Extracellular Interaction of the Voltage-dependent Ca\(^{2+}\) Channel \(\alpha_2\delta\) and \(\alpha_1\) 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 \(\alpha_{1C}\) subunit was investigated. Transiently transfected tsA201 cells processed the \(\alpha_2\delta\) subunit properly as disulfide linkages and cleavage sites between the \(\alpha_2\) and \(\delta\) subunits were shown to be similar to native channel protein. Coimmunoprecipitation experiments demonstrated that in the absence of \(\delta\) subunits, \(\alpha_2\) subunits do not assemble with \(\alpha_1\) subunits. Furthermore, the transmembrane and cytoplasmic sequences in \(\delta\) can be exchanged with those of an unrelated protein without any effect on the association between the \(\alpha_2\delta\) and \(\alpha_1\) proteins. Extracellular domains of the \(\alpha_2\delta\) subunit are also shown to be responsible for increasing the binding affinity of \([3H]PN200-110\) (isopropyl-4-(2,1,3-benzoxadiazol-1-yl)-1,4-dihydro-2,6-dimethyl-5-(([1]H)methoxycarbonyl)-pyridine-3-carboxylate) for the \(\alpha_{1C}\) subunit. Investigation of the corresponding interaction site on the \(\alpha_1\) subunit revealed that although tryptic peptides containing repeat III of native \(\alpha_{1S}\) 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 \(\alpha_1\) 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 posttranslationally cleaved to yield the disulfide-linked 175-kDa protein that is encoded by a single gene that is posttranslationally cleaved to yield the disulfide-linked 175-kDa protein that is encoded by a single gene that is posttranslationally cleaved to yield the disulfide-linked 175-kDa protein. Coimmunoprecipitation experiments demonstrated that in the absence of \(\delta\) subunits, \(\alpha_2\) subunits do not assemble with \(\alpha_1\) subunits. Furthermore, the transmembrane and cytoplasmic sequences in \(\delta\) can be exchanged with those of an unrelated protein without any effect on the association between the \(\alpha_2\delta\) and \(\alpha_1\) proteins. Extracellular domains of the \(\alpha_2\delta\) subunit are also shown to be responsible for increasing the binding affinity of \([3H]PN200-110\) (isopropyl-4-(2,1,3-benzoxadiazol-1-yl)-1,4-dihydro-2,6-dimethyl-5-(([1]H)methoxycarbonyl)-pyridine-3-carboxylate) for the \(\alpha_{1C}\) subunit. Investigation of the corresponding interaction site on the \(\alpha_1\) subunit revealed that although tryptic peptides containing repeat III of native \(\alpha_{1S}\) 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 \(\alpha_1\) subunit.

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Coexpression of mRNA encoding the Ca\(^{2+}\) channel \(\alpha_2\delta\) subunit has been shown to modify properties of the \(\alpha_1\) 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. However, the physical structures and molecular interactions that mediate these effects are entirely unknown.

Interaction sites on the pore-forming \(\alpha\) subunit have been identified for several voltage-dependent ion channel auxiliary subunits, including the Ca\(^{2+}\) and K\(^{+}\) channel \(\beta\) subunits. The binding site of the Ca\(^{2+}\) channel \(\beta\) subunit has been localized to a region of approximately 18 amino acids in the \(\alpha_{1}\) subunit I-II cytoplasmic linker (15), and the corresponding interaction site on the \(\beta\) subunit has also been described (16). Interaction sites between K\(^{+}\) channel \(\alpha\) and \(\beta\) 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 \(\alpha\) 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 \(\alpha_1\) subunit and have also shown that this region is responsible for modulation of dihydropyridine binding affinity to the \(\alpha_1\) subunit.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—tsA201 cells (SV40 large T antigen transformed HEK 293 cells) (Cell Genetics, 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 \(\mu\)g/ml streptomycin. Transfections were performed using the calcium phosphate method on 50–70% confluent cells. Generally 30 \(\mu\)g of each channel subunit DNA (for 150-mm dish) was added to 1.25 ml of 250 \(\mu\)M sterile filtered CaCl\(_2\). An equal volume of 2 \(\times\) sterile HEBS (274 mM NaCl, 40 mM HEPES, 12 mM dextrose, 10 mM KCl, 1.4 mM NaH\(_2\)PO\(_4\), 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 \(\alpha_{1S}\) 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 Rokaz initiation start consisting of CCACCAGTTG (where the methionine start site is underlined) was created in the forward primer along with a KpnI site for insertion into polylinker of pCDNA3. The reverse primer contained an in-frame termination site and a XbaI site for ligation.

