Structural Characterization of the Dihydropyridine-sensitive Calcium Channel $\alpha_2$-Subunit and the Associated $\delta$ Peptides*

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Upon disulfide bond reduction, the $\alpha_2$-subunit of the dihydropyridine-sensitive Ca\textsuperscript{2+} channel undergoes a characteristic mobility shift on sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis with the concurrent appearance of the three $\delta$ peptides $\delta_1$ (25,000 Da), $\delta_2$ (22,000 Da), and $\delta_3$ (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 $\alpha_2$-subunit and the $\delta$ peptides 1, 2, and 3, respectively. Characterization of the $\delta$ 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 $\delta$ peptides reveals three identical sequences that match the predicted amino acid sequence of the $\alpha_2$-subunit starting at Ala$^{935}$. Enzymatic deglycosylation of the reduced $\alpha_2$$\delta$ 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 ($\delta$) peptide, consistent with a single transmembrane domain in the carboxyl ($\delta$) region. The data support a model of the $\alpha_2$-subunit in which the propeptide is processed into two chains that remain attached through disulfide linkages.

Voltage-dependent Ca\textsuperscript{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 with high pH in the presence of reducing agents is able to extract the larger amino-terminal peptide but not the smaller carboxyl ($\delta$) peptide, consistent with a single transmembrane domain in the carboxyl ($\delta$) region. The data support a model of the $\alpha_2$-subunit in which the propeptide is processed into two chains that remain attached through disulfide linkages.

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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

The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; PMSF, phenylmethylsulfonyl fluoride; [193]TID, 3-(trifluoromethyl)- 3-((m-[(10)]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, 0.503 mM sucrose, 0.5 mM EDTA, pH 7.1). All membranes were prepared in the presence of protease inhibitor mix A: aprotonin (0.10μg/ml), aprotinin (100 μg/ml), benzamidine (100 μg/ml), leupeptin (0.5 μg/ml), pepstatin A (1.0 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: (0.363 mM sucrose, 20 mM Tris maleate, pH 7.0, 100 μg/ml benzamidine, 155 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 mM sucrose, 0.75 mM benzamidine, and 0.1 mM PMSF). The membranes were centrifuged for 30 min at 35,000 r/min using a Beckman Ti-45 rotor (142,413 × g) in a Beckman JA-21 rotor for 30 min at 100,000 r/min. The final pellets were 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 mM NaCl, 0.5 mM 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 skeletal 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 mM sucrose, 0.1% digitonin, 0.75 mM benzamidine, 0.1 mM PMSF) containing 0.5 mM NaCl, buffer D with no sodium chloride, and finally eluted with buffer D containing 0.5 M NaCl and 0.07 M benzamidine. The WGA-Sepharose column 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. (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 Tris-Cl, 0.02% SDS, 0.0015 units of enzyme for endoglycosidase H digestion, and 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 cm × 30 cm). Proteins were separated with an isocratic buffer containing 50 mM sodium citrate, pH 5.5, 0.1 mM 2-mercaptoethanol, 0.02% SDS, 0.015% units of enzyme for endoglycosidase H, and 0.1% crystal violet. The fractions were monitored by absorbance of the effluent 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. Some samples were reduced prior to electrophoresis by 1% mercaptoethanol. Molecular weight standards were run in parallel to samples on all gels with prestained molecular weight 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-300efi data system software from Hoefer.

**Affinity-purified Antibodies**—Six subunit-specific antibodies were affinity purified from rabbit sera that had been immunized with the SDS-PAGE-purified nondenatured form of the a2-subunit (175,000 Da) of the rabbit skeletal muscle dihydropyridine receptor (GP31). The affinity purification was performed as reported by Sharp and Campbell (27). Briefly, 200 μg of purified dihydropyridine receptor was separated in 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 a2- and δ-subunits of the dihydropyridine receptor. The immobilized δ peptide was cut from the membrane as an individual 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, incubating, and washing solution.

