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Primary Structure of the γ Subunit of the DHP-Sensitive Calcium Channel from Skeletal Muscle

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Affinity-purified, polyclonal antibodies to the γ subunit of the dihydropyridine (DHP)-sensitive, voltage-dependent calcium channel have been used to isolate complementary DNAs to the rabbit skeletal muscle protein from an expression library. The deduced primary structure indicates that the γ subunit is a 25,058-dalton protein that contains four transmembrane domains and two N-linked glycosylation sites, consistent with biochemical analyses showing that the γ subunit is a glycosylated hydrophobic protein. Nucleic acid hybridization studies indicate that there is a 1200-nucleotide transcript in skeletal muscle but not in brain or heart. The γ subunit may play a role in assembly, modulation, or the structure of the skeletal muscle calcium channel.

THE DHP-SENSITIVE Ca^{2+} CHANNEL from skeletal muscle consists of four subunits: α_1 (170 kD), α_2 (175 kD, nonreduced; 150 kD, reduced), β (52 kD), and γ (32 kD) (*1*). The α_1 subunit contains the binding sites for the three classes of Ca^{2+}-channel blockers (DHPs, phenylalkylamines, and benzothiazepines) (*2*) and is a substrate for protein kinases, as is the β subunit. The α_2 and γ subunits are glycoproteins and bind wheat germ agglutinin. Upon disulfide bond reduction, the α_2 subunit undergoes a characteristic mobility shift by SDS–polyacrylamide gel electrophoresis (PAGE) analysis with the concurrent appearance of the δ peptides (19 to 30 kD). The cDNAs for the α_1, α_2, and β subunits have been isolated and characterized (*3–5*). The α_1 subunit, with 24 putative membrane-spanning segments, is the principal transmembrane subunit of the complex and has significant sequence homology with several other members of the voltage-dependent ion channel family, including the α subunit of the Na^+ channels and the *Drosophila* K^+ channel (*3, 4, 6*). The transmembrane properties of the α_2 and β subunits are quite different from the α_1 subunit, with the α_2 subunit predicted to have as many as three transmembrane domains (*4*) and the β subunit to have none (*5*). Although active DHP-sensitive Ca^{2+} channels have been reconstituted in lipid bilayers (*7*) and the microinjection of an expression plasmid carrying the α_1 subunit cDNA restores a DHP-sensitive Ca^{2+} current and excitation-contraction coupling in dysgenic muscle (*8*), it is unknown which subunits are required for a native functional DHP-sensitive Ca^{2+} channel.

To isolate cDNA clones for the γ subunit of the DHP-sensitive Ca^{2+} channel, affinity-purified guinea pig polyclonal antiserum, specific for the gel-purified γ subunit (Fig. 1) (*9*), was used to screen expression libraries constructed from rabbit back skeletal muscle RNA. Overlapping cDNA clones were isolated to determine the nucleotide sequence encoding the protein (Fig. 2A) (*10*).

The 1171-nucleotide (nt) cDNA sequence contains a 666-nt open reading frame coding for 222 amino acids (Fig. 2B). The deduced amino acid sequence indicates a calculated molecular weight of 25,058, which is in approximate agreement with the observed molecular mass of 32 kD for the glycosylated (*1*) and 20 kD for the chemically deglycosylated forms (*11*), determined by SDS-PAGE. The deduced amino acid sequence agrees with the authentic NH_2-terminus of the γ subunit, as determined by protein sequencing of the purified skeletal muscle protein (*12*). An analysis of the predicted amino acid sequence of the γ subunit for local hydrophobicity revealed four putative transmembrane domains (Fig. 2C). These segments are designated I (residues 11 to 29), II (residues 105 to 129), III (residues 140 to 155), and IV (residues 180 to 204). Unlike many of the transmembrane segments in the α_1 subunit (*3*), none of the above predicted segments contain any charged residues. The length of the predicted transmembrane segments varies from 16 to 25 amino acids. The NH_2-terminal sequence does not resemble a hydrophobic signal sequence.