Cell Membrane Preparation—tSA201 cells were harvested 48 h after transfection by washing twice 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). After a 15-min incubation on ice, swollen cells were disrupted by five strokes with a Dounce homogenizer. Lysed cells were 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 (±)

Calcium Channel αδ and α1 Subunit Interactions

FIG. 1. Comparison of native skeletal muscle αδ subunit and αδα expressed transiently in tSA201 cells. Skeletal muscle triads (100 μg) (Triads) or total membranes of tSA201 cells transfected with either the full-length αδ subunit (αδα) or αδ protein (αδ) (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 αδ 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 αδ 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 α2 and δ subunits was investigated by Western blot analysis of membranes from cells transfected with the full-length αδ subunit. In nonreducing conditions, an antibody directed against the α2 subunit recognized a protein of 175 kDa in both skeletal muscle triads and in membranes prepared from tSA201 cells transfected with the full-length αδ subunit (Fig. 1). One apparent difference between native and transfected αδ subunit is that the transfected αδ 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 α2 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 αδ subunit shifted to an apparent molecular mass of 150 kDa. Likewise, there was a noticeable shift in molecular mass of the transfected αδ protein, suggesting that the cleavage site and disulfide linkages are similar to native protein.

To test the involvement of the extracellular domain of the αδ subunit in the interaction with the α1 subunit, communoprecipitation experiments were performed using cells transfected with both α1C subunit and either the full-length αδ subunit or any one of the truncated αδ subunit constructs (Fig. 2A). Cell membranes were solubilized in 1% digitonin and 1 mM NaCl prior to immunoprecipitation. The full-length αδ subunit assembles with the α1C subunit as demonstrated by its communoprecipitation with an anti-α1C antibody and detection by Western blot analysis with an anti-α2 antibody (Fig. 2B). No αδ protein was immunoprecipitated from control untransfected cells. In addition, no αδ protein was precipitated from cells in the absence of the α1 subunit (data not shown). Truncation of 450 extracellular amino-terminal amino acids of the αδ subunit abolished the ability of this protein to assemble with the α1C subunit, despite its abundance in the starting material. Likewise, the α2 subunit expressed in the absence of the δ subunit was also

2 The abbreviations used are: PMSF, phenylmethanesulfonyl fluoride; [3H]PN200-110, isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-[4H]methoxycarbonyl)-pyridine-3-carboxylate; WGA, wheat germ agglutinin.
unable to coimmunoprecipitate with the \( \alpha_{1C} \) subunit.

We also investigated the role of the transmembrane domain of the \( \delta \) subunit in assembly with the \( \alpha_{1C} \) subunit (Fig. 2B). Substitution of the transmembrane domain from adhalin, an unrelated type I transmembrane protein (recently renamed \( \sigma \)-sarcoglycan), did not appear to alter the ability of the protein to assemble with the \( \alpha_{1C} \) subunit. In this chimera, the 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 \( \alpha_{1} \) subunit.

The region of the \( \alpha_{2}\delta \) subunit responsible for modulation of dihydropyridine binding to the \( \alpha_{1C} \) subunit was also investigated. Although \( [\text{H}]\text{PN200–110} \) binding to whole cell tsA201 cell membranes was often low and nonsaturable when \( \alpha_{1C} \) 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 \( \alpha_{1C} \) 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 \( \alpha_{1C} \) 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 \( \alpha_{1} \) subunit (data not shown). The binding affinity, however, was not affected by the differences in transfection efficiency.

