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Extracellular Interaction of the Voltage-dependent Ca^{2+} Channel $\alpha_2\delta$ and α_1 Subunits*

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The role of the extracellular domain of the voltage-dependent Ca^{2+} channel $\alpha_2\delta$ subunit in assembly with the α_{1C} subunit was investigated. Transiently transfected tsA201 cells processed the $\alpha_2\delta$ subunit properly as disulfide linkages and cleavage sites between the α_2 and δ subunits were shown to be similar to native channel protein. Coimmunoprecipitation experiments demonstrated that in the absence of δ subunits, α_2 subunits do not assemble with α_1 subunits. Furthermore, the transmembrane and cytoplasmic sequences in δ can be exchanged with those of an unrelated protein without any effect on the association between the $\alpha_2\delta$ and α_1 proteins. Extracellular domains of the $\alpha_2\delta$ subunit are also shown to be responsible for increasing the binding affinity of [^{3}H]PN200-110 (isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-([^{3}H]methoxycarbonyl)-pyridine-3-carboxylate) for the α_{1C} subunit. Investigation of the corresponding interaction site on the α_1 subunit revealed that although tryptic peptides containing repeat III of native α_{1S} subunit remain in association with the $\alpha_2\delta$ subunit during wheat germ agglutinin chromatography, repeat III by itself is not sufficient for assembly with the $\alpha_2\delta$ subunit. Our results suggest that the $\alpha_2\delta$ subunit likely interacts with more than one extracellular loop of the α_1 subunit.

The $\alpha_2\delta$ subunit has been identified in every voltage-dependent Ca^{2+} channel purified to date from various mammalian tissues, including skeletal muscle (1, 2), brain (3, 4), and heart (5, 6). Structurally, the $\alpha_2\delta$ subunit is a heavily glycosylated 175-kDa protein that is encoded by a single gene that is post-translationally cleaved to yield the disulfide-linked α_2 and δ proteins (7, 8). Experimental evidence supports a single transmembrane topology of the $\alpha_2\delta$ subunit in which all but the transmembrane sequence and 5 carboxyl-terminal amino acids are extracellular (9–11).

Coexpression of mRNA encoding the Ca^{2+} channel $\alpha_2\delta$ subunit has been shown to modify many properties of the α_1 subunit, including increasing the macroscopic current amplitude (12, 13), accelerating the activation (14) and inactivation kinetics, and shifting the voltage dependence of activation to more hyperpolarizing potentials.[1] However, the physical structures and molecular interactions that mediate these effects are entirely unknown.

Interaction sites on the pore-forming α subunit have been identified for several voltage-dependent ion channel auxiliary subunits, including the Ca^{2+} and K^+ channel β subunits. The binding site of the Ca^{2+} channel β subunit has been localized to a region of approximately 18 amino acids in the α_1 subunit I-II cytoplasmic linker (15), and the corresponding interaction site on the β subunit has also been described (16). Interaction sites between K^+ channel α and β subunits have been mapped to the amino-terminal A and B box (17, 18) near the cytoplasmic region that is also responsible for the subfamily-specific assembly of α subunit multimers (19, 20).

Unlike the previously described cytoplasmic interactions, assessment of interactions between two transmembrane proteins has generally been more challenging. Transmembrane proteins such as the Ca^{2+} channel $\alpha_2\delta$ subunit are often extensively glycosylated, which may preclude the use of bacterial, insect, or *in vitro* expression systems because glycosylation is frequently species-dependent. Likewise, the expression and correct formation of disulfide linkages is also difficult to reproduce in an *in vitro* expression system. Also, although there are reports of successful uses of the two-hybrid yeast expression system to map interaction sites of two transmembrane proteins (21), these have often been performed on a more limited basis after initial investigations localized interaction domains using mammalian expression systems. Using transiently transfected human tsA201 cells, we have implicated the extracellular domain of the $\alpha_2\delta$ subunit in the assembly with the α_1 subunit and have also shown that this region is responsible for modulation of dihydropyridine binding affinity to the α_1 subunit.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—tsA201 cells (SV40 large T antigen transformed HEK 293 cells) (Cell Genesis, Foster City, CA) were maintained at 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Transfections were performed using the calcium phosphate method on 50–70% confluent cells. Generally 30 μg of each channel subunit DNA (for 150-mm dish) was added to 1.25 ml of 250 mM sterile filtered $CaCl_2$. An equal volume of 2 × sterile HEBS (274 mM NaCl, 40 mM HEPES, 12 mM dextrose, 10 mM KCl, 1.4 mM Na_2HPO_4, adjusted to final pH 7.05) was added drop by drop to the Ca^{2+}/DNA mixture with constant agitation. The precipitate was allowed to form for 30 min and added dropwise to the plated cells. The medium was changed the next day.

