Calcium Currents Are Enhanced by $\alpha_2\delta$-1 Lacking Its Membrane Anchor*\textsuperscript{[5]}

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Background: We examined the role of membrane anchoring of voltage-gated calcium channel $\alpha_2\delta$ subunits.

Results: We used a truncated $\alpha_2\delta$-1 construct ($\alpha_2\delta$-1C-term), which still increases Ca\textsubscript{v}2.1/\beta1b currents, despite being mainly secreted.

Conclusion: The effect of $\alpha_2\delta$-1C-term on calcium currents does not involve secretion and subsequent re-binding to the plasma membrane.

Significance: C-terminal membrane anchoring of $\alpha_2\delta$ is not essential for calcium current enhancement.

The accessory $\alpha_2\delta$ subunits of voltage-gated calcium channels are membrane-anchored proteins, which are highly glycosylated, possess multiple disulfide bonds, and are post-translationally cleaved into $\alpha_2$ and $\delta$. All $\alpha_2\delta$ subunits have a C-terminal hydrophobic, potentially trans-membrane domain and were described as type I transmembrane proteins, but we found evidence that they can be glycosylphosphatidylinositol-anchored. To probe further the function of membrane anchoring in $\alpha_2\delta$ subunits, we have now examined the properties of $\alpha_2\delta$-1 constructs truncated at their putative glycosylphosphatidylinositol anchor site, located before the C-terminal hydrophobic domain ($\alpha_2\delta$-1C-term). We find that the majority of $\alpha_2\delta$-1C-term is soluble and secreted into the medium, but unexpectedly, some of the protein remains associated with detergent-resistant membranes, also termed lipid rafts, and is extrinsically bound to the plasma membrane. Furthermore, heterologous co-expression of $\alpha_2\delta$-1C-term with Ca\textsubscript{v}2.1/\beta1b results in a substantial enhancement of the calcium channel currents, albeit less than that produced by wild-type $\alpha_2\delta$-1. These results call into question the role of membrane anchoring of $\alpha_2\delta$ subunits for calcium current enhancement.

Voltage-gated Ca\textsuperscript{2+} (Ca\textsubscript{v})\textsuperscript{4} channels comprise an $\alpha_1$ subunit, which forms the pore and determines the main functional and pharmacological attributes of the channel (1). For the high voltage-activated channels, the $\alpha_1$ subunit is associated with an intracellular $\beta$ subunit, which is required for the channel to reach the plasma membrane (2–4), and an $\alpha_2\delta$ subunit, whose functions are less well understood but which also influences trafficking of the channel (5–7). Genes encoding 10 $\alpha_1$, four $\beta$, and four $\alpha_2\delta$ subunits have been identified (1, 8, 9).

The topology of the $\alpha_2\delta$ protein was initially determined for skeletal muscle $\alpha_2\delta$-1 but is likely to generalize to all $\alpha_2\delta$ subunits (10, 11). The $\alpha_2\delta$ subunits were predicted to be type I transmembrane proteins, as they have an N-terminal signal peptide sequence and a C-terminal hydrophobic and potentially transmembrane region (12). From the early studies of $\alpha_2\delta$-1 purified from skeletal and cardiac muscle, it was identified that the $\alpha_2$ subunit is disulfide-bonded to a transmembrane $\delta$ subunit (13). However, both subunits are the product of a single gene, encoding the $\alpha_2\delta$ protein, which is post-translationally glycosylated and further processed with the formation of disulfide bond(s) and subsequent proteolytic cleavage into $\alpha_2$ and $\delta$ (12).

In terms of function, the $\alpha_2$ moiety of $\alpha_2\delta$ was found to play a role in enhancement of calcium currents (11), and we showed that the von Willebrand factor-A domain in $\alpha_2$ is essential for its trafficking function (6, 14). In contrast, the transmembrane $\delta$ subunit was reported to function by modifying the voltage-dependent properties of the channels (10, 11).

