the nucleotide sequences encoding the parts of peptides 1 and 2 indicated. (b) The closed box represents the protein coding region. Numbers indicate nucleotide positions. Triangles indicate primers A–C used for primer extension (A) and polymerase chain reactions (B, C). The amplified cDNA sequence differs from the γ subunit cDNA at five positions contributed by the DNA of primer B and C (nt 4, 5, 12, 264 and 267) suggesting that less than perfect matches between template and degenerated primers led to product synthesis.

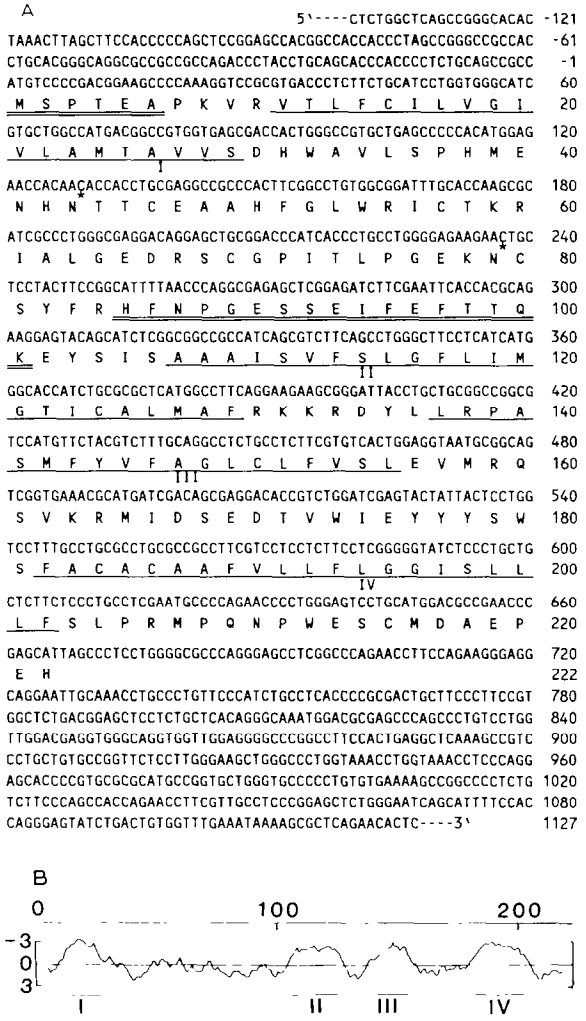

Fig. 3. Nucleotide and deduced amino acid sequence (A) and hydropathicity profile (B) of the γ subunit. (A) The two peptides determined by sequence analysis and the predicted transmembrane segments I–IV are underlined twice and once, respectively. Predicted *N*-glycosylation sites are indicated (*). (B) Hydropathy profile computed according to [22]; the window size is 19 residues, plotted at one residue intervals.

peptide sequences. The translation initiation codon at position 1 is the first ATG triplet in frame that appears downstream of the stop codon TAG (nt −75 to −73). The surrounding nucleotide sequence agrees with the consensus sequence for eukaryotic initiation codons. An analysis of the amino acid sequence of the γ subunit for local hydropathicity (Fig. 3B) reveals the presence of four typical membrane spanning regions (segments of at least 19 residues with an average hydropathicity index greater than 1.6) [22] which have been designated segment I (amino acids 11–29), segment II (amino acids 107–129), segment III (amino acids 137–155) and segment IV (amino acids 182–202). The γ subunit does not contain a signal peptide sequence. Thus it seems reasonable to assume that the amino- and carboxy-

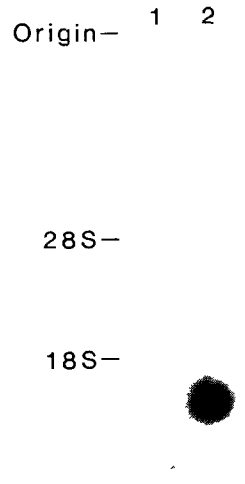

Fig. 4. RNA blot analysis of rabbit RNA with a γ subunit cDNA probe. Samples of 10 μg poly(A)$^+$ RNA from heart (lane 1) and skeletal muscle (lane 2) were hybridized with the amplified cDNA fragment (nt 4–266).

terminus are localized intracellularly whereas the hydrophobic segments I–IV cross the cell membrane. This model is consistent with the two potential *N*-glycosylation sites (Asn-43 and Asn-79) being located on the extracellular site. In vivo the γ subunit is highly glycosylated [3]. This glycosylation accounts for the difference in molecular mass between the natural γ subunit (32000 Da) and the predicted mature unglycosylated polypeptide (25058 Da).

3.2. *Northern blot analysis*

The tissue distribution of the mRNA for the γ subunit was analyzed by RNA blots of polyadenylated [poly(A)$^+$]RNA from several rabbit tissues (Fig. 4). The probe derived from the coding sequence hybridized to a [poly(A)$^+$]RNA species of skeletal muscle with an estimated size of 1300 nt, which is consistent with the size of the cloned cDNA (1268 excluding the 80mer oligo-d(A) tract). No γ subunit specific hybridization was observed with [poly(A)$^+$]RNA from heart, trachea, lung, aorta or brain (14 h exposure).

4. DISCUSSION

The primary structure of the γ subunit lacks significant homology with any protein in the NBRF PIR protein and the GenBank nucleotide sequence databases. The protein with the best score in the protein database was the multidrug resistance protein 2 (mdr2 or *P*-glycoprotein) [23]. 34.1% of the amino acids are identical or conservatively substituted within the amino acid residues 105–148 and 86–129 of mdr2 and of the γ subunit, respectively. Interestingly mdr2 binds calcium channel blockers [24]. Although the γ subunit does not

bind these compounds by itself [2–5,9] it cannot be excluded that the similar amino acid sequences are indirectly involved in calcium channel blocker binding to the CaCB receptor and the mdr2 protein, respectively.

In vivo, the skeletal muscle CaCB receptor functions both as voltage sensor in excitation contraction coupling [15,16] and as a calcium channel [15–17]. The similar tissue-specific expression of the mRNA encoding the skeletal muscle CaCB receptor (α_1 subunit) [9,12], β [13] and γ subunit suggests that the three proteins contribute to these functions. With the cDNAs for the α_1, β and γ subunit available further studies on the structural requirements of these functions will be facilitated.

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