Construction of Plasmids for Mammalian Cell Transfection—The cDNA encoding the rat brain $\alpha_2\delta$ subunit and truncated forms were all transferred to pcDNA3 (Invitrogen) and have been described previously (10). The α_{1S} repeat III was created by polymerase chain reaction utilizing a forward primer beginning at nucleotide 2544 and a reverse

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[1] R. Felix and K. P. Campbell, unpublished observations.

primer beginning at nucleotide 3489. A Kozak initiation start consisting of CCACCATGG (where the methionine start site is underlined) was created in the forward primer along with a *Kpn*I site for insertion into polylinker of pcDNA3. The reverse primer contained an in frame termination site and a *Xba*I site for ligation.

Cell Membrane Preparation—tsA201 cells were harvested 48 h after transfection by washing two times with 10 ml of phosphate-buffered saline and collected by centrifugation at 3,000 rpm for 5 min. Cell membranes were prepared immediately by resuspending cell pellet from one 150-mm plate in 20 ml of ice-cold hypotonic lysis buffer (10 mM Tris, pH 7.4 with 0.64 mM benzamidine, and 0.23 mM PMSF).[2] After a 15-min incubation on ice, swollen cells were disrupted by five strokes with a Dounce homogenizer. Lysed cells were centrifuged at 3,000 rpm for 10 min at 4 °C. The supernatant was then centrifuged at 35,000 rpm for 37 min to collect the membranes. The membrane pellet was resuspended in 1 ml of Buffer I (0.3 M sucrose, 20 mM Tris, pH 7.4, 1.0 mM PMSF, and 0.75 mM benzamidine) and passed through a 28 gauge needle.

Binding Assays—Binding assays were performed in 50 mM Tris, pH 7.4, 0.23 mM PMSF, 0.64 mM benzamidine, and 1.0 mg/ml bovine serum albumin (binding buffer) in a final assay volume of 500 μl. For saturation analysis, 0.05–2 nM (+)-[^{3}H]PN200-110 (Amersham Corp.) were incubated in the dark with 80 μg of membrane protein for 60 min at 37 °C. Nonspecifically bound ligand was determined by the addition of 50 μM nitrendipine. Specific binding sites were determined by subtracting nonspecific binding from total binding. Radiolabeled membranes were washed three times with 5 ml of ice-cold binding buffer on a GF/B glass fiber filter (Whatman) using a Brandel cell harvester (Brandel, Gaithersburg, MD). Data were fitted by a single-site binding model applying nonlinear regression analysis using GraFit software (Trithacus Software, Staines, UK).

Immunoprecipitation—Cell membranes (500 μg) were solubilized in 1 ml of total volume of 1% (w/v) digitonin and 1 M NaCl (final concentrations) for 1 h at 4 °C on a rolling platform. Protease inhibitors were added at a concentration of 0.23 mM PMSF and 0.64 mM benzamidine. Solubilized protein was isolated by centrifugation at 50,000 rpm for 15 min at 4 °C and subsequently diluted 2-fold with ice-cold double distilled H_20. Solubilized protein was added to 30 μl of protein G-Sepharose that had been preincubated overnight with sheep 41 antiserum (α_{1C} II-III loop) (4) or IIF7 ascites (2).

