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Dual Function of the Voltage-Dependent Ca^{2+} Channel $\alpha_2\delta$ Subunit in Current Stimulation and Subunit Interaction

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Summary

Voltage-dependent Ca^{2+} channels are modulated by complex interactions with the $\alpha_2\delta$ subunit. In vitro translation was used to demonstrate a single transmembrane topology of the $\alpha_2\delta$ subunit in which all but the transmembrane sequence and 5 carboxy-terminal amino acids are extracellular. The glycosylated extracellular domain is required for current stimulation, as shown by coexpression of truncated $\alpha_2\delta$ subunits with α_{1A} and β_4 subunits in Xenopus oocytes and deglycosylation with peptide-N-glycosidase F. However, coexpression of the transmembrane domain-containing δ subunit reduced the stimulatory effects of full-length $\alpha_2\delta$ subunits and substitution of a different transmembrane domain resulted in a loss of current stimulation. These results support a model whereby the $\alpha_2\delta$ transmembrane domain mediates subunit interactions and the glycosylated extracellular domain enhances current amplitude.

Introduction

The highly glycosylated $\alpha_2\delta$ subunit is present in Ca^{2+} channel complexes purified from skeletal muscle (Curtis and Catterall, 1984), brain (Witcher et al., 1993), and heart (Cooper et al., 1987; Tokumaru et al., 1992). These $\alpha_2\delta$ subunits are derived from a single gene (Ellis et al., 1988) the product of which is post-translationally cleaved to yield the disulfide-linked α_2 and δ proteins (De Jongh et al., 1990; Jay et al., 1991). The brain expresses $\alpha_2\delta_b$, an alternatively spliced form of the skeletal muscle $\alpha_2\delta_a$, which differs only by an insertion of 7 amino acids and a deletion of a 19 amino acid segment between the first and second hydrophobic domains (Kim et al., 1992).

Functional coexpression of the $\alpha_2\delta$ subunit with α_{1A} in Xenopus oocytes results in an almost 10-fold stimulation of current amplitude (Mori et al., 1991). Similar effects have been recorded in other expression systems when $\alpha_2\delta$ is coexpressed with various channel subtypes (Mikami et al., 1989; Hullin et al., 1992; Williams et al., 1992; Brust et al., 1993). The mechanism of this current stimulation is unknown, but the $\alpha_2\delta$ subunit may facilitate or stabilize plasma membrane incorporation of the Ca^{2+} channel complex, as demonstrated by the effects of coexpression of $\alpha_2\delta$ on ω-conotoxin GVIA binding capacity (Brust et al., 1993). In addition, expression of $\alpha_2\delta$ increases the binding affinity of cell surface receptors for ligands such as ω-conotoxin GVIA (Brust et al., 1993), suggesting that channel structure is modified by conformational changes induced by $\alpha_2\delta$ subunit coassembly. The importance of the $\alpha_2\delta$ subunit for Ca^{2+} channel function is highlighted by the Caenorhabditis elegans *unc-36* $\alpha_2\delta$ subunit mutant, which yields the same phenotype as the *unc-2* α_1 subunit loss of function mutant (Schafer and Kenyon, 1995).

To understand how the $\alpha_2\delta$ subunit interacts with the other channel subunits, it is important to establish its transmembrane topology, since the β subunit is entirely cytoplasmic and the α_1 subunit contains extensive intracellular domains. Hydropathy analysis of the cloned $\alpha_2\delta$ subunit shows the presence of three hydrophobic regions suggesting 3 potential transmembrane domains, approximately equally spaced in the protein, with the third hydrophobic domain being only five amino acids from the carboxyl terminus (Ellis et al., 1988). As shown in Figure 1A, the three-transmembrane model predicts large intra- and extracellular domains of the $\alpha_2\delta$ subunit. Experimentally, the hydrophobic probe [^{125}I] TID (3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine) labels both the α_2 and δ proteins (Takahashi et al., 1987; Jay et al., 1991), supporting the presence of transmembrane domains in each subunit. However, hydrophobic labeling with TID may be misleading since it has been reported to incorporate into nontransmembrane proteins such as the lumenal protein calsequestrin (Mitchell et al., 1988).

An alternative to the three-transmembrane model was suggested by alkaline extraction of $\alpha_2\delta$ from skeletal muscle membranes. In the presence of reducing agents, α_2 is extracted from membranes with high pH, while δ remains membrane associated, suggesting a single transmembrane domain in the δ subunit as depicted in Figure 1B (Jay et al., 1991). Immunocytochemical experiments with site-directed antibodies were also used to demonstrate the single-transmembrane model (Brickley et al., 1995). Yet, the limited experimental data in support of the single-transmembrane topology of the $\alpha_2\delta$ subunit has resulted in the continued depiction of the $\alpha_2\delta$ subunit as a three-transmembrane protein (Wierzbicki et al., 1993; Isom et al., 1994).

Our present results support a single transmembrane model of the $\alpha_2\delta$ subunit and identify the transmembrane domain as necessary for functional interaction with the α_1 subunit. The nature of this topology, in which all but 5 amino acids are extracellular, suggests that there are no direct interactions of the $\alpha_2\delta$ subunit with cytoplasmic regulatory proteins such as the β subunit. Functional studies of the expressed full-length and truncated $\alpha_2\delta$ subunits also demonstrate that, in addition to the necessity of the transmembrane domain for subunit interaction, the structural integrity of the extracellular glycosylated domain is critical for the enhancement of current amplitude.

