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Structural Characterization of the Dihydropyridine-sensitive Calcium Channel α_2-Subunit and the Associated δ Peptides*

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Upon disulfide bond reduction, the α_2-subunit of the dihydropyridine-sensitive Ca^{2+} channel undergoes a characteristic mobility shift on sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis with the concurrent appearance of the three δ peptides δ_1 (25,000 Da), δ_2 (22,000 Da), and δ_3 (17,000 Da). Densitometric scanning of Coomassie Blue-stained gels shows a stoichiometric ratio of 1.0:0.31:0.47:0.08 for the α_2-subunit and the δ peptides 1, 2, and 3, respectively. Characterization of the δ peptides using antibodies, photoincorporation of a hydrophobic probe, and lectin staining shows them to be antigenically similar hydrophobic glycoproteins. Amino-terminal sequence analysis of the δ peptides reveals three identical sequences that match the predicted amino acid sequence of the α_2-subunit starting at Ala^{935}. Enzymatic deglycosylation of the reduced α_2·δ complex produces individual core peptides of 105,000 and 17,000 Da, respectively. Treatment of skeletal muscle membranes with high pH in the presence of reducing agents is able to extract the larger amino-terminal peptide but not the smaller carboxyl (δ) peptide, consistent with a single transmembrane domain in the carboxyl (δ) region. The data support a model of the α_2-subunit in which the propeptide is processed into two chains that remain attached through disulfide linkages.

Voltage-dependent Ca^{2+} channels in excitable membranes are essential for many cellular functions, including muscle contraction and secretory processes (1, 2). Three types of voltage-dependent calcium channels have been distinguished by their electrophysiological and pharmacological properties (3, 4). A variety of organic compounds, including 1,4-dihydropyridine derivatives, are known to modulate ion flux through slow L-type calcium channels (5, 6). Dihydropyridine-sensitive Ca^{2+} channels are highly enriched in the transverse tubule system in skeletal muscle (7, 8), in which they have been suggested to act as a voltage sensor in the process of excitation-contraction coupling (9). The dihydropyridine-sensitive Ca^{2+} channel receptor from rabbit skeletal muscle consists of four subunits: α_1 (170,000 Da), α_2 (175,000 Da nonreduced; 150,000 Da reduced), β (52,000 Da), and γ (32,000 Da) (10-12). All four subunits have been cloned recently through cDNA isolation (13-16). Although many functional roles have been assigned to the α_1-subunit, including channel formation, sensing of the transmembrane voltage, and pharmacological agent binding, complete receptor function is thought to result only from a heterotetrameric complex involving each of the subunits. The increased channel activity measurements following coexpression of cardiac α_1 and skeletal muscle α_2 cDNA clones in a heterologous expression system (17) support a functional role for the α_2-subunit.

The two characteristic biochemical properties of the α_2-subunit are its high level of glycosylation and its apparent mobility (size) difference on SDS[1]-PAGE in the absence or presence of disulfide bond reducing agents (10-12). The first of these characteristics is what presumably allows the purification of the dihydropyridine-sensitive Ca^{2+} channel receptor complex using WGA-Sepharose. The second characteristic, involving a mobility shift on gel analysis coupled with increasingly higher yields of purified receptor resulting in better detection of low molecular weight components, has allowed the discovery of the α_2-subunit-associated δ peptides (18-21). The disulfide-dependent association of the α_2·δ complex is supported immunologically. Polyclonal antibodies (GP13) produced against the nonreduced 175,000-Da form react with both the 150,000-Da form of the α_2-subunit and the δ peptides on immunoblots prepared from reduced gels, and purified antibodies against the δ peptides react with the 175,000-Da form on nonreduced gels (21).

In this study we sought to characterize the observed δ peptides and determine whether they were primary subunits of the dihydropyridine-sensitive Ca^{2+} channel receptor. Our results show that the δ peptides are not true subunits because they do not represent unique translation products but instead represent the carboxyl-terminal peptide of a proteolytically processed α_2-subunit. In addition, the two-chain complex appears to remain anchored in the membrane through the δ segment, indicating a revised model of the α_2-subunit.

EXPERIMENTAL PROCEDURES

Preparation of KCl-washed Microsomal Membrane Vesicles—Heavy microsomes were prepared by a modification of the method of Mitchell *et al.* (22). Rabbit back and hind limb muscles were dissected

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; PMSF, phenylmethylsulfonyl fluoride; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; PVDF, polyvinylidene difluoride; HPLC, high pressure liquid chromatography.

