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Subunit structure of dihydropyridine-sensitive calcium channels from skeletal muscle

(calcium antagonists/protein phosphorylation/glycoproteins/ion channels)

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ABSTRACT **Purified dihydropyridine-sensitive calcium channels from rabbit transverse-tubule membranes consist of three noncovalently associated classes of subunits: α (167 kDa), β (54 kDa), and γ (30 kDa). Cleavage of disulfide bonds reveals two distinct α polypeptides and an additional component, δ. The α_1 subunit, a 175-kDa polypeptide that is not N-glycosylated, contains the dihydropyridine binding site, cAMP-dependent protein kinase phosphorylation site(s), and substantial hydrophobic domain(s). α_2, a 143-kDa glycoprotein, has none of the properties characteristic of α_1 but binds lectins and contains about 25% N-linked carbohydrate. α_2 is disulfide-linked to δ, a 24- to 27-kDa glycopeptide. β (54 kDa) contains a cAMP-dependent phosphorylation site but is not N-glycosylated and does not have a hydrophobic domain. γ (30 kDa) has a carbohydrate content of about 30% and extensive hydrophobic domain(s). Precipitation with affinity-purified anti-α_1 antibodies or α_2-specific lentil lectin-agarose demonstrated that $\alpha_1\alpha_2\beta\gamma\delta$ behaves as a complex in the presence of digitonin or 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, whereas the $\alpha_2\delta$ complex dissociates from $\alpha_1\beta\gamma$ in the presence of Triton X-100. A model for subunit interaction and membrane insertion is proposed on the basis of these observations.**

Dihydropyridine (DHP)-sensitive calcium channels have been solubilized from skeletal muscle transverse tubules (T-tubules) by treatment with digitonin (1–3) or 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (4) and purified by conventional methods using high-affinity binding of radiolabeled derivatives of the DHP calcium channel antagonists nitrendipine or PN200-110 as a specific assay (1–4). The channel protein purified in the presence of digitonin contained three classes of noncovalently associated polypeptides when analyzed by NaDodSO$_4$/PAGE without reduction of disulfide bonds: α (160 kDa), β (53 kDa), and γ (33 kDa) (1). Photoreactive calcium antagonists specific for the allosterically coupled DHP, benzothiazepine, and phenylalkylamine binding sites can all be covalently attached to polypeptide(s) of 145–170 kDa in T-tubule membranes, suggesting that α subunits of the purified calcium channel contain all three drug receptor sites (5, 6). A complex of the α, β, and γ polypeptides mediates DHP-sensitive calcium conductance when reconstituted in phospholipid vesicles or planar bilayers (7, 8). The α and β polypeptides are good substrates for cAMP-dependent protein kinase (9), and phosphorylation of these subunits is accompanied by an increased probability of calcium-channel opening (8). These results are consistent with the conclusion that a complex of α, β, and γ classes of polypeptides is sufficient to form a functional calcium channel that can be modulated by agonist and antagonist drugs and by cAMP-dependent phosphorylation in a manner similar to native calcium channels.

In previous studies in this laboratory (1, 10), the α subunit of the calcium channel was found to behave anomalously in NaDodSO$_4$/PAGE after reduction of disulfide bonds. This behavior was initially ascribed to partial reduction of intrapeptide disulfide bonds whose cleavage and reformation resulted in a variable fraction of the protein with smaller apparent size. We now describe use of subunit-specific antibodies and a battery of specific labeling methods to show that the α-protein band contains two calcium channel subunits, α_1 and α_2, that have similar size but clearly different properties. We propose a model of DHP-sensitive calcium channel structure in which the α_1 subunit is the central transmembrane component with three independently associated subunits. Preliminary reports of some aspects of this work have been presented (11, 12).

MATERIALS AND METHODS

Materials. [^{3}H]Azidopine (53 Ci/mmol; 1 Ci = 37 GBq), [^{3}H]PN200-110 (70 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), ^{125}I-labeled Bolton–Hunter reagent, and endoglycosidase F were obtained from New England Nuclear. Neuraminidase was from Sigma and lentil lectin-agarose from Vector Laboratories (Burlingame, CA).