Results

Membrane Insertion of the $\alpha_2\delta$ Subunit

To study the transmembrane topology of the $\alpha_2\delta$ subunit, full-length and truncated rat brain $\alpha_2\delta_b$ were produced by

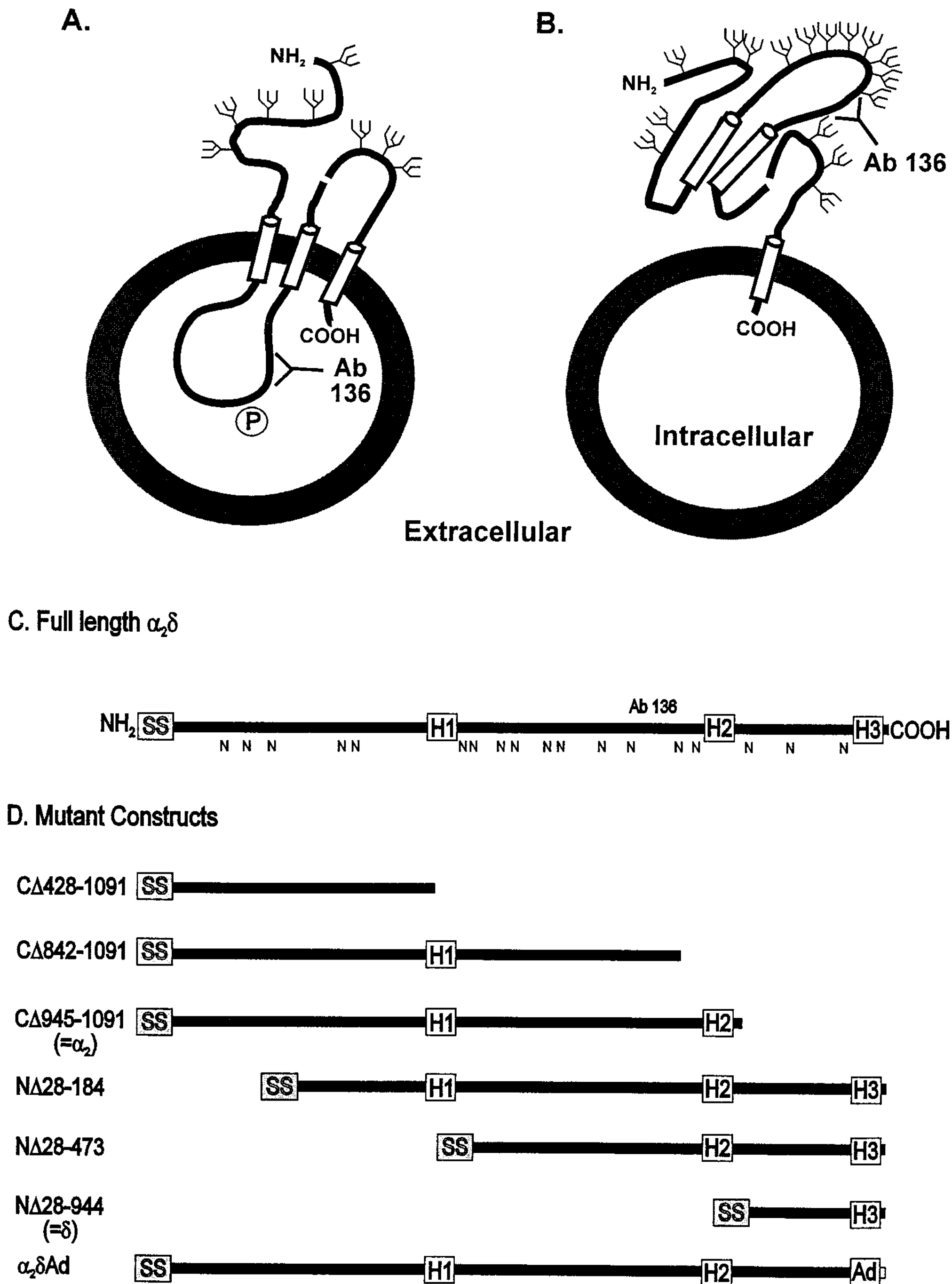

Figure 1. Topological Models of the $\alpha_2\delta$ Subunit

Schematic representation of the three-transmembrane (A) and single-transmembrane (B) models of the $\alpha_2\delta$ subunit. The location of putative N-linked glycosylation sites and a protein kinase C phosphorylation site is shown. Also labeled is the Ab 136 antibody recognition site. (C) Diagram of full-length rat brain $\alpha_2\delta_b$ subunit and (D) $\alpha_2\delta_b$ constructs used in the membrane association assay and expressed in Xenopus oocytes for functional assay. SS denotes signal sequence; N indicates putative N-linked glycosylation sites; and H1 through H3 indicate hydrophobic regions predicted to be transmembrane domains by hydropathy analysis.

in vitro translation in the presence of canine microsomal membranes. The $\alpha_2\delta_b$ subunit, henceforth referred to as $\alpha_2\delta$, is produced as an uncleaved 150 kDa glycosylated protein in the presence of membranes (Figure 2A). Since membranes are limiting in the in vitro translation system, large amounts of unglycosylated and nonmembrane-associated $\alpha_2\delta$ protein are also made. These proteins have largely been removed by an early centrifugation, although they appear faintly as a 110 kDa band. Cleavage of full-length in vitro translated $\alpha_2\delta$ into α_2 and δ

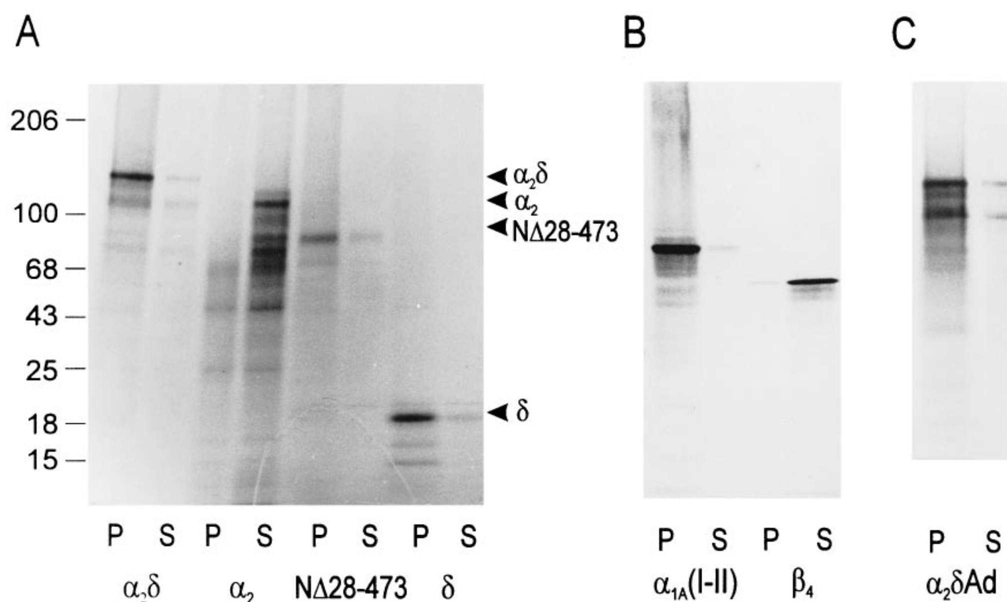

Figure 2. Alkaline Stable Membrane Insertion of Full-Length and Truncated $\alpha_2\delta$ Subunits

(A) $\alpha_2\delta$ subunits in vitro translated in the presence of canine microsomal membranes were alkaline extracted followed by separation of the membrane-associated protein (P) from the alkaline-extractable supernatant (S) and electrophoresed on SDS-polyacrylamide gels. Loss of the third hydrophobic domain (α_2) results in a shift to a primarily alkaline-extractable form, while the third hydrophobic domain, in δ, is sufficient for alkaline-inextractable membrane insertion.