and homogenized in 7.5 volumes of buffer A (20 mM sodium pyrophosphate, 20 mM sodium phosphate monohydrate, 1 mM magnesium chloride, 0.303 M sucrose, 0.5 mM EDTA, pH 7.1). All membranes were prepared in the presence of protease inhibitor mix A: aprotinin (0.5 μg/ml), benzamidine (100 μg/ml), iodoacetamide (185 μg/ml), leupeptin (0.5 μg/ml), pepstatin A (0.5 μg/ml), and PMSF (40 μg/ml). The homogenate was centrifuged for 15 min at 9,000 rpm in a Beckman JA-10 rotor (14,000 × g). The supernatants were filtered through six layers of cheesecloth and recentrifuged for 30 min at 14,000 rpm in a Beckman JA-14 rotor (30,100 × g). The membrane pellets were then resuspended with buffer 1: (0.303 M sucrose, 20 mM Tris maleate, pH 7.0, 100 μg/ml benzamidine, 185 μg/ml iodoacetamide, and 40 μg/ml PMSF) and washed with KCl wash buffer (0.6 M KCl, 50 mM Tris-HCl, pH 7.4, 0.303 M sucrose, 0.75 mM benzamidine, and 0.1 mM PMSF). The membranes were centrifuged for 30 min at 35,000 rpm in a Beckman Ti-45 rotor (142,413 × g), washed a second time, and finally resuspended in buffer 1 and frozen in liquid nitrogen prior to storage at −135 °C.

Purification of the Dihydropyridine Receptor—The microsomal membranes were solubilized using 1% digitonin in a buffer containing 50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.5 M sucrose, and protease inhibitor mix A. Receptor purification involved sequential chromatography steps using WGA-Sepharose and DEAE-cellulose. The solubilized proteins were first passed through a XA7-Sepharose monoclonal antibody affinity column for removal and purification of the skeketal muscle ryanodine receptor as described previously (23). The solubilized dihydropyridine receptor was then bound to the WGA-Sepharose column followed by washes with solubilization buffer, buffer D (50 mM Tris-HCl, pH 7.4, 0.5 M sucrose, 0.1% digitonin, 0.75 mM benzamidine, 0.1 mM PMSF) containing 0.5 M NaCl, buffer D with no sodium chloride, and finally eluted with buffer D containing 300 mM *N*-acetylglucosamine. The WGA eluate was loaded onto a DEAE-cellulose column that was equilibrated with buffer D. The DEAE-cellulose column was eluted with a series of increasing salt concentrations (0, 25, 50, 75, and 100 mM NaCl) in buffer D. The 75 mM NaCl fraction was highly enriched for the dihydropyridine receptor and was used for further experimental studies. Protein was determined by the method of Lowry *et al.* (24) as modified by Peterson (25) using bovine serum albumin as a standard. Detergent-solubilized protein samples were quantitated by the same method after addition of 1% deoxycholate in 0.1 M NaOH and precipitation with 5% trichloroacetic acid.

SDS-Gel Filtration and SDS-PAGE—Purified receptor samples were denatured with 1% SDS and injected onto a Beckman HPLC gel filtration system containing two TSK3000SW columns and one TSK4000SW column (each column was 7.6 × 300 mm). Proteins were separated with an isocratic buffer containing 50 mM sodium phosphate, pH 6.0, 100 mM NaCl, 0.1% SDS at a flow rate of 0.5 ml/min at room temperature. Absorbance of the effluent was monitored at 280 nm, and fractions were collected at 30-s intervals. Fractions were pooled and analyzed by SDS-PAGE, either directly or after concentration using Centricon 10 microconcentrators (Amicon). SDS-PAGE was performed using the discontinuous buffer system of Laemmli (26) with a stacking gel of 3% acrylamide and a separating gel linear gradient of 5–16% acrylamide. Samples were reduced prior to electrophoresis by 1% 2-mercaptoethanol. Molecular weight standards were run in parallel to samples on all gels with prestained standards used on gels for transfer. Gels were either stained with Coomassie Blue or transferred (27) to Immobilon PVDF membranes. The Coomassie Blue-stained gels were scanned with a Hoefer model GS-300 scanning densitometer. The density data were analyzed using the GS-350H data system software from Hoefer.

Affinity-purified Antibodies—δ_2-Subunit specific antibodies were affinity purified from guinea pigs that had been immunized with the SDS-PAGE-purified nonreduced form of the α_2-subunit (175,000 Da) of the rabbit skeletal muscle dihydropyridine receptor (GP13). The affinity purification was performed as reported by Sharp and Campbell (21). Briefly, 200 μg of purified dihydropyridine receptor was separated by preparative SDS-PAGE and electrophoretically transferred to Immobilon PVDF membranes. A vertical strip was cut from the membranes and stained with polyclonal serum to identify the bands corresponding to the α_2- and δ-subunits of the dihydropyridine receptor. The immobilized δ_2 peptide was cut from the membrane as an individual horizontal strip, blocked in TBS-BLOTTO (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5% nonfat dry milk), incubated overnight with the polyclonal serum, washed, and the bound antibody eluted with acid (50 mM glycine-HCl, pH 2.5). The eluted affinity-purified antibody was then neutralized to pH 7.4 by adding 1 M Tris-HCl, pH 8.0, and was stored at 4 °C.

Immunoblotting and WGA Staining—Immunoblots were blocked with TBS-BLOTTO and then incubated overnight at 4 °C with guinea pig antibodies in the same solution. The membranes were washed three times for 5 min each in TBS-BLOTTO and incubated with horseradish peroxidase-linked rabbit anti-guinea pig antibody for 1 h at room temperature. The membranes were washed three times again and developed using 4-chloro-1-naphthol as substrate. The procedure was modified for the staining of the membranes with peroxidase-conjugated WGA by using TBS, 0.05% Tween, as the blocking, incubation, and washing medium.