Trypsin Digestion of Triads and Sucrose Gradient Fractionation—Rabbit skeletal muscle triads (22) were resuspended to a concentration of 4 mg/ml in Buffer I. Trypsin concentrations were varied from 1:1000 to 1:10 (trypsin:protein), and the incubation time was variable between 0 and 160 min at 37 °C. The reaction was terminated by the addition of 1 mM PMSF. Trypsin digested triads were then solubilized in 1% digitonin and 0.5 M NaCl for 1 h at 4 °C followed by centrifugation at 70,000 rpm for 30 min. Solubilized protein was added to WGA-Sepharose (Sigma) and incubated for 3 h at 4 °C. Bound protein was eluted in 300 mM *N*-acetylglucosamine in Buffer I. For sucrose gradient fractionation, samples were concentrated to 600 μl in a YM100 (Amicon) concentration unit. Samples were loaded onto 16-ml linear gradients of 5–20% (w/w) sucrose in 100 mM NaCl, 50 mM Tris, pH 7.4, and 0.1 mM PMSF. Gradients were centrifuged in a Beckman SW 28 rotor with SW 28.1 buckets for 2 h at 150,000 rpm at 4 °C. Gradients were then fractionated (1.2 ml) from the top with an Isco gradient fractionator, and 100 μl of each was analyzed on a 5–16% SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue or immunoblotted with monoclonal antibody IIF7 or rabbit 136 polyclonal against the Ca^{2+} channel α_2 subunit (23).

Determination of Monoclonal Antibody Recognition Epitopes—Monoclonal antibodies IIF7 and IIC12 (2) were used to screen 2×10^4 clones of α_{1S} subunit epitope library in Y1090 *Escherichia coli*. Inserts were amplified from pure phage positives by polymerase chain reaction using primers directed to λgt11 phage arms. These were directly inserted into a T-vector (made from Bluescript Sk$^-$ plasmid) for sequencing. All inserts were sequenced using either the dideoxy chain termination method Sequenase II (U. S. Biochemical Corp.) or automated sequencer (Applied Biosystems, Inc.).

[2] The abbreviations used are: PMSF, phenylmethanesulfonyl fluoride; [^{3}H]PN200-110, isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-([^{3}H]methoxycarbonyl)-pyridine-3-carboxylate; WGA, wheat germ agglutinin.

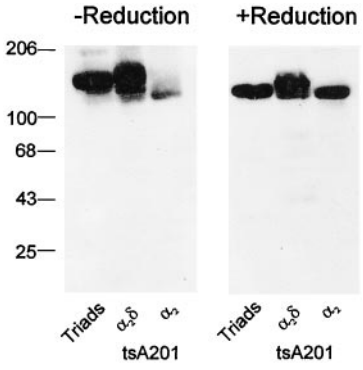

FIG. 1. **Comparison of native skeletal muscle $\alpha_2\delta$ subunit and $\alpha_2\delta$ expressed transiently in tsA201 cells.** Skeletal muscle triads (100 μg) (*Triads*) or total membranes of tsA201 cells transfected with either the full-length $\alpha_2\delta$ subunit ($\alpha_2\delta$) or α_2 protein (α_2) (200 μg each) were subjected to SDS-polyacrylamide gel electrophoresis on a 5–16% gradient gel under nonreducing and reducing conditions. Transfer was stained with polyclonal antibody against the $\alpha_2\delta$ subunit (Rabbit 136) and developed using enhanced chemiluminescence (Amersham Corp.). Molecular mass markers appear on the *left*.