Purification of T-Tubule Membranes and Calcium Channels. Rabbit skeletal muscle T-tubule membranes were prepared according to Rosemblatt *et al.* (13). Calcium channels were purified from T-tubules or skeletal muscle microsomes as described (1). Protein concentrations were determined by the method of Peterson (14).

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. NaDodSO$_4$-denatured proteins were analyzed in a discontinuous gel system, according to Laemmli (15), consisting of a stacking gel of 3% acrylamide and a running gel containing a 5–15% (wt/vol) acrylamide gradient (1). Proteins in sample buffer (1) were treated with either 20 mM *N*-ethylmaleimide (MalNEt) to alkylate sulfhydryl groups or 20 mM dithiothreitol (DTT) to reduce disulfide bonds, as indicated below each gel lane in the figures. The gels were silver-stained according to the method of Oakley *et al.* (16). In some cases, the gel was soaked in 0.1% (wt/vol) $K_2Cr_4O_7$ and 0.013% H_3PO_4 for 10 min before silver staining.

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Abbreviations: DHP, dihydropyridine; T-tubule, transverse tubule; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; MalNEt, *N*-ethylmaleimide; DTT, dithiothreitol; TID, 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine; WGA, wheat germ agglutinin.

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Immunological Methods. Polyclonal antibodies (PAC-10) were obtained from the ascites fluids of a SJL/J mouse (17) immunized with purified skeletal muscle calcium channel and were purified by protein A-Sepharose chromatography. Anti-α_1 antibodies were affinity-purified from PAC-10 by the method of Olmsted (18). The purified calcium channel was treated with 0.5% (wt/vol) Triton X-100. After removal of dissociated $\alpha_2\delta$ subunits by adsorption to lentil lectin-agarose, the remaining subunits were separated by $NaDodSO_4$/PAGE and transblotted onto a nitrocellulose sheet. The portion containing the α_1 subunit was cut out and used for affinity purification. A blank nitrocellulose strip was processed identically and used as a control.

RESULTS AND DISCUSSION

Polypeptide Composition of the DHP-Sensitive Calcium Channel. Calcium channels were solubilized and purified by previously reported methods (1) and then analyzed by $NaDodSO_4$/PAGE and silver staining (Fig. 1, lanes 1 and 2). Under alkylating conditions (lane 1), three classes of polypeptides that have been designated α (167 kDa), β (54 kDa), and γ (30 kDa) (1) were detected. When disulfide bonds were cleaved with DTT (lane 2), the α band split into two clearly resolved protein populations of 175 kDa and 143 kDa. Reduction also led to the appearance of a new family of low molecular mass polypeptides (24–27 kDa) clearly distinct from the γ subunit. These polypeptides were poorly visualized by the silver stain but were labeled clearly by lectins (see Fig. 3). The 175-kDa and 143-kDa polypeptides showed different coloration when gels were silver-stained, suggesting that they were distinct molecular species. Furthermore, these two polypeptides could be distinguished immunochemically. In immunoblots, a polyclonal antibody (PAC-10) against the native calcium channel selectively labeled the 167-kDa band before reduction of disulfide bonds (Fig. 1, lane 3) but only the 175-kDa polypeptide after reduction (lane 4). No immunolabeling was observed with preimmune serum (lane 5) or with PAC-10 that had been preabsorbed with purified calcium channel (lane 6).

Since these observations indicate that the 175-kDa and 143-kDa components are different polypeptides, we designate them α_1 and α_2, respectively. The 24- to 27-kDa components appear to be disulfide-linked to the α_2 subunit. The 24-kDa peptide may be proteolytically derived from the 27-kDa peptide (20), so we refer to them collectively as the δ subunit. In all of our figures, the migration positions of the α protein band and the β and γ subunits under alkylating conditions are indicated on the scale labeled MalNEt, and the migration positions of all the subunits under reducing conditions are indicated on the scale labeled DTT.