(B) Demonstration of alkaline extractability as an indicator of transmembrane domains. Stable membrane incorporation of Ca^{2+} channel α_{1A} construct consisting of the first and second repeats containing 12 transmembrane domains and inability of the Ca^{2+} channel β_4 subunit to form stable membrane associations.

(C) Demonstration of a functional transmembrane domain in the $\alpha_2\delta$Ad chimera in which the transmembrane domain of $\alpha_2\delta$ has been replaced by that of adhalin. Molecular weight markers appear on the left.

subunits does not occur in the in vitro translation system, as it does in vivo (De Jongh et al., 1990; Jay et al., 1991), as there is no difference in the migration of $\alpha_2\delta$ subunit in the presence and absence of reducing agents (data not shown).

Microsomal membranes were alkaline extracted to determine which proteins had stably integrated into the membrane through well-defined transmembrane regions, as previously reported to establish topogenic sequences in human band 3 and P-type ATPases (Bamberg and Sachs, 1994; Tam et al., 1994). Full-length $\alpha_2\delta$ remains associated with the membrane after alkaline extraction, confirming the presence of at least one transmembrane domain as suggested by sequence hydropathy analysis (Figure 2A).

To determine which of the three hydrophobic regions function as transmembrane domains, the full-length $\alpha_2\delta$ subunit (Figure 1C) was truncated at the amino and carboxyl termini (Figure 1D). The 26 amino acid signal sequence was retained in all constructs. Deletion of amino acid sequences including the first (NΔ28–473) (Figure 2A) hydrophobic domain has no effect on membrane association, suggesting that its presence is not critical for membrane association. However, carboxy-terminal deletion of the δ protein (α_2), which includes only the third hydrophobic domain, results in the inability of the protein to retain its membrane association. This demonstrates that neither the first nor the second hydrophobic domain functions as a membrane anchor and that the third hydrophobic domain is necessary for membrane insertion. Furthermore, the third hydrophobic domain alone is sufficient for stable membrane incorporation as shown by the membrane association of the δ protein. These data support the model depicted in Figure 1B whereby the third hydrophobic region in δ is the sole transmembrane domain. Demonstration that this method can be used to identify integral membrane proteins is shown in Figure 2B, as the cytoplasmic β_4 subunit is shown to be completely extractable from the membrane fraction, while a protein consisting of the first and second repeats of the α_{1A} subunit remains membrane associated during alkaline treatment.

Distribution of Glycosylation on the $\alpha_2\delta_b$ Subunit

Comparison of the molecular weight of in vitro translated recombinant proteins in the presence and absence of canine microsomal membranes demonstrates the extent of glycosylation and gives further evidence in support of the single transmembrane model. Two truncated proteins, CΔ428–1091 and CΔ842–1091, are shown to contain ~12 and ~28 kDa of carbohydrate, respectively (Figure 3A). These constructs differ only by the presence of additional amino acid sequence between the first and second hydrophobic domains, which would be either intracellular in model A (Figure 1A) or extracellular in model B (Figure 1B). The additional mass contributed by the oligosaccharide in the larger of these two proteins, CΔ428–1091, confirms that the region between the first

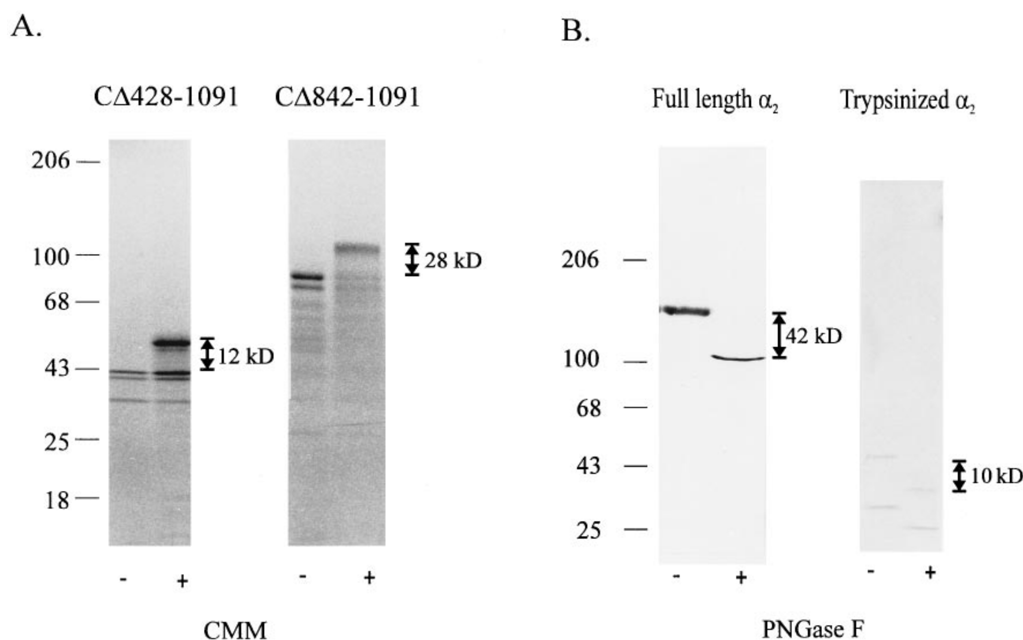

Figure 3. Glycosylation of In Vitro–Translated and Native $\alpha_2\delta$ Subunits

(A) Constructs CΔ428–1091 and CΔ842–1091, which differ only in the amount of sequence between the first and second hydrophobic domains, were in vitro translated in the presence (+) and absence (−) of canine microsomal membranes (CMM). The extent of glycosylation is shown to the right of each lane as the molecular weight difference between the glycosylated and unglycosylated forms.