RESULTS

Because of the extensive post-translational processing events involved in the formation of the $\alpha_2\delta$ subunit (*N*-linked glycosylation, disulfide linkages, and subunit cleavage), we chose to utilize the mammalian tsA201 cell line for expression. The endogenous proteolytic cleavage between the α_2 and δ subunits was investigated by Western blot analysis of membranes from cells transfected with the full-length $\alpha_2\delta$ subunit. In nonreducing conditions, an antibody directed against the α_2 subunit recognized a protein of 175 kDa in both skeletal muscle triads and in membranes prepared from tsA201 cells transfected with the full-length $\alpha_2\delta$ subunit (Fig. 1). One apparent difference between native and transfected $\alpha_2\delta$ protein is that the transfected $\alpha_2\delta$ protein ran as a broader band. This may reflect larger amounts of incompletely processed forms including untrimmed glycosylation and immature noncleaved protein that often result from transient expression. Cells expressing only the α_2 subunit produced a protein migrating at 150 kDa in both the presence and the absence of reducing agents, which is consistent with the addition of more than 40 kDa of *N*-linked oligosaccharide. When identical cell membranes were electrophoresed in reducing conditions, native skeletal muscle $\alpha_2\delta$ subunit shifted to an apparent molecular mass of 150 kDa. Likewise, there was a noticeable shift in molecular mass of the transfected $\alpha_2\delta$ protein, suggesting that the cleavage site and disulfide linkages are similar to native protein.

To test the involvement of the extracellular domain of the $\alpha_2\delta$ subunit in the interaction with the α_1 subunit, coimmunoprecipitation experiments were performed using cells transfected with both α_{1C} subunit and either the full-length $\alpha_2\delta$ subunit or any one of the truncated $\alpha_2\delta$ subunit constructs (Fig. 2*A*). Cell membranes were solubilized in 1% digitonin and 1 M NaCl prior to immunoprecipitation. The full-length $\alpha_2\delta$ subunit assembles with the α_{1C} subunit as demonstrated by its coimmunoprecipitation with an anti-α_{1C} antibody and detection by Western blot analysis with an anti-α_2 antibody (Fig. 2*B*). No $\alpha_2\delta$ protein was immunoprecipitated from control untransfected cells. In addition, no $\alpha_2\delta$ protein was precipitated from cells in the absence of the α_1 subunit (data not shown). Truncation of 450 extracellular amino-terminal amino acids of the $\alpha_2\delta$ subunit abolished the ability of this protein to assemble with the α_{1C} subunit, despite its abundance in the starting material. Likewise, the α_2 subunit expressed in the absence of the δ subunit was also