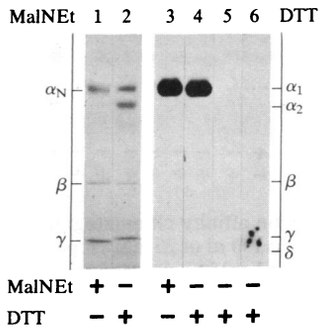

FIG. 1. Polypeptide composition of the DHP-sensitive calcium channel. Lanes 1 and 2: purified calcium channels were analyzed by $NaDodSO_4$/PAGE and silver staining with or without reduction of disulfide bonds as indicated below each lane. Lanes 3–6: polypeptides separated by $NaDodSO_4$/PAGE with or without reduction of disulfide bonds, as indicated below each lane, were electrophoretically transferred to nitrocellulose sheets and immunolabeled by incubation with PAC-10 (lanes 3 and 4), preimmune serum (lane 5), or PAC-10 that had preabsorbed with purified calcium channel (lane 6), followed by incubation with ^{125}I-labeled protein A, washing, and autoradiography as described (19).

Differential Labeling of Calcium Channel Subunits. [^{3}H]-PN200-110 and [^{3}H]azidopine have been shown to covalently label a 145- to 170-kDa polypeptide in T-tubule membranes (5, 6) and purified calcium channels (3) that presumably corresponds to one of the two α subunits. In our preparations, [^{3}H]azidopine was incorporated by UV photolysis into a polypeptide that migrated as a band at 167 kDa before reduction of disulfide bonds (Fig. 2, lane 1) and 175 kDa after reduction of disulfide bonds (lane 2). The electrophoretic behavior of this polypeptide identifies it as the α_1 subunit. No labeling was observed in the presence of 2 μM PN200-110 (lane 3).

In previous work, ^{32}P (from [γ-^{32}P]ATP) was stoichiometrically incorporated into the α-protein band and the β subunit by the catalytic subunit of cAMP-dependent protein kinase (9). Comparison of the electrophoretic mobility of the phosphorylated bands before and after reduction of disulfide bonds (Fig. 2, lanes 4 and 5) showed that the α_1 subunit is a good substrate for this enzyme, whereas the α_2 subunit is not labeled. The β subunit was more weakly labeled at the low ATP concentrations used (9), while no phosphorylation of the γ or δ subunits was detected.

Ion channel-forming polypeptides should contain hydrophobic segments, which may be detected by use of the hydrophobic probe 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID). This photoreactive compound partitions into free detergent micelles and detergent associated with the major hydrophobic domains of integral membrane proteins and is specifically incorporated into these regions by photolysis (22). The α_1 and γ subunits were prominently labeled by [^{125}I]TID, with a much lower level of incorporation into α_2 and δ (Fig. 2, lanes 6 and 7). The β subunit was not detectably labeled. Quantitation of [^{125}I]TID in excised protein bands showed that the α_1 and γ subunits incorporated 10-fold more TID per unit mass than the α_2 or

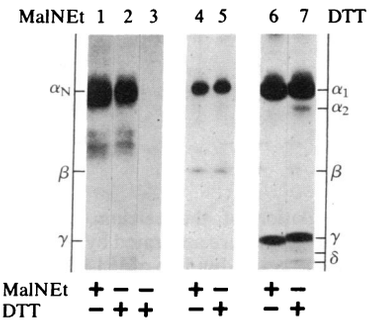

FIG. 2. Differential labeling of calcium channel subunits. (*Left*) Photoaffinity labeling. T-tubule membranes (0.4 mg/ml) in 25 mM Hepes/1 mM $CaCl_2$ adjusted to pH 7.5 with Tris base were incubated with 6 nM [^{3}H]azidopine in the absence (lanes 1 and 2) or in the presence (lane 3) of 2 μM PN200-110 and were irradiated for 15 min at 4°C with a 30-watt UV source (λ_{max} 356 nm). The membranes were solubilized in 1% digitonin/10 mM Hepes/185 mM NaCl/0.5 mM $CaCl_2$/0.1 mM phenylmethanesulfonyl fluoride/1 μM pepstatin A adjusted to pH 7.5 with Tris base, and calcium channels were partially purified by chromatography on WGA-Sepharose (1) and analyzed by $NaDodSO_4$/PAGE and fluorography. (*Center*) Phosphorylation. Purified calcium channel was incubated with 0.3 μM cAMP-dependent kinase catalytic subunit and 0.12 μM carrier-free [γ-^{32}P]ATP for 15 min at 37°C as described (9) (lanes 4 and 5). (*Right*) Hydrophobic labeling. [^{125}I]TID (15 Ci/mmol) was prepared and purified calcium channel was labeled with [^{125}I]TID (100 μCi/ml) in a buffer containing 0.1% digitonin, as previously described (21) (lanes 6 and 7).