On the basis of the local hydrophobicity and the lack of an NH_2-terminal signal sequence, the predicted secondary structure of the γ subunit includes four transmembrane segments separating intracellularly located NH_2- and COOH-terminals. Consistent with biochemical studies (*1*), the two potential N-linked glycosylation sites (Fig. 2B) reside on the extracellular face of the membrane. Of the six consensus phosphorylation sites (Fig. 2B), only Ser^2 and Ser^{214} are predicted to be intracellular. The observed decreased mobility of the γ subunit on SDS-PAGE in the presence of reducing agents, and the resistance of the protein to proteolytic digestion in the absence of disulfide bond reduction, indicate that disulfide bonds play a major role in determining the secondary structure of the native protein. The deduced primary structure of the γ subunit contains ten cysteine residues, one in the COOH-terminal intracellular segment, five within the hydrophobic transmembrane segments, and the remaining four in the first extracellular loop between

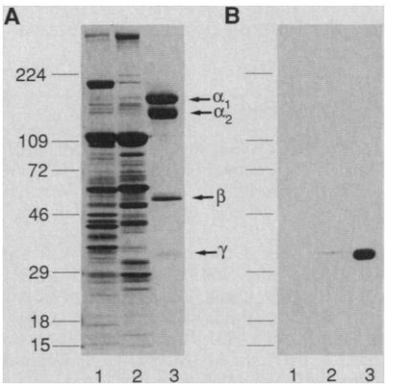

Fig. 1. Rabbit skeletal muscle DHP receptor subunit composition and γ subunit antibody probe specificity. (**A**) Coomassie blue–stained, reduced SDS-acrylamide gel showing crude (100 μg of microsomes) (lane 1), enriched (100 μg of triads) (lane 2), and purified (20 μg of DHP receptor) (lane 3) preparations of DHP-sensitive Ca^{2+} channel (*20*). (**B**) Immunoblot of identical samples as in (A), stained with GP16 polyclonal antiserum that had been affinity-purified (*9*). Arrows show the positions of the four subunits; the positions of the prestained molecular weight standards (BRL) are on the left ($M_r \times 10^{-3}$).

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segments I and II. The selection of clone λSkMCaChG.2, which encodes the COOH-terminal 15 amino acids, with affinity-purified antibodies against the purified γ subunit, indicates that significant proteolytic processing of the COOH terminal of the γ subunit does not take place, in contrast to the α_1 subunit of the receptor (*13*).

To determine the tissue-specific expression of γ-related mRNAs, polyadenylated RNA was isolated from different rabbit tissues for RNA blot analysis (Fig. 3). A prominent band of approximately 1200 nt was detected in RNA from skeletal muscle. A weaker signal of similar size was also detected in lung, while no γ-specific hybridization was observed in brain, heart, kidney, or liver. When the polyclonal antiserum to the γ subunit was used as a probe of immunoblots prepared from protein extracts of the same tissues, immunoreactive bands were present only in the skeletal muscle samples. Although the presence of γ-like subunits in other tissues cannot be ruled out, our data indicate a distribution of the γ subunit that is consistent with it contributing to the unique properties of the skeletal muscle DHP-sensitive Ca^{2+} channel and its postulated role in excitation-contraction coupling (*14*).

A comparison of the deduced amino acid sequence of the γ subunit with sequences in the Swiss-Protein and GenBank nucleotide databases revealed no significant homologies (*15*). The highest initial [init (n)] score was obtained with the Na^+ channel protein from the eel *Electroplax* (*16*) [init (n) = 107, 15% identity in a 133–amino acid overlap, γ subunit residues 40 to 168 versus eel Na^+ channel residues 462 to 593]. Although these database searches did not reveal homologous proteins, the presence of small (30 to 40 kD) glycoprotein subunits in other ion channel complexes (*17*) suggests that these may be homologous in structure and function to the γ subunit.

A functional role for the γ subunit is suggested by a report of stable expression of the α_1 subunit in tissue culture cells (*18*). Although the expression of the α_1 subunit alone was able to produce DHP-sensitive Ca^{2+} currents, activation was much slower than in the native tissue. Thus the γ subunit may contribute to channel activation. Skeletal muscle–like Ca^{2+} channel activity may be a result of a multisubunit complex containing four subunits, α_1, α_2, β, and γ. This notion is supported by data on the coexpression of α_1 and α_2 cDNA clones (*19*). Higher channel activity was reported when the cardiac α_1 and skeletal α_2 mRNAs were coinjected, suggesting that subunits other than α_1 can play a role in the assembly, modulation, or structural formation of the channel.

Now that the cloning and characterization of cDNAs encoding each of the four subunits of the rabbit skeletal muscle DHP-sensitive Ca^{2+} channel has been completed, it should be possible to introduce these cDNAs into heterologous expression systems to determine the functional contribution of each subunit to the formation of this Ca^{2+} channel.

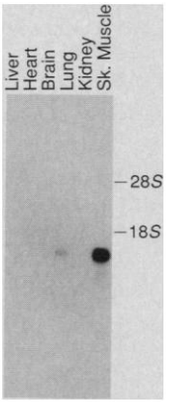
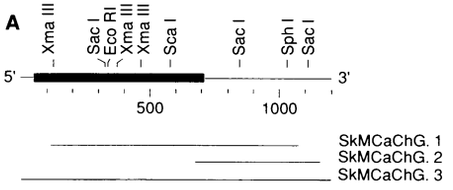


Fig. 3. RNA blot analysis of rabbit RNA with a γ subunit cDNA probe. Polyadenylated RNA (5 μg) from rabbit tissue was electrophoresed in a 1% agarose-formaldehyde denaturing gel (*28*) and transferred to nylon filters (ZetaProbe, Bio-Rad Laboratories, Richmond, California) in 1× SSPE (0.18*M* NaCl, 0.01*M* NaH_2PO_4, 0.05 m*M* EDTA, *p*H 7.0). After baking under vacuum, the filter was washed for 1 hour at 65°C in 0.1× SSPE, 0.1% SDS, and then hybridized with the ^{32}P-labeled SkMCaChG.1 fragment (nt 335 to 1074) for 17 hours at 42°C in 5× SSPE, 5× Denhardt's, 50% deionized formamide, 0.2% SDS, and sonicated herring sperm DNA (200 μg/ml) (*28*). The final washing was at 65°C in 0.2× SSPE, 0.1% SDS, and the filter was exposed to x-ray film for 3 days with one intensifying screen.



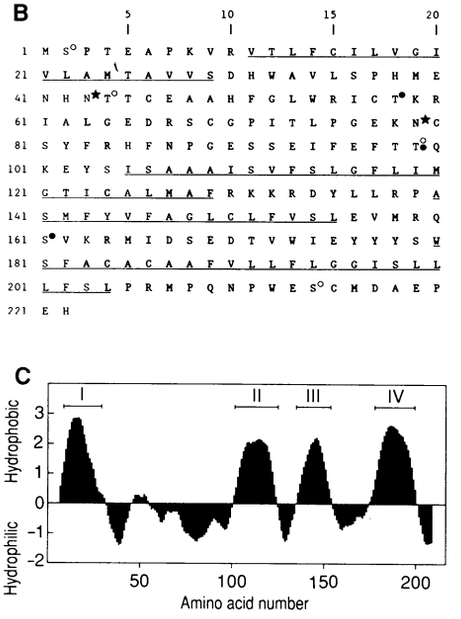


Fig. 2. (**A**) Restriction map and overlapping γ subunit cDNA clones encoding the γ transcript. The protein-coding region is indicated by a solid box. Two clones (λSkMCaChG.1 and λSkMCaChG.2) were isolated from a random primed expression cDNA library constructed in λgt11 from young rabbit back skeletal muscle polyadenylated RNA (*21*), with the affinity-purified antibody as a probe. Additional clones were identified by screening a rabbit back skeletal muscle Okayama-Berg cDNA library (*22*) with a fragment of SkMCaChG.1 (nt 335 to 1074). One of the resulting clones, pSkMCaChG.3, was completely characterized. (**B**) Deduced amino acid sequence of the γ subunit (*23*). The four putative transmembrane segments are underlined. Consensus sites for N-linked glycosylation (star) (*24*), and phosphorylation by protein kinase C (filled circle) (*25*) and casein kinase II (open circle) (*26*) are indicated. (**C**) Hydropathy profile computed according to Kyte and Doolittle (*27*); the window size is 19 residues plotted at 1-residue intervals. The positions of the four predicted transmembrane segments are labeled (I, II, III, and IV).

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Evidence That β-Amyloid Protein in Alzheimer's Disease Is Not Derived by Normal Processing

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The β-amyloid protein (β/A4), derived from a larger amyloid precursor protein (APP), is the principal component of senile plaques in Alzheimer's disease. APP is an integral membrane glycoprotein and is secreted as a carboxyl-terminal truncated molecule. APP cleavage, which is a membrane-associated event, occurred at a site located within the β/A4 region. This suggests that an intact amyloidogenic β/A4 fragment is not generated during normal APP catabolism. Therefore, an early event in amyloid formation may involve altered APP processing that results in the release and subsequent deposition of intact β/A4.

ALZHEIMER'S DISEASE (AD), THE most common form of dementia in the elderly, affects >10% of the population over the age of 65 years. The major neuropathological features of AD are (i) senile plaques, composed of amyloid surrounded by neurites (abnormal neuronal processes including nerve terminals), (ii) amyloid deposits around blood vessels, and (iii) neurofibrillary tangles (NFT), composed of filamentous aggregates in cell bodies of certain populations of neurons (*1, 2*). Plaques are particularly common in certain regions of the brain, including the cerebral cortex, and the severity of these lesions is correlated with the presence of dementia (*3*). The principal component of amyloid in plaques is an ~4-kD peptide, β/A4 (*4*), derived from APP (*5, 6*). Three APP mRNAs derived by alternative splicing of APP pre-mRNA transcripts have been identified that encode integral membrane glycoproteins of 695, 751, and 770 amino acids (*6, 7*). A fourth alternatively spliced transcript has also been reported that encodes a 563–amino acid polypeptide lacking the β/A4 region, APP transmembrane, and cytoplasmic domains (*8*). The 563–amino acid polypeptide, as well as the APP-751 and APP-770 isoforms, contains a domain homologous to the Kunitz serine protease inhibitors (KPI) (*7*). The ~4-kD β/A4 peptide includes between 12 and 14 amino acids of the transmembrane domain and between 24 and 28 amino acids of the adjacent extracellular domain of APP (*4*). Molecular and cellular events responsible for generating the amyloidogenic β/A4 peptide from the proteolysis of APP have not yet been elucidated (*2*).

Studies of cultured cells have documented that APPs have a short intracellular half-life (~20 to 30 min) and are tyrosine-sulfated, N- and O-glycosylated membrane proteins (*9*). APP appears transiently on the cell surface, and proteins lacking the cytoplasmic domain are secreted into the medium. Such COOH-terminal truncated molecules also appear in human cerebrospinal fluid (CSF), suggesting that similar events occur in vivo (*9, 10*). Although the precise sites of APP cleavage in vivo are unknown, knowledge of the normal biology of this cleavage process is crucial for understanding mechanisms that lead to amyloid formation in the brain.

To identify sequences required for the initial cleavage of APP, we transfected genes encoding authentic or hybrid APPs into cultured mammalian cells. Transcription of these genes (which included the SV40 small t antigen's splice and polyadenylation signals at the 3′ end) was controlled by the SV40 promoter/enhancer region (*11*). COS-1 cells, an African green monkey kidney line that constitutively synthesizes SV40 T antigen, were chosen for transfection for two reasons: (i) T antigen expression allows

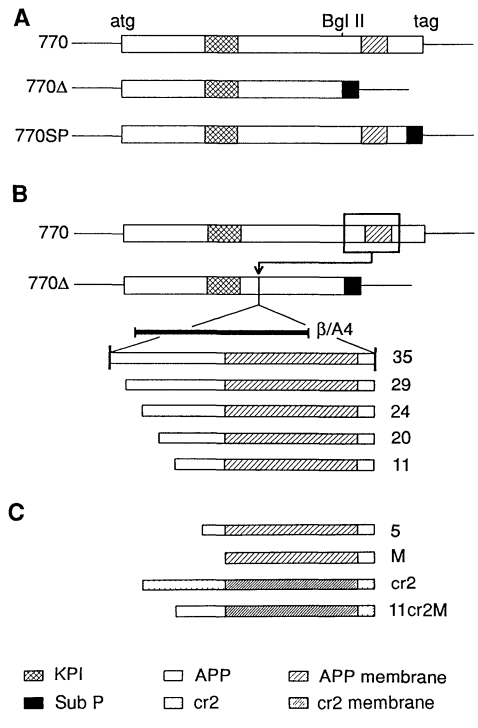

Fig. 1. Cleavage and secretion of APP. (**A**) Construction of authentic, hybrid, and COOH-terminal–deleted APP-770 cDNAs. Plasmids were constructed as described (*16, 17*). Regions labeled atg and tag signify translation initiation and termination sites, respectively. (**B**) Construction of modified APP-770Δ cDNAs. The number at the end of each construct signifies the length (in residues) of the inserted APP extracellular domain. This strategy for construction of plasmids is described (*18, 19*). (**C**) Construction of plasmids p770Δ5, p770ΔM, p770Δcr2, and p770Δ11cr2M. The cr2 regions in p770Δcr2 and p770Δ11cr2M include amino acids 955 to 1005 and 976 to 1005 of the type-2 complement receptor, respectively. The strategy for construction of these plasmids is described (*18, 19*).

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