Radiolabeling of Hydrophobic Segments—Purified dihydropyridine receptor in buffer D with the sodium chloride concentration adjusted to 150 mM was incubated for 30 min on ice with 25 μCi/ml [^{125}I]TID. The mixture was irradiated for 30 min on ice using a Spectroline model ENF-280C handheld ultraviolet lamp operating at 365 nm at a distance of 7 cm from the sample. Incorporation was detected by autoradiography after combined denaturing gel filtration and SDS-PAGE separation.

Amino-terminal Sequence Analysis—Amino-terminal sequence data were obtained from purified dihydropyridine receptor that was separated by both SDS-gel filtration and SDS-PAGE and electroblotted to Immobilon PVDF membranes according to the method of Matsudaira (28). The membranes were stained with Coomassie Blue to visualize the protein bands that were excised with a clean razor. Edman degradation sequence analysis was performed using an Applied Biosystems model 470 sequenator equipped with on-line phenylthiohydantoin derivative analysis. The phenylthiohydantoin derivatives were separated by reverse-phase HPLC over a Brownlee C-18 column (220 × 2.1 mm). Initial yields ranged from 30 to 60%, with repetitive yields of approximately 94%. The reported amino-terminal sequence data were confirmed by analysis of separate receptor samples, prepared in a similar manner.

Enzymatic Deglycosylation of the Receptor—Samples (2–20 μg/30 μl) prepared for endoglycosidase treatment by SDS denaturation were boiled for 3 min in the presence of 0.1% SDS for endoglycosidase H or 1% SDS for glycopeptidase F (*N*-glycosidase F). The samples were diluted 5-fold with concentrated buffer, enzyme, and water to final concentrations of 50 mM sodium citrate, pH 5.5, 0.1 mM 2-mercaptoethanol, 0.02% SDS, 0.0015 units of enzyme for endoglycosidase H, and 50 mM sodium phosphate, pH 7.2, 20 mM EDTA, 2% *n*-octyl glucoside, 0.2% SDS, 1% 2-mercaptoethanol, 3 units of enzyme for glycopeptidase F. Both buffers contained the protease inhibitors benzamidine (0.75 mM) and PMSF (0.1 mM). The reactions were incubated at 37 °C for periods from 30 min to 12 h.

Alkaline Extraction of Membranes—Microsomal membranes were diluted to a concentration of 5 mg/ml with buffer containing 50 mM Tris, 0.1 M sucrose, 5 mM *N*-ethylmaleimide, 0.75 mM benzamidine, and 0.1 mM PMSF. The pH was adjusted to 11 with 10 N NaOH. The mixture was divided equally into two samples, and dithiothreitol (100 mM final) was added to one. The samples were incubated at room temperature for 1 h with gentle end-over-end mixing and centrifuged for 30 min at 55,000 rpm (107,000 × g) in a Beckman TLA-100.3 rotor. The supernatants were removed and adjusted to pH 7.4 with 1 N HCl. The pellets were resuspended in buffer 1, and all samples were centrifuged again for 30 min at 55,000 rpm (107,000 × g) in a Beckman TLA-100.3 rotor. The high pH extract and washed pellet were then analyzed by SDS-PAGE.

Materials—[^{125}I]TID was obtained from Amersham Corp. Peroxidase-conjugated secondary antibodies were from Organon Teknika-Cappel, Malvern, PA. Peroxidase-conjugated wheat germ agglutinin, wheat germ agglutinin-Sepharose, digitonin (prepared as described previously (23)), SDS molecular weight standards, bovine serum albumin, and protease inhibitors were from Sigma. Prestained molecular weight standards were from Bethesda Research Laboratories. Endoglycosidase H, glycopeptidase F, and electrophoretic reagents were from Boehringer Mannheim. DEAE-Cellulose (DE52) was from Whatman. Immobilon PVDF membranes were from Millipore. All other chemicals were of reagent grade quality.

RESULTS

The disulfide-linked association of the α_2-subunit and the δ peptides can be demonstrated biochemically using two size-dependent fractionation steps, in the absence and then presence of disulfide bond reducing agents. In this paper we have used gel filtration under denaturing conditions (0.1% SDS)

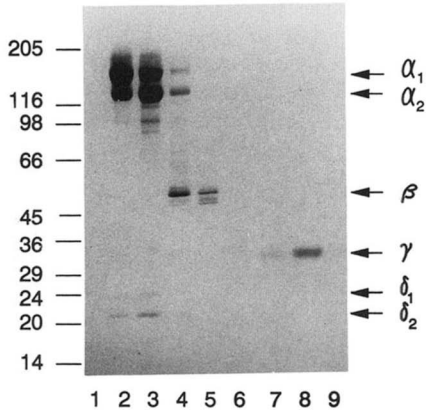

FIG. 1. **Two-dimensional size separation of the dihydropyridine-sensitive Ca^{2+} channel.** Purified receptor (40 μg of the DEAE-cellulose, 75 mM NaCl eluate) was separated by SDS-gel filtration. Sequential pooled fractions, corresponding to *lanes 1–9,* were reduced (1% 2-mercaptoethanol), analyzed by SDS-PAGE, and stained with Coomassie Blue as described under "Experimental Procedures." Individual subunits were clearly resolved with α_1 (170 kDa) peaking in *lane 2,* α_2 (150 kDa), δ_1 (25 kDa), and δ_2 (22 kDa) in *lane 3,* β (52 kDa) in *lane 4,* and γ (32 kDa) in *lane 8.* Molecular weight standards are indicated on the left ($M_r \times 10^{-3}$).