δ subunits, even though, as shown below, nearly all α_1 and γ subunits are associated with an α_2 subunit. These results indicate that the α_1 and γ subunits are the most hydrophobic components of the purified calcium channel complex, suggesting that they are the principal transmembrane polypeptides.

Subunit Glycosylation. Solubilized [^{3}H]DHP receptors specifically bind to various immobilized lectins (23), and affinity chromatography on wheat germ agglutinin (WGA)-Sepharose is the most efficient purification step (1–4). These results imply that at least one subunit is glycosylated. The oligosaccharide chains of the calcium channel were detected by separating the subunits by NaDodSO$_4$/PAGE and probing the resolved polypeptides with ^{125}I-labeled WGA or Con A. ^{125}I-labeled WGA bound to the α-protein band (α_N) and the γ subunit in gels run under alkylating conditions (Fig. 3*A*, lane 1) and to the α_2, γ, and δ subunits in gels run under reducing conditions (Fig. 3*A*, lane 2). ^{125}I-labeled Con A labeled only the α_2 subunit (Fig. 3*B*, lanes 1 and 2). No labeling of α_1 or β was detected with either lectin. An unidentified polypeptide of about 105 kDa was also labeled by radioiodinated WGA in reducing conditions but not by Con A. Since these results indicate that α_2 and δ are glycosylated but α_1 is not, the α_1 subunit and the disulfide-linked $\alpha_2\delta$ complex are independent polypeptides and are not derived from one another.

To determine the extent of glycosylation and the core polypeptide size of the calcium channel subunits, purified channel preparations were labeled with ^{125}I (19), incubated with glycosidases to remove oligosaccharide chains, and analyzed by NaDodSO$_4$/PAGE. Fig. 3*C* presents the change in electrophoretic mobility and apparent molecular mass of the α_1, α_2, β, and γ subunits resulting from sequential deglycosylation with neuraminidase and endoglycosidase F. Both neuraminidase and endoglycosidase F caused reduction in the apparent sizes of α_2 and γ. A plateau was reached after 16 hr with endoglycosidase F, corresponding to core polypeptide sizes of 105 kDa for α_2 and 20 kDa for γ. Poor iodination of the δ subunit prevented estimation of its carbohydrate content by this method. No shift in the mobility of the α_1 or β subunits was noted. Since α_1 and β are particularly sensitive to proteolysis (ref. 1 and unpublished results), the change in mobility of α_2 and γ must result from deglycosylation and not peptide bond cleavage by contaminating proteases.