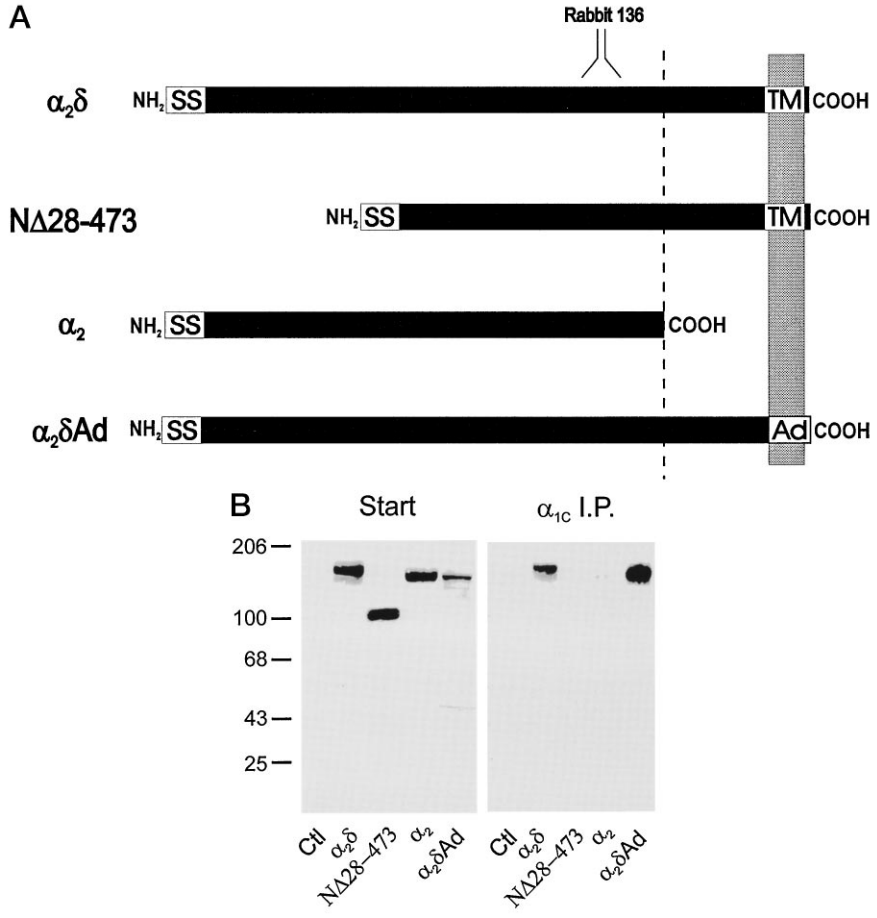

FIG. 2. **Coimmunoprecipitation of full-length and truncated $\alpha_2\delta$ subunits with the α_{1C} subunit expressed in tsA201 cells.** *A*, shown are the full-length rat brain $\alpha_2\delta$ subunit, amino-terminal truncation NΔ28–473, carboxyl-terminal truncation consisting of the α_2 protein, and chimera containing transmembrane of adhalin ($\alpha_2\delta Ad$). The plasma membrane is shown as a *vertical rectangle*. The *vertical dashed line* indicates the cleavage site between the α_2 and δ subunits. Also shown is the antibody recognition site of Rabbit 136 at amino acids 839–856. *B*, cell membranes (500 μg) from tsA201 cells transfected with cDNAs encoding either the α_{1C} subunit alone or in combination with full-length $\alpha_2\delta$ subunit, NΔ29–473, α_2, or $\alpha_2\delta$Ad were solubilized in 1% digitonin and 1 M NaCl and incubated with protein G-Sepharose preincubated with a polyclonal antibody against the α_{1C} subunit (sheep 41). Sepharose beads were washed extensively, and immunoprecipitating protein was resolved using 5–16% SDS-polyacrylamide gel electrophoresis under reducing conditions. Shown are Western blots of the starting solubilized material (200 μg) (*Start*) and the α_{1C} immunoprecipitates (α_{1C} *I.P.*). Both were incubated with a polyclonal antibody against the $\alpha_2\delta$ subunit (Rabbit 136) and developed using enhanced chemiluminescence.

unable to coimmunoprecipitate with the α_{1C} subunit.

We also investigated the role of the transmembrane domain of the δ subunit in assembly with the α_{1C} subunit (Fig. 2*B*). Substitution of the transmembrane domain from adhalin, an unrelated type I transmembrane protein (recently renamed α-sarcoglycan), did not appear to alter the ability of the protein to assemble with the α_{1C} subunit. In this chimera, the 5 cytoplasmic amino acids of the $\alpha_2\delta$ protein were also substituted with adhalin sequence. Therefore, we conclude that neither intracellular or transmembrane sequences of the $\alpha_2\delta$ subunit are required for interaction with the α_1 subunit.

The region of the $\alpha_2\delta$ subunit responsible for modulation of dihydropyridine binding to the α_{1C} subunit was also investigated. Although [^{3}H]PN200-110 binding to whole cell tsA201 cell membranes was often low and nonsaturable when α_{1C} was transfected in the absence of any auxiliary subunit, several experiments resulted in significant and saturable binding that allowed us to determine the binding affinity (K_d) and binding capacity (B_{max}) using saturation analysis (Table I). Cells expressing α_{1C} alone had an average B_{max} of 94.6 ± 51.7 fmol/mg (n = 4).

Coexpression of the full-length $\alpha_2\delta$ subunit with the α_{1C} subunit resulted in a significant increase in binding, most of which could be accounted for by a significant mean increase in the binding affinity (Table I). Binding was saturable in all experiments. There appeared to be little effect of the $\alpha_2\delta$ subunit on B_{max} (B_{max} = 133 ± 73.5 fmol/mg), although there was significant error between experiments in the B_{max} depending on the transfection efficiency. Likewise, Western blot analysis on whole cell membranes from transfected cells showed no effect of coexpression of the $\alpha_2\delta$ subunit on the protein expression of the α_1 subunit (data not shown). The binding affinity, however, was not affected by the differences in transfection efficiency.