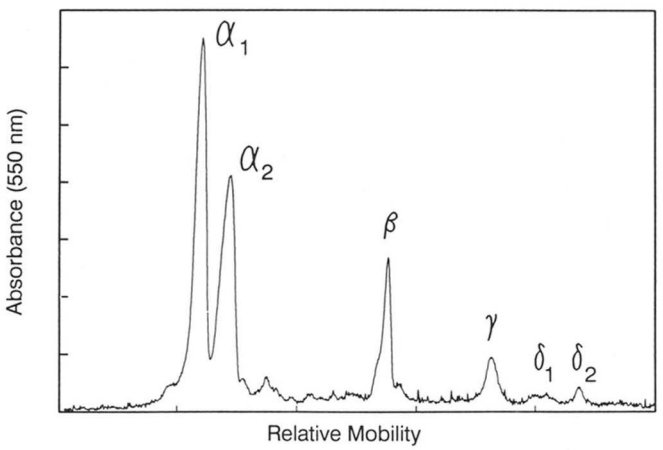

FIG. 2. **Densitometric scan of Coomassie Blue-stained gel containing purified dihydropyridine-sensitive Ca^{2+} channel receptor.** Purified receptor (40 μg) was separated on a single lane of a reducing SDS-PAGE gel. The stained proteins were then scanned, and a plot of the digitized absorbance values is shown. The *top* and *bottom* of the gel are presented from *left* to *right,* respectively, on the *x* axis.

TABLE I

Apparent subunit sizes and their predicted stoichiometries

The area under the curve in Fig. 2 corresponding to each subunit band was integrated manually by addition of the digitized absorbance values and rounded to three significant places. To facilitate comparison, the absorbance areas were divided by the subunit's apparent molecular weight and then normalized relative to the α_1-subunit.

Subunit	Apparent molecular mass	Area	Normalized relative area
	kDa		
α_1	170	15,400	1.00
α_2	150	11,900	0.87
β	52	4,950	1.05
γ	32	2,620	0.90
δ_1	25	604	0.27
δ_2	22	815	0.41

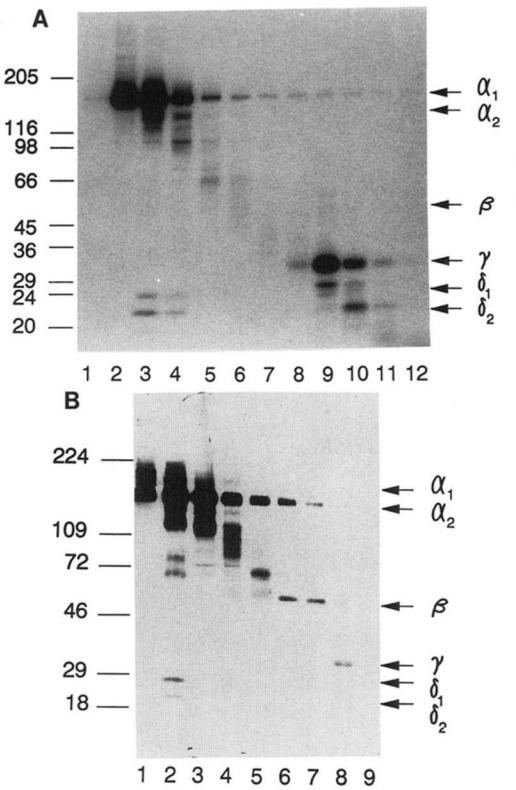

FIG. 3. **Biochemical characterization of the δ peptides.** *A,* autoradiogram of [^{125}I]TID-labeled purified receptor. After photolabeling, the sample was separated two-dimensionally as described under "Experimental Procedures." In an effort to show better resolution of the α_1- and α_2-subunits, gel filtration fractions were pooled into smaller aliquots, and 12 instead of 9 pooled samples were analyzed by SDS-PAGE. The label is observed to incorporate into the δ peptides, which are enriched in *lane 3. B,* WGA peroxidase staining of the separated dihydropyridine-sensitive Ca^{2+} channel receptor subunits. Protein samples similar to Fig. 1 were transferred to PVDF membranes prior to analysis with the conjugated lectin. Increased staining of δ_1 over the δ_2 peptide is observed in *lane 2.* The positions of the subunits, determined by antibody counterstaining of the blot, are indicated on the *right.* Molecular weight markers are indicated on the *left* ($M_r \times 10^{-3}$).

for the first separation, followed by SDS-PAGE in the presence of reducing agents, for separation in the second dimension. When the purified dihydropyridine receptor is subjected to the above two-dimensional analysis (Fig. 1), the α_2 and δ proteins comigrate as a complex during gel filtration (corresponding to *lanes 2* and *3*), with a retention time similar to the α_1-subunit. With SDS-PAGE separation under reducing conditions, the complex is observed to separate into two or more components, the 150,000-Da form of the α_2-subunit and the smaller (< 30,000 Da) δ peptides. Under such conditions the δ peptides are well resolved from the other four subunits as well as from any potentially copurifying proteins of similar molecular weight. Two-dimensional separation was therefore employed to identify unambiguously the δ peptides in all biochemical characterizations.