**Radiolabeling of Hemoglobin 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 [32P]TID. The mixture was irradiated for 30 min on ice using a Spectrolinker UVP-250C handheld UV lamp operating at 254 nm, 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 470A sequencer equipped with on-line phenylthiohydantoin derivatization analysis. The peptides 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, 1% Triton X-100, 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 microsomes were divided equally into two samples, and diethiothreitol (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**—[32P]TID was obtained from Amersham Corp. Peroxidase-conjugated secondary antibodies were from Organon Teknika Corp. (Schenectady, NY). Rabbit anti-rabbit IgG, rabbit anti-chicken IgG, 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 a2-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)
peaking in 3, 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 standards are indicated on the left. Densitometric scan of Coomassie Blue-stained gel. The stained proteins were then scanned, and a plot of the digitized absorbance values is shown. The area under the curve in Fig. 2 corresponding to each subunit was integrated manually by addition of the digitized absorbance areas were divided by the subunit's apparent molecular weight and then normalized relative to the a1-subunit.

**TABLE I**

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Apparent molecular mass (kDa)</th>
<th>Area</th>
<th>Normalized relative area</th>
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<td>a1</td>
<td>170</td>
<td>15,400</td>
<td>1.00</td>
</tr>
<tr>
<td>a2</td>
<td>150</td>
<td>11,900</td>
<td>0.87</td>
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<tr>
<td>b</td>
<td>52</td>
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<td>0.90</td>
</tr>
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<td>δ1</td>
<td>25</td>
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</tr>
<tr>
<td>δ2</td>
<td>22</td>
<td>815</td>
<td>0.41</td>
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</tbody>
</table>

**FIG. 1.** Two-dimensional size separation of the dihydropyridine-sensitive Ca2+ 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 molecular weight and then normalized relative to the a1-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 a2-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.

**FIG. 2.** Densitometric scan of Coomassie Blue-stained gel containing purified dihydropyridine-sensitive Ca2+ 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.

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 a1 and δ proteins comigrate as a complex during gel filtration (corresponding to lanes 2 and 3), with a retention time similar to the a2-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 a2-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 a2-δ complex migrates as a 175,000-Da band, consistent with previous results (10, 18, 21). It is interesting to note that the a2-δ complex migrates slower than the a1-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.
A New Model for the Dihydropyridine Receptor α2-Subunit

chimetry between δ peptides and the α2-subunit, to determine whether more than one δ peptide was disulfide linked to each α2-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 α1-, α2-, 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 α2-subunit there exists 0.31 mol of δ, and 0.47 mol of δ2, consistent with a single δ peptide in each α2 complex.

To detect the presence of hydrophobic stretches of amino acids, the hydrophobic probe [3H]TID was photoincorporated into purified dihydropyridine-sensitive Ca2+ channel receptor (Fig. 3A). The δ peptides were found to be labeled by the probe, as were the α1-, α2-, 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 heightened 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 peroxidasen-conjugated lectins, concanavalin A and wheat germ agglutinin (WGA). Although concanavalin A bound only to the α2-subunit (data not shown), WGA binding was detected for the α2- and γ-subunits as well as the δ peptides (Fig. 3B). 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 α2-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 α2-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 δ1 and δ2 peptides have been shown previously to be antigenically cross-reactive (21). A smaller δ peptide (17,000 Da) can also be detected by immunoblot analysis with prolonged development. The presence of δ1 can also be detected by Coomassie Blue staining of highly loaded gels (Fig. 4). The

**Table II**

A comparison of the measured δ peptide amino-terminal sequence versus the predicted sequence of the α2-subunit

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Yield pmol</th>
<th>Amino acid</th>
<th>Yield pmol</th>
<th>Amino acid</th>
<th>Yield pmol</th>
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</thead>
<tbody>
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<td>50</td>
<td>A</td>
<td>72</td>
<td>A</td>
<td>12</td>
</tr>
<tr>
<td>D</td>
<td>16</td>
<td>D</td>
<td>48</td>
<td>D</td>
<td>6</td>
</tr>
<tr>
<td>M</td>
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</tr>
<tr>
<td>D</td>
<td>12</td>
<td>D</td>
<td>47</td>
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<td>S</td>
<td>5</td>
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**Fig. 5.** Endoglycosidase treatment of the dihydropyridine-sensitive Ca2+ 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 α2-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: α2', for the partial deglycosylation observed with the α2-subunit in lane 2; and α2', γα, and δα, for the core peptides observed in lane 3. The locations of the detected protein bands representing the added enzymes are labeled with *.

The calculated densitometric ratio of the three δ peptides is 4:6:1 (δ1 to δ2 to δ3).

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 α2-subunit starting at Ala25. These data provide direct evidence that the δ peptides represent carboxyl-terminal proteolytic products of the α2-subunit which remain attached to the larger (150,000-Da) amino-terminal peptide through disulfide linkages.