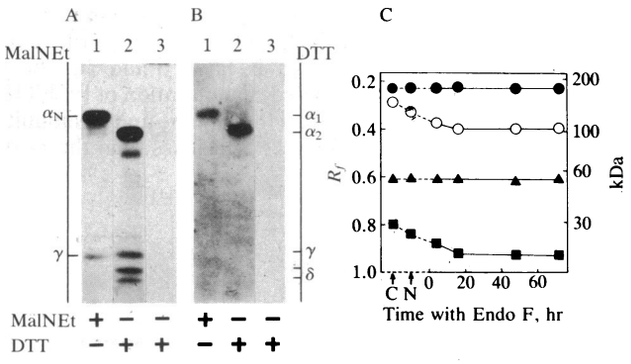

FIG. 3. Glycosylation of the calcium channel subunits. (*A*) Calcium channel subunits were separated by NaDodSO$_4$/PAGE and labeled directly in the gel by binding of ^{125}I-labeled WGA (prepared as in ref. 24) according to the method of Burridge (25) in the absence (lanes 1 and 2) or presence (lane 3) of 200 mM *N*-acetylglucosamine. (*B*) Separated calcium channel subunits were electrophoretically transferred to a nitrocellulose sheet to reduce background and then incubated with ^{125}I-labeled Con A (prepared as in ref. 24) in the absence (lanes 1 and 2) or presence (lane 3) of 100 mM methyl α-D-mannoside. (*C*) ^{125}I-labeled calcium channel was treated with neuraminidase (N, 1 unit) in 50 mM NaCl/25 mM sodium citrate/50 mM NaH$_2$PO$_4$/0.02% digitonin, pH 5.0, at 37°C. After 4 hr, the mixture was boiled for 2 min in the presence of 0.5% NaDodSO$_4$, and 2% 2-mercaptoethanol, and 1 unit of endoglycosidase F (Endo F) with Triton X-100 and EDTA (final concentrations 2% and 50 mM, respectively) was added. After the indicated incubation periods, aliquots were removed, mixed with 3% NaDodSO$_4$ to stop the reaction and applied to a Sephadex G-50 column preequilibrated with sample buffer (1). Material that was eluted at the void volume was analyzed by NaDodSO$_4$/PAGE under reducing conditions and autoradiography, and the R_f and apparent molecular mass of the α_1 (●), α_2 (○), β (▲), and γ (■) subunits were determined. Control samples (C) were incubated for the same time in identical buffers from which enzymes were omitted.

Analysis of Noncovalent Subunit Interactions by Lentil Lectin-Agarose Chromatography. The specific labeling experiments described above establish that α_1 and α_2 are independent polypeptides, with widely different core polypeptide sizes, that comigrate during NaDodSO$_4$/PAGE in nonreducing conditions due to the high carbohydrate content of α_2 and its disulfide linkage to δ. The α_1 subunit has properties expected of the calcium channel, including a DHP binding site, at least one cAMP-dependent phosphorylation site, and an extensive hydrophobic domain(s). It is important to establish whether $\alpha_2\delta$ is a persistent impurity or a specifically associated component of the oligomeric calcium channel complex.

Experiments with ^{125}I-labeled lectins established Con A as a specific probe for the α_2 subunit (Fig. 3*B*). Lentil lectin has the same specificity as Con A, and its lower affinity facilitates elution of bound glycoproteins with methyl α-D-mannoside. The calcium channel was labeled with ^{125}I by the Bolton–Hunter procedure (Fig. 4, lanes 1 and 2). The incorporation of ^{125}I into α_2 was anomalously low, presumably indicating a low content of accessible primary amines. When the calcium channel protein was denatured by treatment with 1% NaDodSO$_4$ and then exchanged into 0.5% Triton X-100 by gel filtration, only α_2 bound specifically to lentil lectin-agarose (Fig. 4, lane 10), while α_1, β, and γ remained in the unbound fraction (Fig. 4, lane 9). This result confirms the conclusion that only α_2 binds to lectins with the specificity of lentil lectin and Con A.