On similar analysis of purified dihydropyridine receptor, but with the second-dimension polyacrylamide gels run under nonreducing conditions, the $\alpha_2 \cdot \delta$ complex migrates as a 175,000-Da band, consistent with previous results (10, 18, 21). It is interesting to note that the $\alpha_2 \cdot \delta$ complex migrates slower than the α_1-subunit on SDS-PAGE analysis, consistent with larger apparent molecular weight but has a longer retention time on SDS-gel filtration, consistent with a smaller size.

Initial characterization of the complex focused on the stoi-

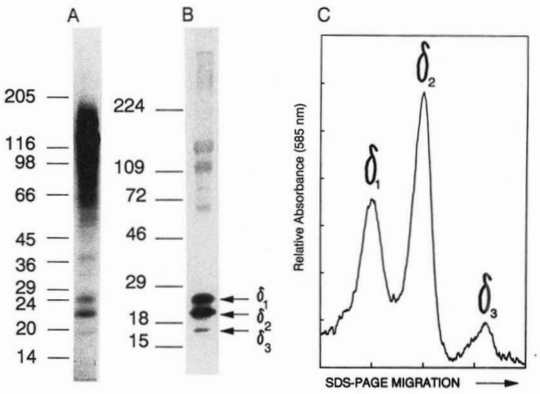

FIG. 4. **Demonstration of a third δ peptide.** *A*, Coomassie Blue staining of an overloaded gel lane. Gel filtration fractions corresponding to *lane 3* in Fig. 1 were pooled and concentrated 5-fold prior to gel electrophoresis. *B*, immunoblot of the same fractions (not concentrated) stained with polyclonal antiserum (GP13) that was affinity purified using the δ_2 peptide as described under "Experimental Procedures." The detected immunoreactivity of the α_2-subunit is an artifact caused by residual contaminating antibodies coupled with prolonged development. *C*, densitometric scan of low molecular weight range of gel in *panel A*.

chiometry between δ peptides and the α_2-subunit, to determine whether more than one δ peptide was disulfide linked to each α_2-subunit. Coomassie Blue-stained gels were scanned densitometrically (Fig. 2), and the absorbance values were used to calculate subunit stoichiometry (Table I). The predicted 1.00:0.87:1.05:0.90 stoichiometry for the α_1-, α_2-, β-, and γ-subunits is in close agreement with previously published data supporting a 1:1:1:1 stoichiometry (10–12). The calculations also predict that for each mol of the reduced 150,000-Da α_2-subunit there exists 0.31 mol of δ_1 and 0.47 mol of δ_2, consistent with a single δ peptide in each α_2 complex.

To detect the presence of hydrophobic stretches of amino acids, the hydrophobic probe [^{125}I]TID was photoincorporated into purified dihydropyridine-sensitive Ca^{2+} channel receptor (Fig. 3*A*). The δ peptides were found to be labeled by the probe, as were the α_1-, α_2-, and γ-subunits. The incorporation of this hydrophobic probe correlates well with the presence of cDNA-predicted hydrophobic regions including transmembrane segments (13–16). The need for two-dimensional separation for unambiguous detection of the δ peptides is highlighted by the labeled fragments observed in *lanes 9* and *10* which would comigrate with the δ-peptides on one-dimensional reducing SDS-PAGE.

To detect the presence of attached oligosaccharides, the δ peptides were stained with the peroxidase-conjugated lectins, concanavalin A and wheat germ agglutinin (WGA). Although concanavalin A bound only to the α_2-subunit (data not shown), WGA binding was detected for the α_2- and γ-subunits as well as the δ peptides (Fig. 3*B*). The subunit identity of the detected bands was confirmed by antibody counterstaining. The multiple bands observed in the 50,000–150,000-Da range were identified as α_2-subunit fragments, and clearly none represented the β-subunit (which peaked in *lanes 4* and *5*, as observed in Fig. 1). Consistent with the carbohydrate specificity of the lectins, the α_2-subunit is predicted to contain both high mannose and complex/hybrid oligosaccharides whereas the γ and δ proteins are predicted to contain only complex/hybrid oligosaccharides.

The δ_1 and δ_2 peptides have been shown previously to be antigenically cross-reactive (21). A smaller δ_3 peptide (17,000 Da) can also be detected by immunoblot analysis with prolonged development. The presence of δ_3 can also be detected by Coomassie Blue staining of highly loaded gels (Fig. 4). The calculated densitometric ratio of the three δ peptides is 4:6:1 (δ_1 to δ_2 to δ_3).