TABLE I

Comparison of K_d and saturable [^{3}H]PN200–110 binding to total microsomes from tsA201 cells transfected with α_{1C} and $\alpha_2\delta$ constructs

tsA201 cells were transfected with the cDNAs encoding Ca^{2+} channel subunits in the combinations indicated. K_d values were calculated from saturation binding data using GraFit. Data are presented as the means ± S.E. Also shown are the number of experiments (separate transfections) in which saturable binding was measured and the total number of experiments that were performed.

Membranes	K_d ± S.E.	Saturable/total
	nM	
α_{1C}	1.34 ± 0.79	4/7
$\alpha_{1C}\alpha_2\delta^a$	0.16 ± 0.08	5/5
$\alpha_{1C}\alpha_2$	0.65 ± 0.26	3/5
α_{1C}NΔ28–473	1.31 ± 0.74	3/5
$\alpha_{1C}\alpha_2\delta$Ad^a	0.21 ± 0.16	4/4

^{a} Paired t test, $p < 0.05$ versus α_{1C}.

As expected, when the α_2 subunit was coexpressed with the α_{1C} subunit, there was no effect on [^{3}H]PN200-110 binding. This is consistent with coimmunoprecipitation experiments that demonstrated the inability of the α_2 subunit to associate with α_1 in the absence of the δ subunit. However, coexpression of the $\alpha_2\delta$Ad chimera, in which the transmembrane domain of the $\alpha_2\delta$ subunit was replaced with that of adhalin, increased [^{3}H]PN200-110 binding affinity to approximately the same extent as full-length $\alpha_2\delta$ protein.

Because the α_1 subunit is very large and difficult to express, we chose an alternative approach to identify regions interacting with the $\alpha_2\delta$ subunit. Our approach was to trypsinize skeletal muscle microsomes containing native dihydropyridine re-

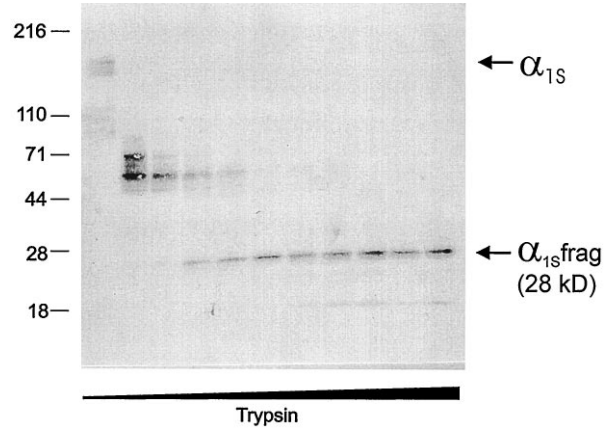

FIG. 3. **Identification of 28-kDa α_{1S} trypsin fragment from WGA-Sepharose eluate.** Skeletal muscle triads were resuspended to a concentration of 4 mg/ml, and trypsin concentrations and incubation times (0 to 160 min) were varied to achieve gradually increasing digestion. Trypsinized triads were then solubilized in 1% digitonin and loaded onto a wheat germ agglutinin-Sepharose column. Bound protein was eluted with 300 mM *N*-acetylglucosamine. Aliquots were separated onto 5–16% SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred to nitrocellulose. Transfer was stained with a monoclonal antibody IIF7 against the α_{1S} IIIS5-S6 region. The *arrows* indicate full-length α_{1S} protein and α_{1S} fragment (*frag*) of 28 kDa.