We have recently obtained evidence that $\alpha_2\delta$ subunits can form GPI-anchored proteins (15). In this study, we wished to further examine the role of membrane anchoring of $\alpha_2\delta$-1 by creating an anchorless $\alpha_2\delta$-1, truncated at the putative GPI-anchor $\omega$-site, which removes the C-terminal hydrophobic domain (Fig. 1, $\alpha_2\delta$-1C-term, construct iii). A similar approach has been taken with GPI-anchored prion protein, which was found to remain associated with lipid rafts despite the loss of membrane anchoring (16). The interaction of a transmembrane form of prion protein with lipid rafts was found to require interaction with glypicans, which are themselves GPI-anchored (17).

We have examined the role of membrane anchoring of $\alpha_2\delta$-1 on its biochemical properties, processing, subcellular localization...

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\textsuperscript{[5]}The abbreviations used are: Ca\textsubscript{v}, voltage-gated calcium; Ab, antibody; DRG, dorsal root ganglion; DRM, detergent-resistant membrane; GPI, glycosylphosphatidylinositol; PNGase, peptide N-glycosidase; WCL, whole cell lysate; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; CFP, cyan fluorescent protein.
tion, and function. We present the surprising evidence that \( \alpha_\delta-1 \Delta C \)-term is still able to produce a significant enhancement of \( Ca_{\text{a},2.1}/B1b \) calcium channel currents following its heterologous expression, indicating that intrinsic membrane anchoring is not essential for this property. Furthermore, we have found that although a large fraction (~75% after 3 days in culture) of \( \alpha_\delta-1 \Delta C \)-term is soluble and secreted into the medium, some of this protein remains extrinsically associated with the external leaflet of the plasma membrane. Future studies will be directed toward identifying the binding partner(s) of \( \alpha_\delta-1 \Delta C \)-term mediating this extrinsic interaction.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology—\( \alpha_\delta-1 \Delta C \)-term was constructed with a C-terminal HA tag, followed immediately by a STOP codon inserted directly after Cys-1059 (thus abolishing the Cys-1059/Gly-1060/Gly-1061-predicted GPI anchor site). A second construct was made with an HA tag between Asn-549 and Asp-550, which was also truncated by a STOP codon immediately after Cys-1059. All mutations were made by standard molecular biological techniques and verified by DNA sequencing.**

**Heterologous Expression of cDNAs—**The calcium channel cDNAs used were rabbit \( Ca_{\text{a},2.1} \) (M64373), rat \( \alpha_\delta-1 \) (M86621), and rat \( \beta1b \) (18). The cDNAs were cloned into the pMT2 vector for expression, unless otherwise stated. tsA-201 cells were transfected with the cDNA combinations stated. The cDNA for green fluorescent protein (mut3 GFP) (19) was also included to identify transfected cells from which electrophysiological recordings were made. Transfection was performed as described previously (20). In control experiments where \( \alpha_\delta \) was omitted, the ratio was made up as stated with empty vector.

**Dorsal Root Ganglion (DRG) Neuron Culture and Transfection—**DRG neurons were isolated from P10 Sprague-Dawley rats and transfected by Amaxa nucleofection as described in the manufacturer’s protocol (program G13, Lonza). Briefly, neurons were dissociated in dissection solution as follows: Hanks’ basal salt solution buffer containing 5 mg/ml Dispase (Invitrogen), 2 mg/ml collagenase type 1A (Worthington), and 0.1 mg/ml DNase, (Invitrogen), for 30 min at 37 °C, and then resuspended in 160 \( \mu \)l of nucleofection buffer (80 \( \mu \)l per sample). 2 \( \mu \)g of total plasmid DNA was used for each transfection condition. For expression, \( \alpha_\delta-1 \) mid-HA and \( \alpha_\delta-1 \Delta C \)-term-HA were used in pcDNA3. Enhanced cyan fluorescent protein (Clontech) was co-transfected with \( \alpha_\delta-1 \) cDNA at a ratio of 1:4. After transfection, DRGs were plated on poly-L-lysine-coated glass coverslips and cultured in DMEM/F-12 medium (Invitrogen) supplemented with 10% FBS and 50 ng/ml NGF. Culture medium was replaced 18 h after transfection.

**Primary Antibodies (Abs)—**The following primary Abs were used: anti-\( \alpha_\delta-1 \) (mouse monoclonal, Sigma); anti-HA (rabbit polyclonal, Sigma, or rat monoclonal, Roche Applied Science); anti-floatillin-1 (mouse monoclonal, BD Biosciences); anti-Akt/PKB (rabbit polyclonal, Cell Signaling Technologies), and anti-GAPDH (mouse monoclonal, Ambion).