TABLE II

A comparison of the measured δ peptide amino-terminal sequence versus the predicted sequence of the α_2-subunit

The measured amino-terminal sequences for the δ peptides are compared with internal sequence predicted from the α_2 cDNA. Initial sequencing reaction yields from samples prepared as described under "Experimental Procedures" were: 10 and 50 pmol, for δ_1; 15, 22, and 72 pmol, for δ_2; and 7.5 and 12 pmol for δ_3. Values shown for the three δ peptides are from the same preparation.

δ_1		δ_2		δ_3		α_2 cDNA amino acid
Amino acid	Yield	Amino acid	Yield	Amino acid	Yield	
	pmol		*pmol*		*pmol*	
A	50	A	72	A	12	A^{935}
D	16	D	48	D	6	D
M	47	M	70	M	8	M
E	15	E	32			E
D	12	D	47			D
D	20	D	62			D
D	44	D	75			D
F	25	F	52			F
T	18	T	21			T
A	30	A	40			A
S	4					S
M	17					M
S	5					S

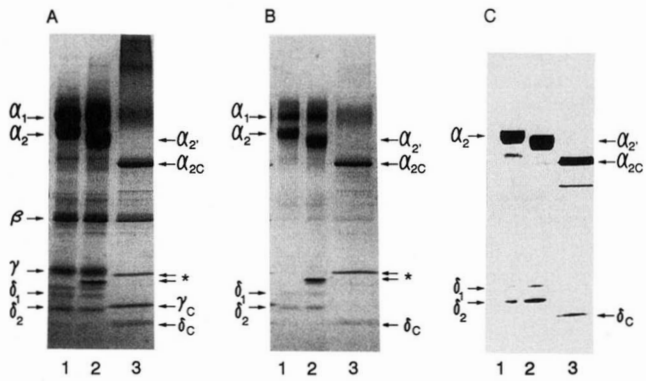

FIG. 5. **Endoglycosidase treatment of the dihydropyridine-sensitive Ca^{2+} channel receptor.** *Lane 1*, control no treatment; *lane 2*, endoglycosidase H treatment; *lane 3*, glycopeptidase F (*N*-glycosidase F) treatment. *A* and *B*, Coomassie Blue-stained gels showing endoglycosidase treatment of purified receptor (*panel A*) and α_2·δ gel filtration peak fractions (*panel B*). *C*, immunoblot of gel identical to gel in *panel A*, stained with polyclonal antiserum GP13. The detected subunits are shown on the *left* of each *panel*. On the *right* of each *panel* the altered mobilities noted for subunits sensitive to the treatment are labeled: $\alpha_{2'}$, for the partial deglycosylation observed with the α_2-subunit in *lane 2*; and α_{2c}, γ_c, and δ_c, for the core peptides observed in *lane 3*. The locations of the detected protein bands representing the added enzymes are labeled with *.

To assess directly the relationship of the three δ peptides, amino-terminal sequence analysis was performed (Table II). Not only did the sequence analysis reveal identical peptide sequences, but the measured sequence matched the predicted α_2-subunit starting at Ala^{935}. These data provide direct evidence that the δ peptides represent carboxyl-terminal proteolytic products of the α_2-subunit which remain attached to the larger (150,000-Da) amino-terminal peptide through disulfide linkages.

The relative intensity of [^{125}I]TID incorporation (Fig. 3*A*) into the two prominent δ peptides paralleled the observed Coomassie Blue staining intensity (Fig. 1) with $\delta_2 > \delta_1$. The inverse intensity staining pattern observed with WGA per-