The relative intensity of [3H]TID incorporation (Fig. 3A) into the two prominent δ peptides paralleled the observed Coomassie Blue staining intensity (Fig. 1) with δ1 > δ. The inverse intensity staining pattern observed with WGA per-
The post-translation products are shown on complex. The predicted sizes of the post-translation products are based upon the cDNA predicted sequence and the determined molecular weight is based upon the cDNA segments. Two of the possible 18 glycosylation sites are not labeled as per changes described under "Discussion."

The calculation of the amino acid length and the associated core molecular weight is based upon the cDNA predictions. Two of the possible 18 glycosylation sites are not labeled as per changes described under "Discussion.

The deglycosylated core peptides had apparent sizes of $\alpha_{2c} = 105,000$ Da, $\gamma_c = 23,000$ Da, and $\delta_c = 17,000$ Da. The agreement between the observed size of the deglycosylated $\delta_c$ core peptide and the size predicted from the $\alpha_{2c}$ cDNA from Ala$^{329}$ 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 $\alpha_{1c}$ and $\beta$-subunit apparent sizes remained unchanged, and the approximate $\gamma$ 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 $\alpha_{2c}$ 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 (β) segment (Fig. 6). In the proposed three-transmembrane region model of the $\alpha_{2c}$-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-
D I S C U S S I O N

Historically, the dihydropyridine-sensitive Ca\textsuperscript{2+} channel receptor was observed to contain a large (140,000–175,000-Da) subunit termed the \( \alpha \)-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 photoactive calcium channel antagonists and whose mobility was insensitive to reducing agents. This distinction gave rise to the nomenclature \( \alpha_2 \), \( \alpha_3 \), and \( \alpha_4 \) for the respective subunits (for reviews, see Refs. 10–12). We have provided evidence that the \( \alpha_2 \)-subunit is composed of two disulfide-linked chains referred to previously as the reduced form of the \( \alpha_2 \) and the \( \delta \)-subunits. To emphasize their relationship to the \( \alpha \)-complex we propose the term \( \delta \)-peptides and not the \( \delta \)-subunits.

Contrary to previous experimental conclusions (20), the size heterogeneity of the \( \delta \)-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 cysteine, result in the observed heterogeneity of the \( \delta \)-peptides. We feel that the results involving alkaline extraction and the pattern and extent of N-linked glycosylation are most consistent with the above data we propose that the \( \delta \)-peptides represent a heterogeneously glycosylated group of identical core peptides (\( \delta_1 \)) with hybrid or complex oligosaccharides, \( N \)-linked at two, one, or zero sites for \( \delta_1 \), \( \delta_2 \), and \( \delta_3 \), respectively.

Interpretation of the experimental results involving the 150,000-Da \( \alpha_2 \)-segment and its transmembrane characteristics remains controversial. Although the 1239-TID incorporation into the 150,000-Da \( \alpha_2 \)-peptide (Fig. 3A) is consistent with a hydrophobic segment within the protein, this method is not specific for transmembrane regions. Under the same experimental conditions significant incorporation is noted into calmodulin (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 \( \alpha_2 \)-subunit is whether the cleavage is physiological or an artifact of membrane isolation. The possibility of this cleavage representing an isoform 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 \( \alpha_2 \)-subunit has been noted to shift with reduction, and the \( \delta \)-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 \( \alpha_2 \)-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 \( \delta \)-peptides are indeed components of the dihydropyridine-sensitive Ca\textsuperscript{2+} channel receptor, although not unique subunits. The evidence supports a two-chain model of the \( \alpha_2 \)-subunit in which the larger, highly glycosylated amino-terminal peptide remains covalently associated by disulfide linkage to the carboxyl (\( \delta \))-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 (\( \delta \))-terminal peptide, clearly providing candidate sites for disulfide linkages. Additionally, our data support a new model of the \( \alpha_2 \)-subunit in which the carboxyl (\( \delta \))-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 \( \alpha_2 \)-subunit was thought to be an integral membrane protein and the \( \delta \)-peptides to be peripheral (18).

The physiological role of the described post-translational modifications of the \( \alpha_2 \)-subunit is still unclear. Similar evidence for proteolytic processing of the carboxyl terminus of the skeletal muscle \( \alpha_2 \)-subunit has been reported recently (22), 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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REFERENCES


A New Model for the Dihydropyridine Receptor \( \alpha_2 \)-Subunit
A New Model for the Dihydropyridine Receptor α₂-Subunit