**Cell Lysis, Cell Surface Biotinylation, and Immunoblotting—**The procedures were modified from those described previously (15, 20). 72 h after transfection, tsA-201 cells were rinsed with phosphate-buffered saline (PBS, pH 7.4, Sigma) and then incubated with PBS containing 1 mg/ml EZ-link Sulfo-NHS-LC-Biotin (Thermo Scientific) for 30 min at room temperature. Cells were then rinsed twice with PBS containing 200 mM glycine to quench the reaction. The cells were scraped, resuspended in cold PBS, and centrifuged at 1000 \( \times \) g at 4 °C for 10 min. The cell pellets were homogenized in PBS, pH 7.4, at 4 °C containing 1% Igepal and protease inhibitors (complete, Roche Applied Science) by five passes through a 23-gauge needle, followed by sonication for 10 s, and were incubated on ice for 45 min. The whole cell lysates (WCL) were then centrifuged at 20,000 \( \times \) g for 25 min at 4 °C, and the pellet was discarded. Aliquots of supernatant were assayed for total protein (Bradford assay, Bio-Rad). WCL corresponding to 20–40 \( \mu \)g of total protein was diluted with Laemmli sample buffer (15) supplemented with 100 mM dithiothreitol, incubated at 60 °C for 10 min, resolved by SDS-PAGE on 3–8% Tris-acetate or 4–12% Bis-Tris gels (Invitrogen), and transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad) by Western blotting (semi-dry, Bio-Rad). 500 \( \mu \)g of the same lysate was used to precipitate biotinylated protein by adding 50 \( \mu \)l of prewashed streptavidin-agarose beads (Thermo Scientific) and overnight incubation at 4 °C. The beads were washed five times with PBS containing 0.1% Igepal and resuspended in an equal volume of 2 \( \times \) Laemmli buffer with 100 mM DTT, followed by 10 min of incubation at 60 °C. The eluted protein was analyzed by immunoblotting, as described above. The following secondary Abs were used for Western blot: goat anti-rabbit coupled to horseradish peroxidase (HRP) and goat anti-mouse coupled to HRP (Bio-Rad). The signal was obtained by HRP reaction with fluorescent product (ECL Plus, GE Healthcare), and membranes were scanned on a Typhoon 9410 phosphorimager (GE Healthcare).

**Deglycosylation with Peptide N-Glycosidase-F (PNGase-F)—**On gel imaging software (rsb.info.nih.gov) was used to draw a box around each band of interest, to quantify the mean gray intensity. The background was subtracted using an equally sized “background” box next to each band. To quantify the cleavage of \( \alpha_\delta \) to \( \alpha_\delta \) and \( \delta \), the \( \alpha_\delta-1 \) and \( \alpha_\delta-1 \Delta C \)-term-HA bands were summed (total = cleaved + uncleaved), from which the % cleavage was calculated.

The proportion of \( \alpha_\delta-1 \Delta C \)-term-HA secreted into the medium 72 h after transfection of tsA-201 cells was quantified by measuring the mean intensity of \( \alpha_\delta-1 \)-associated bands detected by HA Ab in the media and in the WCL. Taking into account the total volume of the media and the WCL for each condition, an estimate of the amount of \( \alpha_\delta-1 \Delta C \)-term-HA protein in each fraction was obtained and expressed as % of the total \( \alpha_\delta-1 \Delta C \)-term-HA in all fractions.
both cases, and the whole reaction volume was analyzed by Western blot.

Collection of Medium—tsA-201 cells were incubated for 72 h post-transfection, and medium was collected and centrifuged (1000 × g) to remove any detached cells. The supernatant was filtered through a 0.22-μm syringe filter (Millipore). The resulting cell-free medium was applied to 3-kDa cutoff filtration column (Amicon) and centrifuged to concentrate the proteins (∼150-fold). Aliquots of the concentrate were diluted in the appropriate amount of Laemmli sample buffer and analyzed by Western blot.