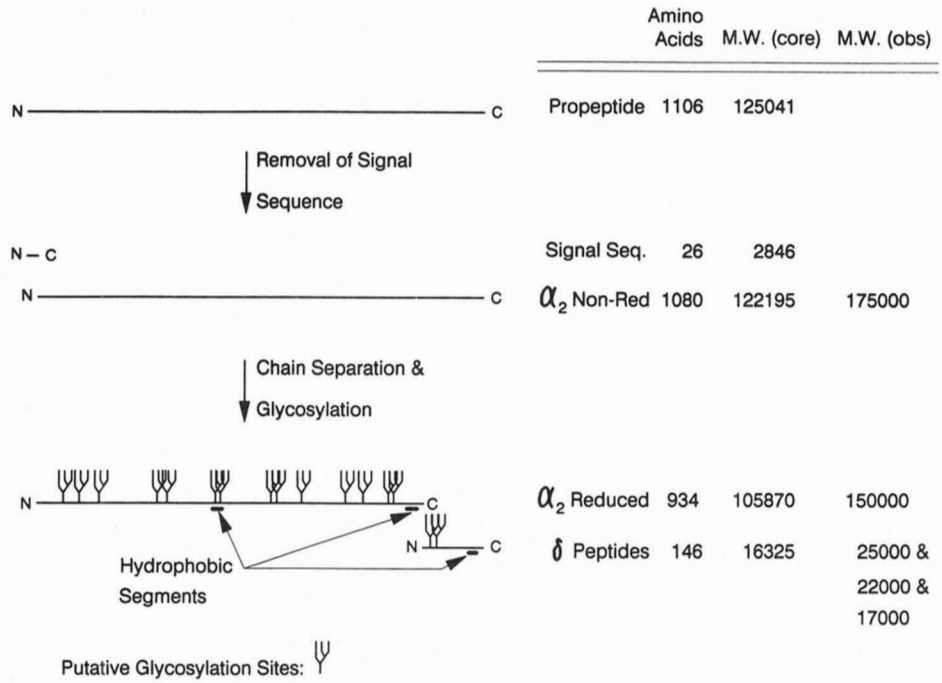

FIG. 6. **Schematic representation of the α_2-subunit transcript and its processing to the observed protein complex.** The predicted sizes of the post-translation products are shown on the *right*. The calculation of the amino acid length and the associated core molecular weight is based upon the cDNA predicted sequence and the determined amino-terminal sequences of both fragments. Two of the possible 18 glycosylation sites are not labeled as per changes described under "Discussion."

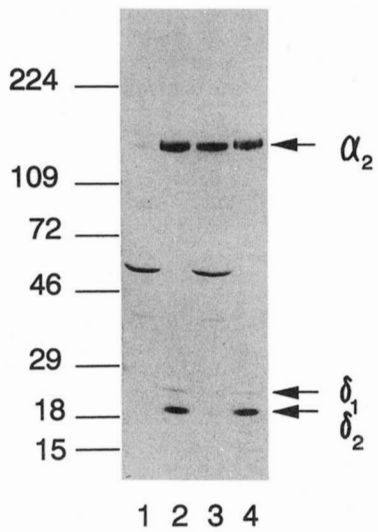

FIG. 7. **Alkaline extraction of skeletal muscle microsomal membranes.** Extraction with pH 11 was performed in the presence of 5 mM *N*-ethylmaleimide (*lanes 1* and *2*, nonreducing conditions) or 5 mM *N*-ethylmaleimide, 100 mM dithiothreitol (*lanes 3* and *4*, reducing conditions). Membranes after pH treatment were separated by centrifugation, and the soluble supernatants (*lanes 1* and *3*) and insoluble pellets (*lanes 2* and *4*) were analyzed by SDS-PAGE. The gel was transferred to a PVDF membrane and stained with polyclonal antiserum GP13. In addition to the detection of α_2, δ_1, and δ_2, a 50-kDa cross-reactive band was detected in the soluble fraction. This band was not labeled by antiserum affinity purified using α_2 or δ proteins but served as a useful marker of the soluble fraction. Examination of identical gels stained with Coomassie Blue showed fractionation of markers such as calsequestrin to the extracted supernatant (*lanes 1* and *3*) and the sarcoplasmic Ca^{2+} ATPase to the treated membrane pellets (*lanes 2* and *4*). In control membranes treated identically, but at neutral pH, α_2, δ_1, and δ_2 were immunologically detectable only in the insoluble pellet fraction (data not shown).

oxidase staining $\delta_1 > \delta_2$ suggested that the size differences observed for the δ peptides may be because of varying levels of glycosylation. To test this hypothesis enzymatic deglycosylation was performed on both the purified receptor and the peak $\alpha_2 \cdot \delta$ SDS-gel filtration fractions (Fig. 5). Endoglycosidase H treatment, which cleaves *N*-linked high mannose oligosaccharides, produced a mobility change in only the α_2-subunit (*lane 2*). Treatment with glycopeptidase F, which cleaves *N*-linked high mannose, hybrid and complex oligosaccharides, produced mobility changes in the α_2- and γ-subunits as well as the δ peptides (*lane 3*). To confirm the identity of the detected bands, identical samples were probed with antibodies.

The deglycosylated core peptides had apparent sizes of α_{2c} = 105,000 Da, γ_c = 23,000 Da, and δ_c = 17,000 Da. The agreement between the observed size of the deglycosylated δ core peptide and the size predicted from the α_2 cDNA from Ala^{935} onward of 16,325 Da (Fig. 6) supports the common core peptide model. The deglycosylation was observed to be complete in 30 min, with no further mobility changes noted with either enzyme with incubation periods as long as 12 h. After deglycosylation with endoglycosidase H and glycopeptidase F, lectin staining analyses were negative with concanavalin A and WGA, respectively (data not shown). The observed mobility changes appeared to be specific for deglycosylation and not proteolysis, as the α_1- and β-subunit apparent sizes remained unchanged, and the approximate γ core peptide size is in close agreement to the cDNA predicted size of 25,058 Da (16).

It has been reported previously that the predicted α_2 amino acid sequence contains 18 potential *N*-glycosylation sites and three putative transmembrane segments (14), with three *N*-glycosylation sites and one transmembrane region existing in the carboxyl (δ) segment (Fig. 6). In the proposed three-transmembrane region model of the α_2-subunit, only 5 of the 15 consensus glycosylation sites within the larger amino-terminal peptide are predicted to be extracellular. The large mobility shift observed with deglycosylation from 150,000 to 105,000 Da is more consistent with a higher number of glycosylation sites, predicted to be possible only with the entire 150,000-Da peptide being extracellular.