(B) Immunoblot analysis of glycosylated fragments of the native $\alpha_2\delta_a$ subunit showing nondigested and partially trypsin–digested skeletal muscle triads in the presence (+) and absence (−) of PNGase F. The extent of glycosylation of the tryptic 42 kDa fragment identified by the $\alpha_2\delta$ anti-peptide antibody (Ab 136) is shown as the difference in molecular weight between the glycosylated and unglycosylated forms. Molecular weight markers are on the left.

and second hydrophobic domains is extracellular and thus is again consistent with a single-transmembrane domain within the δ peptide.

Peptide Antibody Mapping of Glycosylated Fragments of Native $\alpha_2\delta$ Subunit

Experimental evidence from native glycosylated protein also supports a single transmembrane model of the skeletal muscle $\alpha_2\delta_a$ subunit. We produced a peptide antibody (Ab 136) to the region between the first and second hydrophobic domains for the purpose of determining whether tryptic fragments of this region are glycosylated. Complete deglycosylation with Peptide-N-Glycosidase F (PNGase F) results in a shift in the molecular weight of the full-length $\alpha_2\delta$ subunit from 150 kDa to 106 kDa (Figure 3B). Tryptic fragments of native skeletal muscle $\alpha_2\delta$ (44 kDa and 28 kDa) also shift to lower molecular weight species upon deglycosylation with PNGase F (Figure 3B). These tryptic fragments are by necessity confined to the region between the first and second hydrophobic domains, based on in vivo cleavage of α_2 and δ and the Ab 136 antibody recognition site (Figures 1A and 1B). Although some transmembrane proteins have been shown to have carbohydrates associated with cytoplasmic domains (Hart et al., 1989), the extent of glycosylation seen in these tryptic fragments is inconsistent with cytoplasmic glycosylation.

Structural Integrity of the $\alpha_2\delta$ Subunit Is Essential to Its Function

Functionally, we were able to characterize the effect of the full-length and truncated $\alpha_2\delta$ subunits by examining the maximum Ba^{2+} current amplitude resulting from coexpression of these proteins in Xenopus oocytes. All mutant constructs produce proteins at levels similar to full-length $\alpha_2\delta$ subunit when translated in vitro (data not shown). Maximum average current amplitude is attained at +10 mV and is -0.4 ± 0.08 μA (n = 7) in cells expressing α_{1A} and β_4 subunits and increases to -3.4 ± 0.5 μA (n = 6) upon addition of the $\alpha_2\delta$ subunit. Coexpression of $\alpha_2\delta$ subunits results in a statistically significant 8.7-fold increase in current amplitude, which is equivalent to a ratio of R = 8.7 when compared to α_{1A}- and β_4-expressing cells (Figure 4A). There is no shift in the voltage-dependence of activation upon the addition of the $\alpha_2\delta$ subunit, and peak current occurs consistently during depolarizations to 0 mV or +10 mV. In the presence of the β_4 subunit, the kinetic properties of the α_{1A} channel are not significantly altered by the coexpression of the $\alpha_2\delta$ subunit (data not shown).

As the $\alpha_2\delta$ protein appears cleaved in vivo into disulfide-linked α_2 and δ subunits, it is interesting to examine whether either subunit functions independently. Expression of α_2 or δ proteins singly does not cause any increase in current amplitude compared with the α_{1A}- and β_4-expressing cells (R = 0.74 for α_2, and R = 0.87 for δ;

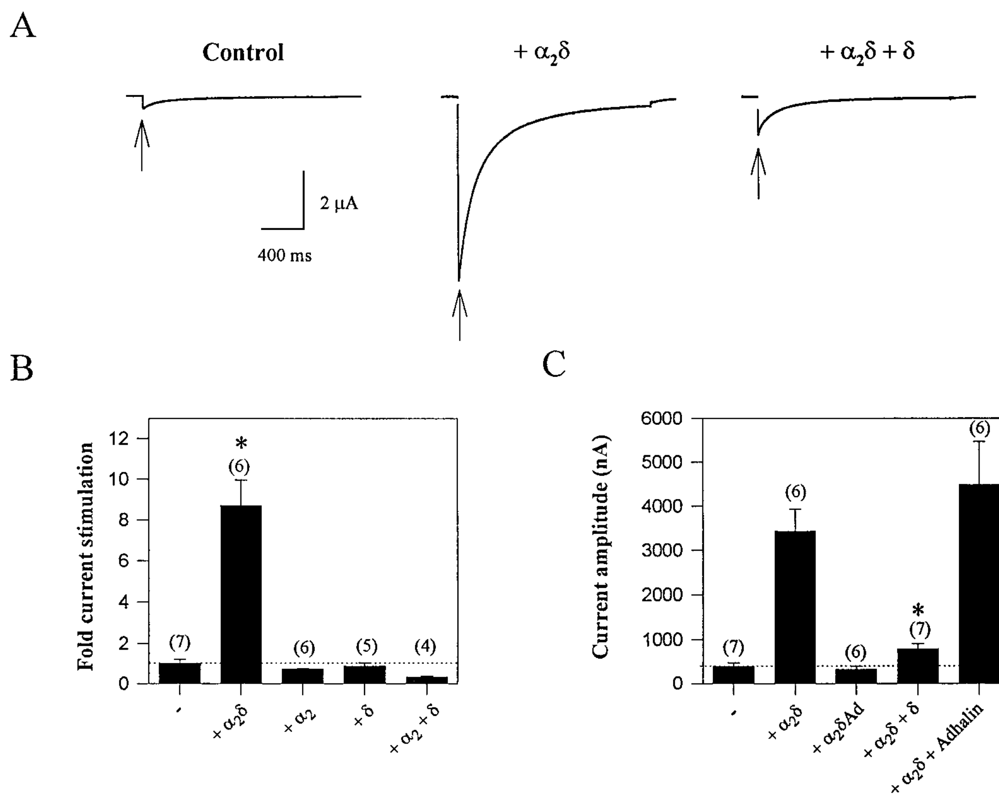

Figure 4. Effect of $\alpha_2\delta$ Mutants on Ba^{2+} Current Amplitude

(A) Current tracings of control (α_{1A}, β_4)–injected oocytes, addition of full-length $\alpha_2\delta$ subunit, and the competition of δ protein with full-length $\alpha_2\delta$ subunit for a depolarization to +10 mV. Arrows indicate time at which maximum current amplitude was measured.