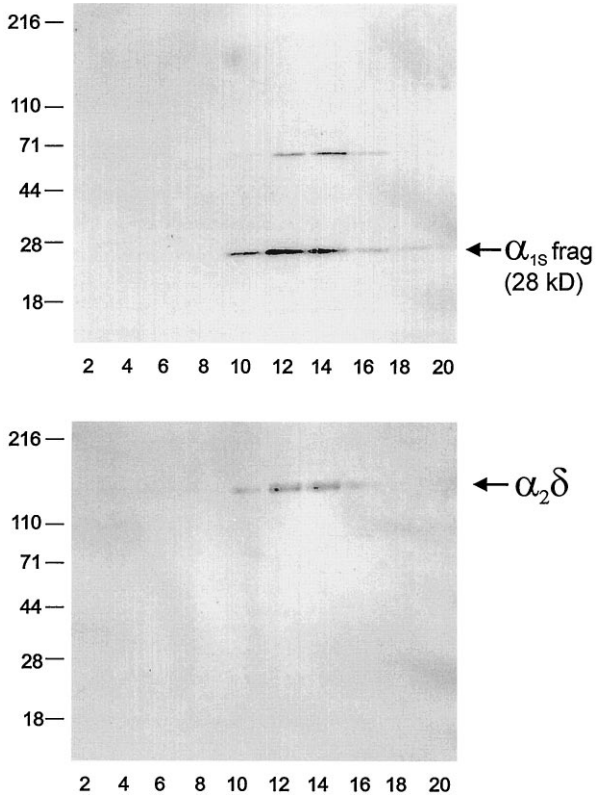

FIG. 4. **Sucrose gradient fractionation of trypsinized skeletal muscle WGA eluate.** Trypsinized skeletal muscle triads were loaded onto WGA-Sepharose and eluted with 300 mM *N*-acetylglucosamine. Samples were loaded onto 5–20% linear sucrose gradients, centrifuged, and fractionated from the top. Alternate fractions were analyzed on a 5–16% SDS-polyacrylamide gradient gel and immunoblotted with either monoclonal antibody IIF7 against the α_{1S} subunit (*top*) or polyclonal antibody against the $\alpha_2\delta$ subunit (Rabbit 136) (*bottom*). Fraction number is listed on the *bottom*, and molecular mass is indicated at the *left*. *Arrows* indicate α_{1S} fragment (*frag*) and full-length $\alpha_2\delta$ subunit.

ceptors and follow the α_{1S} subunit fragments remaining in association with the $\alpha_2\delta$ subunit during WGA affinity chromatography. By taking advantage of the selective ability of the glycosylated $\alpha_2\delta$ subunit to bind WGA, any α_{1S} fragment identified is presumed to bind WGA only through its interaction with the $\alpha_2\delta$ subunit. With increasing concentrations of trypsin, an α_{1S} subunit-specific monoclonal antibody that recognizes an epitope within the first extracellular loop of the IIIS5-IIIS6 linker (amino acid 955–1005) (IIF7) detected 28- and 18-kDa α_{1S} subunit fragments eluted from a WGA-Sepharose column (Fig. 3). Sucrose gradient fractionation was subsequently used to demonstrate cosedimentation of the α_{1S} subunit fragments with the intact full-length $\alpha_2\delta$ subunit (Fig. 4). Multiple tryptic fragments of α_1 did not bind WGA and were identified in the starting material, including carboxyl-terminal fragments (identified by monoclonal antibody IIC12) and fragments of repeat I and II and the II-III loop (identified by polyclonal antibody sheep DHPR) (data not shown).

To test the ability of α_{1S} repeat III to associate with the $\alpha_2\delta$ subunit, we cotransfected tsA201 cells with constructs containing only repeat III and the full-length $\alpha_2\delta$ subunit. Although the $\alpha_2\delta$ subunit and repeat III were well expressed, we were unable to detect stable interactions between these two proteins using coimmunoprecipitation assays after solubilization in 1% digitionin and 1 M NaCl (data not shown). This suggests that expression of the α_{1S} subunit repeat III by itself is not sufficient to form stable interactions with the $\alpha_2\delta$ subunit.