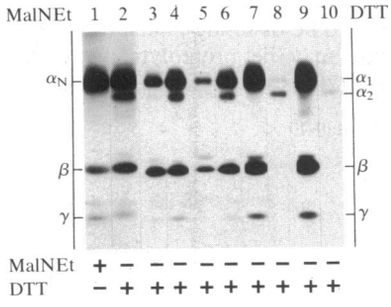

FIG. 4. Lentil lectin affinity chromatography. ^{125}I-labeled calcium channel (100 ng) in 150 μl of 25 mM Hepes/150 mM NaCl/1 mM CaCl$_2$/1 mM MnCl$_2$/0.1 mM phenylmethanesulfonyl fluoride/1 μM pepstatin A (adjusted to pH 7.5 with Tris base) containing detergent as indicated below was incubated for 90 min at 4°C with 50 μl of lentil lectin-agarose, equilibrated in the same buffer, under agitation. The resin was removed by centrifugation and the supernatant wash was collected. The resin was washed three times with 1 ml of buffer and resuspended in 150 μl of buffer containing 0.2 M methyl α-D-mannoside. The eluate was collected after a 90-min batch incubation. Eluate (lanes 4, 6, 8, and 10) and wash (lanes 3, 5, 7, and 9) samples were analyzed by NaDodSO$_4$/PAGE and autoradiography. Lanes 1 and 2: ^{125}I-labeled calcium channel with no treatment. Lanes 3 and 4: 0.1% digitonin. Lanes 5 and 6: 0.1% CHAPS. Lanes 7 and 8: 0.5% Triton X-100. Lanes 9 and 10: samples treated with 1% NaDodSO$_4$ were exchanged into 0.5% Triton X-100 by filtration over a 2-ml Sephadex G-50 column and analyzed in 0.5% Triton X-100.

In contrast to these results after denaturation of the channel subunits, in detergent conditions (0.1% digitonin or 0.1% CHAPS) under which the channel is known to retain DHP binding, allosteric coupling of the three calcium antagonist receptor sites, and ion conductance activity (1–4, 7, 8), an $\alpha_1\alpha_2\beta\gamma$ complex was specifically bound to lentil lectin (Fig. 4, lanes 4 and 6), indicating that all four polypeptides behave as a complex. The presence of some α_1 and β in the unbound fraction (lanes 3 and 5) probably results from partial subunit dissociation during iodination or sample storage.

Although α_1 and α_2 were associated in 0.1% digitonin or CHAPS, in 0.5% Triton X-100 [which causes complete loss of DHP binding activity (10, 26)] the α_2 subunit alone bound specifically to lentil lectin-agarose (Fig. 4, lane 8), while the α_1, β, and γ subunits dissociated and appeared in the unbound fraction (lane 7). Considered together, these results indicate a correlation between retention of the DHP-binding activity of the solubilized calcium channel in various detergents and association of the α_1, α_2, β, and γ subunits.

Analysis of Noncovalent Subunit Interactions with an Antibody Against α_1. The data presented in Fig. 1*B* demonstrate that PAC-10 antibodies recognize only the α_1 subunit of $NaDodSO_4$-denatured calcium channel. However, this polyclonal serum produced by immunization with native calcium channel may contain antibodies that bind only to native conformations of the $\alpha_2\delta$, β, or γ subunits. To eliminate any antibodies with this specificity, a nitrocellulose strip containing α_1 subunit was used as an affinity matrix to purify anti-α_1 antibodies (see *Materials and Methods*). Purified anti-α_1 antibodies specifically precipitated [^{3}H]PN200-110-labeled calcium channel, whereas proteins from PAC-10 that were nonspecifically adsorbed to a bare nitrocellulose strip did not (Fig. 5*A*).

Immunoprecipitation of ^{125}I-labeled calcium channel (Fig. 5*B*) showed that only α_1 was precipitated after $NaDodSO_4$ denaturation (lane 7). In contrast, α_1, α_2, β, and γ were precipitated as a complex in 0.5% digitonin (lane 1) or 0.1% CHAPS (lane 3). A higher concentration of CHAPS (1%) caused dissociation of α_2 from the complex (data not shown).