(B) Fold current stimulation is plotted for α_{1A}- and β_4-expressing cells (−) and addition of either full-length $\alpha_2\delta$ subunit, individual α_2, or δ proteins, or coexpression of α_2 and δ proteins from separate constructs. The addition of the full-length $\alpha_2\delta$ subunit resulted in a significant ($p < .05$) increase in current amplitude when compared to α_{1A}- and β_4-injected cells. The number of oocytes recorded is listed in parentheses above each bar.

(C) Current amplitude in nA is plotted for α_{1A}- and β_4-expressing cells (−), the addition of full-length $\alpha_2\delta$ subunit or $\alpha_2\delta$Ad chimera, and the competition of either δ or adhalin with full-length $\alpha_2\delta$ subunit. Coexpression of δ significantly reduced the stimulatory effect of the full-length $\alpha_2\delta$ subunit ($p < .05$). Coexpression of adhalin had no effect on stimulation by the $\alpha_2\delta$ subunit.

Figure 4B). We also expressed both subunits simultaneously from separate cRNA constructs. Concurrent expression of these two proteins fails to result in any increase in current amplitude ($R = 0.34$), suggesting that cotranslational formation of disulfide-linked tertiary structure is necessary for proper $\alpha_2\delta$ subunit conformation (Figure 4B).

To examine the role of the extracellular domain of the $\alpha_2\delta$ subunit in Ca^{2+} channel function, amino terminal truncations were made in the $\alpha_2\delta$ sequence. The smallest truncation, NΔ28–184, which deleted 156 amino acids and 3 potential N-glycosylation sites, results in a loss of stimulation of the $\alpha_2\delta$ subunit with $R = 0.9$ (data not shown).

The Transmembrane Domain of the $\alpha_2\delta$ Subunit Is Important for Channel Interaction

Having shown that the integrity of the extracellular domain is necessary for enhancement of channel activity by the $\alpha_2\delta$ subunit, we sought to determine if the extracellular domain is sufficient for these functional effects or whether the $\alpha_2\delta$ transmembrane domain is also required for functional activity. We created a chimera ($\alpha_2\delta$Ad) containing the extracellular portion of the $\alpha_2\delta$ subunit fused to the transmembrane domain of adhalin, a component of the dystrophin-associated glycoprotein complex, which shares similar type I transmembrane topology. Comparison of the $\alpha_2\delta$ and adhalin transmembrane domains shows 19% amino acid identity and few conserved amino acid substitutions. Alkaline extraction of in vitro–translated $\alpha_2\delta$Ad protein demonstrates that the transmembrane domain in the chimera incorporates into the membrane (Figure 2C). Substitution of the $\alpha_2\delta$ transmembrane domain with the adhalin transmembrane region, however, results in a significant reduction in the ability of the protein to stimulate channel activity as demonstrated by an R-value of 0.82 as shown in Figure 4C.

Confirmation that the $\alpha_2\delta$ transmembrane domain is involved in the formation of stable interactions with the $\alpha_1\beta$ Ca^{2+} channel complex is demonstrated by the ability of the δ protein to almost completely compete off the stimulatory effects of the full-length $\alpha_2\delta$ subunit (Figure

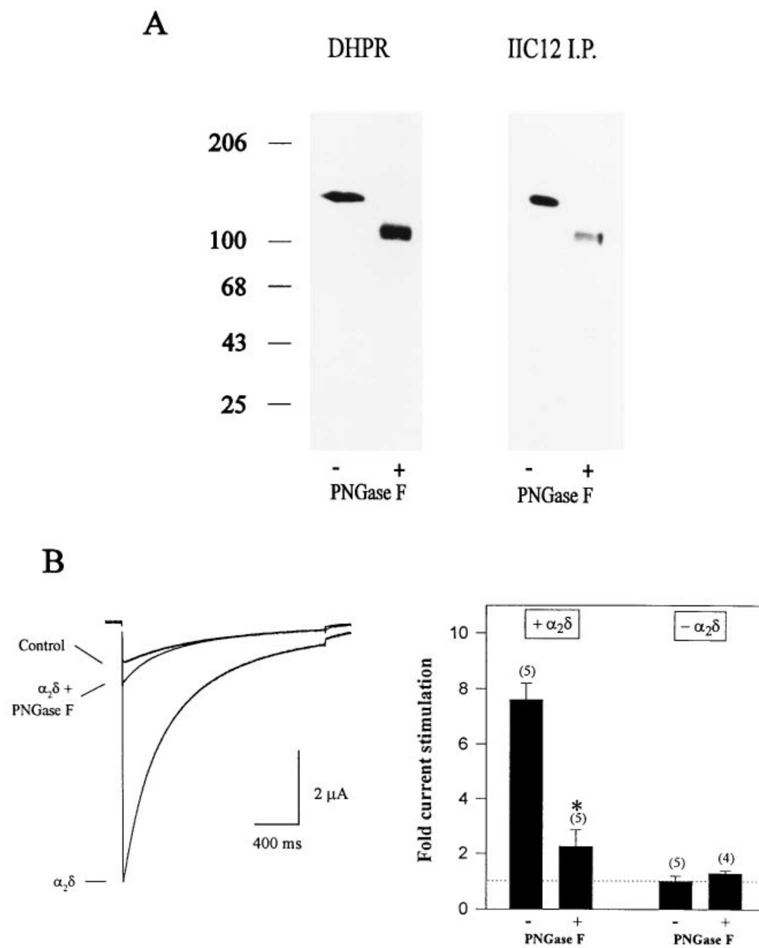

Figure 5. Effects of Deglycosylation on Subunit Interactions and $\alpha_2\delta$ Subunit Function

(A) Immunoblot analysis of purified skeletal muscle dihydropyridine receptor (5 μg) incubated in the presence (+) and absence (−) of PNGase F, demonstrating complete deglycosylation of the $\alpha_2\delta$ subunit. The $\alpha_2\delta$ subunit was detected with affinity-purified Ab 136. Identical quantities of native and deglycosylated receptor were immunoprecipitated with monoclonal antibody IIC12 specific for the α_1 subunit (IIC12 immunoprecipitate), and immunoblotted with Ab 136. Molecular weight markers are on the left.