DISCUSSION

Our data support a model whereby the interaction sites between the $\alpha_2\delta$ and α_1 subunits are entirely extracellular, because transmembrane modifications of the $\alpha_2\delta$ subunit did not appear to alter coassembly with the α_1 subunit. Morever, our data suggest a requirement for nontransmembrane domains of the δ subunit in determining a stable association between the $\alpha_2\delta$ and α_1 proteins, because the α_2 protein by itself could not support interaction. δ may contain the interaction site, or the tertiary structure it confers on α_2 through its disulfide linkages may enable α_2 to directly interact with the α_1 subunit. Low expression of δ expressed alone resulted in our inability to distinguish between these possibilities. However, δ was shown to be able to compete with full-length $\alpha_2\delta$ protein in *Xenopus* oocytes and inhibit its stimulatory effects on current amplitude (10), and expression studies in tsA201 cells demonstrated that coexpression of δ can significantly modulate the biophysical properties of the α_{1C} subunit.[1]

Interestingly, our data are consistent with reports regarding a functionally related pair of proteins, the Na^+,K^+-ATPase α and auxiliary β subunit, in which the interaction sites have also been localized to extracellular domains (24, 25). In this case, the yeast two-hybrid assay was successful in further localizing the site of interaction on the β subunit to the 61 amino acids most proximal to the membrane (21). Although the interaction sites between the voltage-dependent Na^+ channel α and β_1 or β_2 subunits have not been mapped, it is interesting to note that deletion of the β_1 intracellular domain does not alter functional effects of β_1 subunit coexpression (26), suggesting that this interaction may also be in the extracellular domain.

Repeat III of the Ca^{2+} channel α_{1S} subunit appears to interact strongly with the $\alpha_2\delta$ subunit after extensive trypsinization, although we cannot exclude the involvement of other unidentified fragments (especially in repeat IV) based on our inability to recognize small tryptic fragments with specific antibodies. Interestingly, whereas repeat III remains in association with the $\alpha_2\delta$ subunit after extensive trypsinization, we were unable to reconstitute the interaction between this small region and $\alpha_2\delta$ in an expression system. This suggests that

multiple regions of the α_1 subunit may be involved in assembly with the $\alpha_2\delta$ subunit. In analogous studies on the voltage-dependent Na^+ channel, multiple domains within the carboxyl-terminal half of the skeletal muscle Na^+ channel α subunit were shown to be required for functional response of the coexpressed Na^+ channel β_1 subunit on inactivation kinetics (27).

Association of the $\alpha_2\delta$ subunit with the carboxyl-terminal half of the α_1 subunit is consistent with the significant effects that we and others have measured of the $\alpha_2\delta$ subunit on dihydropyridine binding affinity (28). The membrane spanning segments IIIS6 and IVS6 of the α_{1S} subunit have recently been shown to contain amino acids critical for dihydropyridine binding (29), although the S5-S6 extracellular linkers of the III and IV repeats also confer dihydropyridine sensitivity (30). The extracellular domain of the $\alpha_2\delta$ subunit, which is capable of modulating dihydropyridine binding, may be interacting with sites at or near these dihydropyridine binding sites within the III and IV repeats.

Based on several observations regarding the extracellular regions of the α_1 subunit, we can speculate on the exact sites of interaction. Most extracellular loops of the α_1 subunit are small in size, the smallest being only 7 amino acids. The largest extracellular loops, and thus the regions with the highest probability of interacting with the $\alpha_2\delta$ subunit, are the S5-S6 linkers, which also contain the pore. Experimental evidence regarding the folding pattern of the α_1 subunit suggests that the S5-S6 regions of all four repeats closely interact to form the central pore (31). These amino acids near the pore, while neighboring each other in tertiary structure, are far apart in primary structure, and thus it may be difficult to reconstitute the structure of this region by expression of a single repeat. Based on the substantial effects of the $\alpha_2\delta$ subunit on dihydropyridine binding affinity, we predict that the smallest regions of interaction may be within the S5-S6 extracellular loops, particularly of repeat III, because this region copurifies with the $\alpha_2\delta$ subunit on WGA chromatography.

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