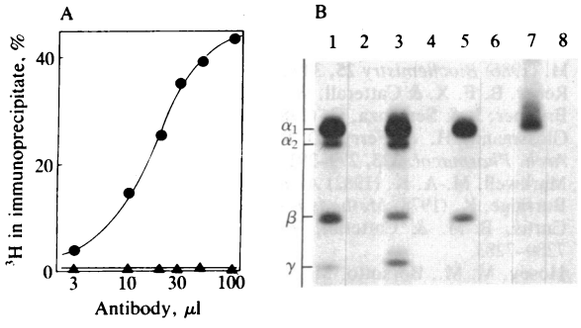

FIG. 5. Immunoprecipitation of calcium channel subunits by anti-α_1 antibodies. Affinity-purified anti-α_1 antibodies (●) and a blank control (▲) were incubated with [^{3}H]PN200-110-labeled skeletal muscle calcium channel in 145 μl of 0.5% digitonin/75 mM NaCl/50 mM NaH_2PO_4/25 mM Tris·HCl, pH 7.4, at 4°C for 4 hr. Antigen–antibody complexes were adsorbed to protein A-Sepharose and washed by centrifugation, and the radioactivity was determined by scintillation counting. Results are expressed as the percentage of input radioactivity. (*B*) ^{125}I-labeled calcium channel was immunoprecipitated as described previously (19), using anti-α_1 antibodies (lanes 1, 3, 5 and 7) or the control preparation (lanes 2, 4, 6 and 8) in immunoassay buffer containing the detergents indicated below, and the immunoprecipitates were analyzed by $NaDodSO_4$/PAGE in the presence of DTT, followed by autoradiography. Lanes 1 and 2: 0.5% digitonin. Lanes 3 and 4: 0.1% CHAPS. Lanes 5 and 6: 1% Triton X-100. Lanes 7 and 8: samples incubated with 1% $NaDodSO_4$ for 2 min at 100°C and exchanged into 0.5% digitonin by gel filtration on a 2-ml Sephadex G-50 column.

In addition, experiments in 1% Triton X-100 (lane 5) showed complete dissociation of the α_2 subunit, in agreement with the results of lentil lectin chromatography. The β subunit and a small fraction of the γ subunit (not easily seen in Fig. 5*B*) were coimmunoprecipitated with α_1 in Triton X-100. The results of these immunoprecipitation experiments have been confirmed by selective elution of α_2 subunits from immune complexes. Purified calcium channels were immunoprecipitated in 0.5% digitonin as in Fig. 5, lane 1. Resuspension in a buffer containing 1% Triton X-100 caused complete release of the $\alpha_2\delta$ dimer and partial release of γ without loss of β from the precipitate (data not shown). These results show that the $\alpha_2\delta$ dimer is specifically associated with a complex of the α_1, β, and γ subunits in detergents that preserve the functional properties of the calcium channel. We tentatively conclude that $\alpha_2\delta$, β, and γ all interact independently with the "central" subunit α_1. The differing degrees of stability in Triton X-100 suggest subunit binding to α_1 with increasing affinity in the order $\alpha_2\delta < \gamma < \beta$.

An Oligomeric Model for the Subunit Structure of the DHP-Sensitive Calcium Channel. Since the initial report from this laboratory (1), studies on the polypeptide composition of the T-tubule calcium channel using digitonin extraction and similar purification techniques have confirmed both the existence of the α, β, and γ classes of subunits and the anomalous behavior of the α protein band on disulfide reduction (2, 3, 8). In contrast to these results, purification of the calcium channel by a different procedure yielded only 140-kDa and 32- to 33-kDa polypeptides that were reported to be noncovalently associated (4), although subsequent immunochemical analyses suggested that these two polypeptides were disulfide-linked (20). These observations were inconsistent with the finding by the same authors that photoaffinity labeling of T-tubules identified, after disulfide reduction, a single 170-kDa band as containing the calcium antagonist binding sites (6). This apparent discrepancy led to the proposal that their purified protein consisting of a disulfide-linked dimer of 140-kDa and 32- to 33-kDa polypeptides resulted from proteolytic nicking of this single 170-kDa polypeptide labeled in T-tubules and represented the entire functional calcium channel including cAMP-dependent phosphorylation sites (27) and calcium antagonist binding sites (4). Our present results require an alternative interpretation of these data. The disulfide-linked dimer observed in these previous studies is likely to correspond to the $\alpha_2\delta$ disulfide-linked glycoprotein dimer, which we show here to be a peripherally associated component of the calcium channel that has neither cAMP-dependent phosphorylation sites, DHP binding sites, nor extensive hydrophobic domains. Instead, these functional components of the calcium channel reside in the α_1, β, and γ subunits of the purified calcium channel. While the $\alpha_2\delta$ complex appears to be quite resistant to proteolysis, the β subunit (1) and, to a lesser degree, the α_1 subunit (unpublished results) are susceptible to proteolytic degradation during typical purification procedures, and special precautions are required to recover them intact (1). This differential susceptibility to proteolysis may explain why α_1 and β were not detected in preparations made from frozen and thawed muscle and solubilized in the stronger detergent CHAPS (4).