Previous work with erythrocyte membranes has shown that if membranes are extracted with strong alkaline solutions, the membrane cytoskeleton and other peripheral membrane pro-

teins are solubilized, leaving the integral membrane proteins with the bilayer (29). To test the hypothesis that the 150,000-Da peptide is a peripheral membrane protein, skeletal muscle microsomal membranes were extracted with alkaline pH (Fig. 7). In the absence of reducing agents the entire complex pelleted with the extracted membranes, but under conditions of disulfide bond reduction, the larger α_2 fragment but not the δ peptides was solubilized. Although the extraction of the 150,000-Da peptide was not complete, possibly because of intravesicular trapping, the data suggest that the larger amino-terminal segment remains associated with the membrane by disulfide linkages to the smaller δ segment, which is an integral membrane protein possessing a single transmembrane domain.

DISCUSSION

Historically, the dihydropyridine-sensitive Ca^{2+} channel receptor was observed to contain a large (140,000–175,000-Da) subunit termed the α-subunit. Subsequently it was shown that the protein whose mobility on SDS-PAGE shifted with reduction was distinct from the protein that was labeled by photoreactive calcium channel antagonists and whose mobility was insensitive to reducing agents. This distinction gave rise to the nomenclature α_2 and α_1 for the respective subunits (for reviews, see Refs. 10–12). We have provided evidence that the α_2-subunit is composed of two disulfide-linked chains referred to previously as the reduced form of the α_2- and the δ-subunits. To emphasize their relationship to the α_2 complex we propose the term δ peptides and not the δ-subunits.

Contrary to previous experimental conclusions (20), the size heterogeneity of the δ peptides does not appear to result from additional proteolytic processing but is rather the result of variable *N*-linked glycosylation. Modifications in the consensus sites for *N*-glycosylation, eliminating sites containing proline in either the (+1) or (−1) position (30), allow us to reject two of the previously proposed sites within the α_2 cDNA predicted sequence (Asn^{671} and Asn^{1055}), thus leaving just two predicted *N*-glycosylation sites in the carboxyl (δ) segment. Consistent with the above data we propose that the δ peptides represent a heterogenously glycosylated group of identical core peptides (δ_c) with hybrid or complex oligosaccharides, *N*-linked at two, one, or zero sites for δ_1, δ_2, and δ_3, respectively.

Interpretation of the experimental results involving the 150,000-Da α_2 segment and its transmembrane characteristics remains controversial. Although the ^{125}I-TID incorporation into the 150,000-Da peptide (Fig. 3*A*) is consistent with hydrophobic segments within the protein, this method is not specific for transmembrane regions. Under the same experimental conditions significant incorporation is noted into calsequestrin (data not shown), a protein determined to not contain transmembrane regions but to contain a hydrophobic segment. We feel that the results involving alkaline extraction and the pattern and extent of *N*-linked glycosylation are most consistent with the entire peptide segment positioned on the extracellular face of the lipid bilayer. The possibility of covalent attachment of this segment to a hydrophobic anchor has not been disproved experimentally, but the linkage would have to be labile in alkaline thiol solutions.

An important question that still remains concerning the processed model of the α_2-subunit is whether the cleavage is physiological or an artifact of membrane isolation. The possibility of this cleavage representing an isolation artifact appears unlikely. Extreme care is taken at all stages of receptor purification to avoid proteolysis; and in all probed samples to date, even from freshly prepared membranes, the α_2-subunit has been noted to shift with reduction, and the δ peptides have been immunologically detectable. It is curious to note that no consensus proteolytic site containing 2 or more adjacent positively charged residues occurs within 40 residues in the predicted α_2 sequence, near the point of observed cleavage. The site does, however, follow the (−3,−1) rule used to identify the site of cleavage between the signal peptide and the mature protein for secretory proteins (31).

In this report we have shown that the δ peptides are indeed components of the dihydropyridine-sensitive Ca^{2+} channel receptor, although not unique subunits. The evidence supports a two-chain model of the α_2-subunit in which the larger, highly glycosylated amino-terminal peptide remains covalently associated by disulfide linkage to the carboxyl (δ)-terminal peptide. Although we have not yet identified the specific amino acids involved in the disulfide linkage, 11 cysteine residues exist in the amino-terminal peptide, and 9 exist in the carboxyl (δ)-terminal peptide, clearly providing candidate sites for disulfide linkages. Additionally, our data support a new model of the α_2-subunit in which the carboxyl (δ) fragment is the integral membrane protein to which the larger amino fragment is covalently attached. This model is in contrast to previous models in which the reduced α_2-subunit was thought to be an integral membrane protein and the δ peptides to be peripheral (18).

The physiological role of the described post-translational modifications of the α_2-subunit is still unclear. Similar evidence for proteolytic processing of the carboxyl terminus of the skeletal muscle α_1-subunit has been reported recently (32), but parallel mechanisms are not favored because of the different locations, relative to the membrane, of the proteolytic sites. A more detailed analysis of the individual subunit's structure and their interactions with one another is required before processes like excitation-contraction coupling can be understood at the molecular level.

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