(B) Current tracings at 0 mV depolarization of untreated control (α_{1A}, β_4)-injected oocytes and α_{1A}-, β_4-, $\alpha_2\delta$-injected oocytes, and α_{1A}-, β_4-, $\alpha_2\delta$-injected oocytes treated with PNGase F. At right are fold stimulation of current amplitude (over α_{1A}, β_4) with (+) and without (−) treatment with PNGase F. The reduction in current amplitude is significant only for the α_{1A}-, β_4-, $\alpha_2\delta$-injected cells.

4C). The full-length $\alpha_2\delta$ subunit stimulates the maximum current amplitude by only R = 1.99 in the presence of δ compared with R = 8.6 in its absence, which represents a 77% reduction in current stimulation. To demonstrate the specificity of the δ protein competition, we show that coinjection of another unrelated cRNA, full-length adhalin, does not result in a reduction in current amplitude.

N-Glycosylation of the $\alpha_2\delta$ Subunit Is Not Required for Maintenance of α_1 Subunit Interactions

No role has yet been assigned to the carbohydrates of the heavily glycosylated $\alpha_2\delta$ subunit. In an effort to characterize the interaction between $\alpha_2\delta$ and α_1 subunits of the skeletal muscle dihydropyridine receptor, we sought to determine whether glycosylation is necessary for maintaining stable subunit interactions. Skeletal muscle dihydropyridine receptor was completely deglycosylated with PNGase F in nonreducing conditions. Equivalent amounts of deglycosylated and glycosylated skeletal muscle dihydropyridine receptor were immunoprecipitated with a monoclonal antibody previously shown to recognize the α_{1S} subunit (Leung et al., 1987) at its carboxyl terminus (C. A. Gurnett and K. P. Campbell, unpublished data). Both glycosylated and deglycosylated $\alpha_2\delta$ subunits remain associated with the α_1 subunit as demonstrated by their coimmunoprecipitation with antibody IIC12 (Figure 5A). However, there appears to be a 57.3% ± 10.9% (n = 3) reduction in the amount of $\alpha_2\delta$ precipitated when the complex was deglycosylated. This suggests that carbohydrate may play a role in stabilizing the interaction between Ca^{2+} channel α_1 and $\alpha_2\delta$ subunits.

Deglycosylation Results in Reduced Current Stimulation by the $\alpha_2\delta$ Subunit

Since amino terminal truncations of the $\alpha_2\delta$ subunit abolished current stimulation, we decided to determine whether the extracellular carbohydrate was required for the functional increase in current amplitude. We previously demonstrated that complete deglycosylation of the skeletal muscle $\alpha_2\delta$ subunit could be achieved in nonreducing conditions with the enzyme PNGase F (Figure 5A). Using similar treatment conditions, deglycosylation of intact oocytes expressing α_{1A}, β_4, and $\alpha_2\delta$ subunits results in a significant reduction of current amplitude. The current amplitude is reduced by a factor of 67% from -7.1 ± 0.6 μA (n = 5) to −2.3 ± 0.65 μA (n = 5). In contrast, identical treatment of oocytes expressing only the α_{1A} and β_4 subunits does not have an effect on current amplitude when compared with untreated control cells (Figure 5B). Control cells expressing α_{1A} and β_4 have a maximum current amplitude of −1.05 ± 0.21 μA (n = 4) compared with −1.34 ± 0.11 μA (n = 5) for cells treated with PNGase F.

Figure 6. Functional Domain Model of the $\alpha_2\delta$ Subunit

The $\alpha_2\delta$ subunit is depicted as a type I transmembrane protein, with the other two hydrophobic regions forming a hydrophobic core of the protein. The sole transmembrane domain is located in the δ protein. Shown are the extracellular glycosylated domain of the $\alpha_2\delta$ subunit that mediates enhancement of current amplitude and the transmembrane domain in the δ subunit that mediates interaction with the α_1 subunit (arrows). The approximate locations of the 18 putative N-glycosylation sites on the $\alpha_2\delta$ subunit are indicated by the branched model carbohydrate. Also shown are the interaction sites between the α_1 and β subunits (De Waard et al., 1994; Pragnell et al., 1994).

Discussion

Our results support a single transmembrane model of the $\alpha_2\delta$ subunit whereby the third hydrophobic domain in δ is necessary and sufficient for stable membrane incorporation (Figure 6). The almost entirely extracellular localization of the $\alpha_2\delta$ subunit is consistent with a topology required to account for the nearly 50 kDa of glycosylation, but is inconsistent with the 3 transmembrane domains predicted by hydropathy analysis (Ellis et al., 1988). Numerous cases exist, however, in which prediction of transmembrane domains by hydropathy analysis has been misleading for several proteins, including the glutamate receptor (Hollmann et al., 1994; Bennett and Dingledine, 1995) and the P-glycoprotein transporter (Skach et al., 1993). Significantly, the transmembrane topology, which leaves only 5 residues in the cytoplasm, suggests that there are no direct interactions of the $\alpha_2\delta$ subunit with β subunits, G proteins, or protein kinases. Thus, the recent observation that coexpression of the entirely cytoplasmic β subunit is required to demonstrate the maximal effects of the $\alpha_2\delta$ subunit on channel activity (De Waard and Campbell, 1995) cannot be easily explained by simple direct structural interaction between the $\alpha_2\delta$ and β subunits.

Interestingly, the $\alpha_2\delta$ subunit has recently been genetically linked to familial malignant hyperthermia (Iles et al., 1994), a disease that had previously been shown to involve mutations in the ryanodine receptor, a key component in excitation–contraction coupling (Gillard et al., 1992). With only five cytoplasmic amino acids, the $\alpha_2\delta$ subunit is unlikely to directly interact with the ryanodine receptor and thus cannot be the intermediary between the cell membrane voltage-sensor and the intracellular sarcoplasmic reticulum Ca^{2+} pool. Though mutations in the $\alpha_2\delta$ subunit responsible for disease in these families have not been identified, once identified, they may be shown to indirectly affect Ca^{2+} channels by altering the voltage-sensing function or through interactions that structurally modify the II-III loop of the α_{1S} subunit (Tanabe et al., 1990).

The effects of N-glycosylation on ion channel activity have varied tremendously, being virtually without effect in the Shaker and Kv1.1 K^+ channels (Deal et al., 1994; Santacruz-Toloza et al., 1994), but resulting in larger currents in the ROMK1 inwardly rectifying K^+ channel (Schwalbe et al., 1995). Glycosylation appears important for the function of the $\alpha_2\delta$ subunit, since the current stimulation was almost entirely abolished by deglycosylation. Although deglycosylation does not cause complete complex dissociation, glycosylated residues may increase channel stability at the plasma membrane, as suggested by the reduction in the amount of $\alpha_2\delta$ subunit remaining in association with the α_1 subunit after deglycosylation. Since the $\alpha_2\delta$ subunit is predicted to contain complex oligosaccharide (Jay et al., 1991) and thus also contains sialic acid residues, some of the effects of the $\alpha_2\delta$ subunit may also be due to surface charge, as has been demonstrated for the voltage-dependent Na^+ channel (Recio-Pinto et al., 1990). Unfortunately, the low pH required for effective desialidation with neuraminidase is incompatible for use on live oocytes, and the surface charge effect could not be tested. Likewise, it was predicted that progressive amino-terminal trunca-

tions with gradual deletions of potential N-glycosylation sites would result in larger and larger reductions in current amplitude. However, the smallest N-terminal truncation of the $\alpha_2\delta$ subunit tested (NΔ28–184) completely abolished its functional activity. Further investigation of the precise mechanism of glycosylation may involve site-directed mutagenesis at various combinations of the 18 potential N-glycosylation sites.

Demonstration that amino-terminal truncations involving the extracellular region of the $\alpha_2\delta$ subunit result in a dramatic loss of function suggests that the overall integrity of the extracellular domain is important for function. Similar effects were seen in truncations of the β subunit of the voltage-dependent Na^+ channel (Chen and Cannon, 1996) where any perturbation of the extracellular region resulted in a loss of function. The interaction between the Ca^{2+} channel α_1 and $\alpha_2\delta$ subunits, however, differs critically from the Na^+/K^+-ATPase α and β subunit interaction, since in the latter case the ectodomain is sufficient for assembly and substitution of the transmembrane sequence with that of another protein has no effect on assembly (Hamrick et al., 1993).

Transmembrane domain interactions have previously been described for several cell surface receptors, but our data are the first report of a transmembrane domain interaction between ion channel subunits. Failure of the adhalin transmembrane domain to substitute for the $\alpha_2\delta$ transmembrane domain demonstrated that this region functions not only as a hydrophobic membrane anchor, but also determines specific interactions between the $\alpha_2\delta$ subunit and the α_1 subunit. This was confirmed by the ability of the δ protein to compete with the full-length $\alpha_2\delta$ subunit for functional interaction with the α_1 subunit. Examination of the $\alpha_2\delta$ transmembrane sequence did not reveal any previously identified structural motifs, although those identified have been quite heterogeneous. For example, glycine rich sequences have been shown to be important for the α-helical interaction of the α and β chains of the class II major histocompatibility complex (Cosson and Bonifacino, 1992) and dimerization of glycophorin A (Lemmon et al., 1994), but charged residues within the transmembrane sequences are required for specific interactions of the T cell antigen receptor components (Manolios et al., 1990). Interestingly, there is slight homology between the transmembrane domains of the $\alpha_2\delta$ subunit and the voltage-gated Na^+ channel β_1 subunit (Isom et al., 1992), which suggests that this region may be involved in Na^+ channel subunit interaction as well.

A transmembrane interaction between the α_1 and $\alpha_2\delta$ subunits would be consistent with our inability to identify the interaction sites using in vitro protein association assays, as used for the identification of the α_1 subunit interaction domain (AID; Pragnell et al., 1994) and the β interaction domain (BID; De Waard et al., 1994). It may prove difficult, however, to identify the corresponding transmembrane domain of the α_1 subunit that forms the interaction, as there are 24 transmembrane domains in the α_1 subunit. Since the $\alpha_2\delta$ subunit does not appear to alter the voltage dependence of activation, it is unlikely that the $\alpha_2\delta$ subunit interaction involves the charged residues of the S4 voltage sensor. Further description of the physical interaction sites on these two proteins may be provided by biochemical analysis of recombinant subunit assembly, but such experiments have so far been hindered by difficulties in expressing the full-length α_1 subunit. Knowledge of the transmembrane topology and interaction sites of the $\alpha_2\delta$ subunit will be useful in designing future experiments to determine whether the mechanism of current stimulation by the $\alpha_2\delta$ subunit is through channel stabilization or changes in single channel activity or both.

Our data support a dual role for the $\alpha_2\delta$ subunit. The extracellular domain, which is particularly sensitive to structural modification, provides the necessary carbohydrate for either channel stabilization or surface charge effects that results in increased current amplitude, whereas the transmembrane domain specifies the interaction with the Ca^{2+} channel α_1 subunit. Identification of a transmembrane domain interaction between Ca^{2+} channel subunits suggests that other ion channel subunits may be similarly engaged in transmembrane associations. It will be interesting to identify the relationship of these subunit interactions to other channel transmembrane functions such as the pore and voltage-sensor.

Experimental Procedure

Construction of Truncated $\alpha_2\delta$ Subunits

All constructs were made in the full-length rat brain $\alpha_2\delta_b$ subunit (Kim et al., 1992) in pBluescript and transferred to plasmid pcDNA3 (Invitrogen) for expression. To facilitate formation of amino terminal truncations, a unique MluI site was created 2 amino acids after the end of the $\alpha_2\delta$ signal sequence by polymerase chain reaction mutagenesis, which altered the nucleotide sequence from TCCCTT to ACGCGT beginning at nucelotide base pair 90 and created 2 amino acid substitutions (F27Y and P28A). (All numbers indicate amino acid [aa] sequence unless otherwise indicated.) All of the constructs described hereafter contain these substitutions, which were shown not to alter the function of $\alpha_2\delta$ subunits expressed in Xenopus oocytes (C. A. Gurnett and K. P. Campbell, unpublished data). Polymerase chain reaction–amplified fragments were inserted at the MluI site and the indicated downstream restriction site: Bst XI (NΔ28–184), AflII (NΔ28–473), and BstEII (δ). CΔ945–1091, the α_2 protein, was formed by insertion of polymerase chain reaction fragment into an upstream BamHI and polylinker EcoRI. A stop codon in the antisense oligonucleotide primer truncated the protein at L943, which is at the site of cleavage between α_2 and δ. CΔ428–1091 and CΔ842–1091 were created by digestion at two internal XbaI sites and self-ligation of complete and partial digestion products with an XbaI site in the polylinker. CΔ428–1091 is truncated at D428, which precedes the first hydrophobic domain by 17 aa; and CΔ842–1091 is truncated at D842, which precedes the second hydrophobic domain by 63 aa (see Figure 1D). The $\alpha_2\delta$Ad construct was created by polymerase chain reaction mutagenesis using four primers. The forward primer for the first reaction began at nucleotide 2662 in $\alpha_2\delta$, which includes a unique BamHI site; and the reverse primer included 24 bases of the dystrophin-associated glycoprotein adhalin (Roberds et al., 1993) followed by 18 bases complementary to the $\alpha_2\delta$ sequence starting at nucleotide 3408. The second reaction amplified 35 amino acids of adhalin and included an EcoRI site at the 3′ end for subcloning. A third reaction, using both of these amplification products, resulted in the chimera that included $\alpha_2\delta$ sequence followed by 8 extracellular, 22 transmembrane, and 5 cytoplasmic amino acids of the adhalin protein sequence with an in-frame termination codon. These amino acids were inserted after G1064 in the $\alpha_2\delta$ sequence through upstream BamHI and polylinker EcoRI restriction sites.

Antibody Preparation

A 19 aa peptide (peptide 54) representing the highly conserved residues 839–856 of $\alpha_2\delta$ sequence was prepared by the Howard

Hughes Medical Institute Biopolymers Facility (University of Texas Southwestern) with a cysteine residue at the amino terminus for coupling to bovine serum albumin and keyhole limpet hemocyanin through the bifunctional agent m-maleimidobenzoic acid-N-hydroxysuccinimide ester as described previously (Gurnett et al., 1995). Polyclonal antibodies were made by injection of peptide 54 into Rabbit 136 and then affinity purified using immobilized strips of bovine serum albumin–conjugated peptide as described previously (Sharp et al., 1987). Monoclonal antibody IIC12 was described previously (Leung et al., 1987).

Alkaline Extraction of In Vitro–Translated Proteins

In vitro–translated protein was made using T7 RNA polymerase in the in vitro translation lysate system (Promega) coupled to canine microsomal membranes (Promega). Proteins were made in a total volume of 25 μl, with added 2.5 μl canine microsomal membranes for 2 hr at 30°C. In vitro translate and membranes were centrifuged at 14,000 rpm for 10 min at 4°C to separate the membrane–associated fraction from the soluble protein. The small membrane pellet was resuspended in 40 μl 0.1 M Na_2CO_3 (pH 11.5), incubated on ice for 1 hr, and centrifuged for 30 min at 50,000 rpm in a TL 100.1 rotor. Alkaline extracted membranes were resuspended in phosphate-buffered saline, and excess phosphate-buffered saline was added to neutralize the alkaline extract.

Trypsin Digestion and Deglycosylation of Native Proteins

Skeletal muscle triads (Sharp et al., 1987) were digested with a 10:1 protein-to-trypsin ratio for 30 min at 37°C in 0.3 M sucrose, 20 mM Tris (pH 7.4). Digestion was stopped by the addition of 1.0 mM phenylmethylsulfonyl fluoride and 50 μl soybean trypsin inhibitor (10 mg/ml) per 150 μg of trypsinized triads and incubated on ice for 30 min. Following complete trypsin inhibition, deglycosylation buffer was added to give a final concentration of 20 mM $NaHPO_4$ (pH 7.5), containing 50 mM EDTA. Samples were then boiled for 2 min in 1% SDS. After addition of 2% Triton, 5 U of PNGase F (Oxford Glycosystems) were added per 150 μg trypsinized triads and incubated overnight at 37°C in the presence of protease inhibitors (76.8 nM aprotinin, 1 mM benzamidine, 1.1 μM leupeptin, 0.7 μM pepstatin A, and 0.23 mM phenylmethylsulfonyl fluoride). Preparation of purified dihydropyridine receptor was described previously (Leung et al., 1987). Purified receptor was deglycosylated as above, but in the absence of denaturing agents (i.e., SDS) and then loaded onto protein G beads that had been preincubated with the monoclonal antibody IIC12, which is specific for the α_{1S} subunit (Leung et al., 1987). Deglycosylation of intact Xenopus oocytes was performed for 12 hr at room temperature followed by overnight incubation at 15°C in oocyte media with the addition of 20 mM $NaHPO_4$ (pH 7.5) and 10 U PNGase F. Control cells were treated identically but were without added enzyme.

Ca^{2+} Channel Expression in Xenopus Oocytes and Electrophysiological Recordings

Stage V and VI oocytes were prepared and maintained as described previously (De Waard et al., 1994). The follicle membranes from isolated oocytes were enzymatically digested with 2 mg/ml collagenase IA (Sigma) to facilitate the injection and recording procedures. cRNAs were transcribed in vitro using T7 RNA polymerase or SP6 RNA polymerase in the case of α_{1A} cDNA. Oocytes were injected with 50 nl of various subunit composition at the following cRNA concentrations: 0.7 μg/μl α_{1A}; 0.1 μg/μl β_4; and 0.7 μg/μl $\alpha_2\delta$ wild-type, mutant, or chimeric constructs. Ba^{2+} currents were recorded by two-electrode voltage-clamp with a Dagan TEV-200. Both voltage and current electrodes were filled with 3 M KCl and had tip resistances of 0.5 MΩ. The bath solution contained 40 mM $Ba(OH)_2$, 50 mM NaOH, 2 mM KCl, 1 mM niflumic acid, 0.1 mM EGTA, 5 mM HEPES (pH 7.4) with methanesulfonic acid. Recordings were filtered at 0.5 kHz, sampled at 5 kHz, and analyzed by pClamp 6 (Axon Instruments). Leak and capacitance currents were subtracted on-line by a P/6 protocol. Peak barium currents were measured for test potentials of 0 or 10 mV from a holding potential of −90 mV.

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