On the basis of present knowledge of the structure of the DHP-sensitive calcium channel, and in analogy with current models of the structure of voltage-sensitive sodium channels (reviewed in ref. 28), we propose a model (Fig. 6) based on a central ion channel-forming element interacting with three other noncovalently associated subunits. The α_1 subunit, which contains the calcium antagonist binding sites, cAMP-dependent phosphorylation sites, and the largest hydrophobic domains, is proposed to be the central ion channel-forming component of the complex. Its apparent molecular

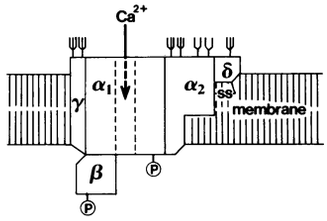

FIG. 6. Proposed model for calcium channel structure. Sites of cAMP-dependent phosphorylation (P), glycosylation (ψ and ψ), and interaction with the membrane are illustrated.

mass of 175 kDa from $NaDodSO_4$/PAGE is likely to be a reasonable approximation of the true polypeptide size, since no N-glycosylation was detected. This calcium channel subunit is therefore enough to contain four homologous transmembrane domains analogous to those of the rat brain sodium channel α subunit, whose mRNA alone encodes a functional ion channel (29, 30). Like α_1, the sodium channel α subunit also contains cAMP-dependent phosphorylation sites (28) and extensive hydrophobic domains that are efficiently labeled by TID (21).

The β subunit is also a substrate for cAMP-dependent kinase (9), but hydrophobic labeling indicates that it does not interact with the membrane phase and it is not a glycoprotein. It is probably therefore tightly associated with an intracellular domain of α_1.

The γ subunit of 30 kDa interacts independently with α_1, contains at least one transmembrane segment, and consists of approximately 30% carbohydrate. All these properties are similar to those of the β_1 subunit of the rat brain and skeletal muscle sodium channels (28). A polypeptide of similar size appears to be associated with the apamin-sensitive, calcium-activated potassium channel (31), and it is interesting to speculate that this subunit may be a conserved constituent of voltage- or calcium-dependent ion channels.

The $\alpha_2\delta$ dimer appears to interact more weakly with α_1, although the conditions necessary to achieve dissociation result in a loss of DHP-binding activity. The 105-kDa core polypeptide of α_2 contains a heavily glycosylated extracellular domain but displays weak hydrophobic labeling, indicating a limited intramembrane domain. For this reason it seems unlikely that the ion channel is formed jointly by α_1 and α_2 at their zone of interaction.

The proposed model assumes a complex containing one of each subunit type. Our present results and previous data showing quantitative binding of solubilized calcium channels to Con A (23) suggest that each complex contains at least one α_1 and one α_2 subunit but do not specify the stoichiometry of any subunits. α_1 and α_2 appear to be present in approximately equal amounts on silver stained gels, and the α_1 and β subunits incorporate approximately one mole of ^{32}P per mole of complex (9). A complete hydrodynamic analysis of the skeletal muscle calcium channel has not been reported. However, a size of 370 kDa determined for the rat ventricular muscle DHP receptor (32) is within reasonable range of the predicted size of the complex represented in Fig. 6 (416 kDa). Thus, an assumption of one mole of each subunit in the complex is plausible but requires direct experimental verification.

Our model emphasizes a central role for the α_1 subunit in calcium channel function. Further studies of pharmacological and physiological modulation of reconstituted calcium channels lacking individual subunits or of calcium channels specified by mRNA for individual subunits will be necessary to ascribe a functional role to the other subunits in the complex.

Note Added in Proof. A recent report (33) shows that a verapamil derivative labels α_1, but not α_2, subunits. In contrast, another recent paper (34) reports only a single intact α_2 subunit for calcium channels purified from frozen muscle, as found by Borsotto *et al.* (4).

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