Preparation of Triton X-100-insoluble Membrane Fractions (DRMs)—All steps were performed on ice. Confluent tsA-201 cells from two 175-cm² flasks were taken up in Mes-buffered saline (MBS, 25 mM Mes, pH 6.5, 150 mM NaCl, and complete protease inhibitor mixture (Roche Applied Science)) containing 1% (v/v) Triton X-100 (Thermo Scientific), resuspended by 10 passages through a 1-ml Gilson pipette tip, and left on ice for 1 h. An equal volume of 90% (w/v) sucrose in MBS was then added. The sample was transferred to a 13-ml ultracentrifuge tube and overlaid with 10 ml of discontinuous sucrose gradient, consisting of 35% (w/v) sucrose in MBS (5 ml) and 5% (w/v) sucrose in MBS (5 ml). The sucrose gradients were centrifuged at 33,000 rpm for 18 h at 4 °C (Beckman SW40 rotor). 1-ml fractions were subsequently harvested from the top to the bottom of the tube. When necessary, protein fractions from the gradient were washed free of sucrose by dilution into 25 ml MBS (5 ml). The sucrose gradients were centrifuged as above.

Collection of Medium—DRG cultures were incubated with monoclonal rat anti-HA Ab (1:250, Roche Applied Science) for 1 h at 37 °C in medium containing (in mM) the following: 145 NaCl; 5 KCl; 2 CaCl₂; 1 MgSO₄; 10 Hepes; 10 glucose, pH 7.4. Neurons were washed with PBS, fixed with 4% paraformaldehyde in PBS for 5 min and then blocked for 30–60 min with PBS supplemented with 10% goat serum. Secondary Ab (anti-rat Alexa Fluor 555, 1:500, Invitrogen) was applied for 2 h at room temperature. Samples were mounted and scanned as above.

Electrophysiology—Calcium channel currents were recorded in tsA-201 cells by whole cell patch-clamp recording, essentially as described previously (23). The internal (pipette) and external solutions and recording techniques were similar to those previously described (24). The patch pipette solution contained in mM: cesium aspartate, 140; EGTA, 5; MgCl₂, 2; CaCl₂, 0.1; K₂ATP, 2; Hepes, 10; pH 7.2, 310 mosm with sucrose. The external solution for recording Ba²⁺ currents contained in mM: tetrathylammonium bromide, 150; KCl, 3; NaHCO₃, 1.0; MgCl₂, 1.0; Hepes, 10; glucose, 4; BaCl₂, 1 or 5 as indicated, pH 7.4, 320 mosm with sucrose. Pipette of resistance 2–4 meqohms were used. An Axopatch 1D amplifier (Axon Instruments, Burlingame, CA) was used, and data were filtered at 1–2 kHz and digitized at 5–10 kHz. Current records were subjected to leak and residual capacitance current subtraction (P/8 protocol). Analysis was performed using PCLAMP9 (Molecular Devices) and Origin 7 (Microcal Origin, Northampton, MA).

Current-voltage (I-V) plots were fit with a modified Boltzmann equation as described previously (25), for determination of the voltage for 50% activation (V₅₀,act). Where data are given as mean ± S.E., statistical comparisons were performed using either Student’s t test or analysis of variance with post hoc test, as appropriate.

RESULTS

Expression of α₂δ-1ΔC-term—An anchorless α₂δ-1 construct (α₂δ-1ΔC-term-HA) was made with a C-terminal HA tag (Fig. IA, construct iii), to monitor expression. It was expressed in tsA-201 cells, and expression was compared with WT α₂δ-1 (Fig. 1A, construct i) and α₂δ-1 mid-HA (Fig. 1A, construct ii) in the WCL (Fig. 1B, lanes 2–4). The presence of a mid-HA tag in this position within α₂δ-1 does not affect the function of the full-length α₂δ-1 (see Fig. 4A). Similar expression levels and a similar level of N-linked glycosylation, as shown by treatment with PNGase-F, were observed (Fig. 1B, top two panels, α₂δ-1 Ab).

As found previously for heterologous expression of WT α₂δ subunits (15, 26), both α₂δ-1 mid-HA and α₂δ-1ΔC-term-HA were only partially cleaved into α₂ and δ (Fig. 1B, lanes 6–8). Partial cleavage is the reason that the α₂δ-1 Ab recognizes two bands in reduced samples. These can best be distinguished following deglycosylation and have molecular masses of ