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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 add hybridization studies indicate that there is a 1200-nudeoride transcript in skeletal muscle but not in brain or heart. The γ subunit may play a role in assemblv. 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, phenylalkyiamines, 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-tcrminal sequence docs 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

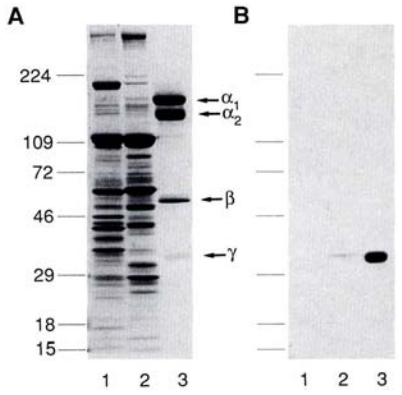

Fig. 1. Rabbit skeletal muscle DHP receptor subunit composition and γ subunit antibody probe specificity. (**A**) Coomassie blue-stained, reduced SDS-acrylamide gel showing crude (100 μg of microsomes) (lane 1), enriched (100 μg of triads) (lane 2), and purified (20 μg of DHP receptor) (lane 3) preparations of DHP-sensirive Ca^{2+} channel (*20*). (**B**) Immunoblot of identical samples as in (A), stained with GP16 polyclonal anriserum 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 x 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 ammo acid sequence of the γ subunit with sequences in the Swiss-Protein and GenBank nucleotide databases revealed no significant homologies (*15*). The highest initial [ink (n)] score was obtained with the Na^+ channel protein from the eel *Electroplax* (*16*) [ink (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.

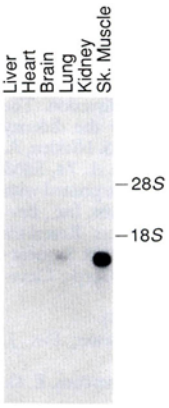

Fig. 3. RNA blot analysis of rabbit RNA with a γ subunit cDNA probe. Polyadenylated RNA (5 μg) from rabbit tissue was electrophoresed in a 1% agarose-formaldehyde denaturing gel (28) and transferred to nylon filters (ZetaProbe, Bio-Rad Laboratories, Richmond, California) in 1 × SSPE (0.18*M* NaCl, 0.01*M* 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.

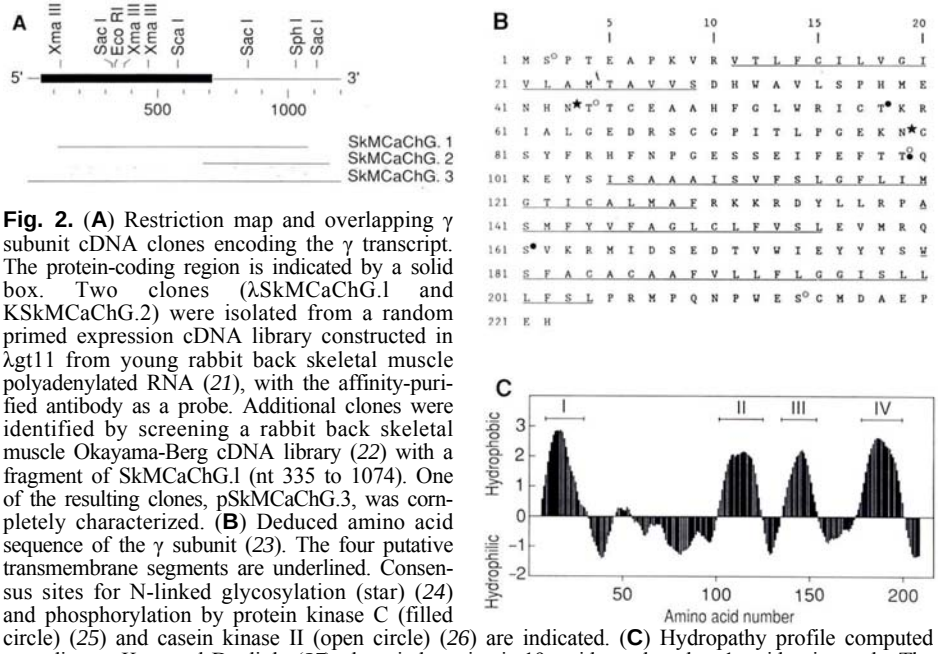

Fig. 2. (**A**) Restriction map and overlapping γ subunit cDNA clones encoding the γ transcript. The protein-coding region is indicated by a solid box. Two clones (λSkMCaChG.1 and KSkMCaChG.2) were isolated from a random primed expression cDNA library constructed in λgt11 from young rabbit back skeletal 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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