As expected, when the \( \alpha_{2} \) subunit was coexpressed with the \( \alpha_{1C} \) subunit, there was no effect on \( [\text{H}]\text{PN200–110} \) binding. This is consistent with communoprecipitation experiments that demonstrated the inability of the \( \alpha_{2} \) subunit to associate with \( \alpha_{1} \) in the absence of the \( \delta \) subunit. However, coexpression of the \( \alpha_{2}\delta \) chimera, in which the transmembrane domain of the \( \alpha_{2} \) subunit was replaced with that of adhalin, increased \( [\text{H}]\text{PN200–110} \) binding affinity to approximately the same extent as full-length \( \alpha_{2}\delta \) protein.

Because the \( \alpha_{1} \) 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-


Receptors and follow the \( \alpha_{1S} \) subunit fragments remaining in association with the \( \alpha\delta \) subunit during WGA affinity chromatography. By taking advantage of the selective ability of the glycosylated \( \alpha\delta \) subunit to bind WGA, any \( \alpha_{1S} \) fragment identified is presumed to bind WGA only through its interaction with the \( \alpha\delta \) subunit. With increasing concentrations of trypsin, an \( \alpha_{1S} \) 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 \( \alpha_{1S} \) subunit fragments eluted from a WGA-Sepharose column (Fig. 3). Sucrose gradient fractionation was subsequently used to demonstrate cosedimentation of the \( \alpha_{1S} \) subunit fragments with the intact full-length \( \alpha\delta \) subunit (Fig. 4). Multiple tryptic fragments of \( \alpha_{1S} \) 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 \( \alpha_{1S} \) repeat III to associate with the \( \alpha\delta \) subunit, we cotransfected tsA201 cells with constructs containing only repeat III and the full-length \( \alpha\delta \) subunit. Although the \( \alpha\delta \) subunit and repeat III were well expressed, we were unable to detect stable interactions between these two proteins using communoprecipitation assays after solubilization in 1% digitonin and 1 M NaCl (data not shown). This suggests that expression of the \( \alpha_{1S} \) subunit repeat III by itself is not sufficient to form stable interactions with the \( \alpha\delta \) subunit.

**DISCUSSION**

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

Interestingly, our data are consistent with reports regarding a functionally related pair of proteins, the Na\(^+\),K\(^+\)-ATPase \( \alpha \) and auxiliary \( \beta \) 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 \( \beta \) subunit to the 61 amino acids most proximal to the membrane (21). Although the interaction sites between the voltage-dependent Na\(^+\) channel \( \alpha \) and \( \beta_1 \) or \( \beta_2 \) subunits have not been mapped, it is interesting to note that deletion of the \( \beta_1 \) intracellular domain does not alter functional effects of \( \beta_1 \) subunit coexpression (26), suggesting that this interaction may also be in the extracellular domain. Repeat III of the Ca\(^{2+}\) channel \( \alpha_{1S} \) subunit appears to interact strongly with the \( \alpha\delta \) 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 \( \alpha\delta \) subunit after extensive trypsinization, we were unable to reconstitute the interaction between this small region and \( \alpha\delta \) in an expression system. This suggests that
multiple regions of the α1 subunit may be involved in assembly with the αδ subunit. In analogous studies on the voltage-dependent Na+ channel, multiple domains within the carboxy-terminal half of the skeletal muscle Na+ channel α subunit were shown to be required for functional response of the coexpressed Na+ channel β1 subunit on inactivation kinetics (27).

Association of the αδ subunit with the carboxy-terminal half of the α1 subunit is consistent with the significant effects that we and others have measured of the αδ subunit on dihydropyridine binding affinity (28). The membrane spanning segments IIIS6 and IVS6 of the α1S 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 αδ 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 α1 subunit, we can speculate on the exact sites of interaction. Most extracellular loops of the α1 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 αδ subunit, are the S5-S6 linkers, which also contain the pore. Experimental evidence regarding the folding pattern of the α1 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, and 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 αδ 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 αδ subunit on WGA chromatography.

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REFERENCES
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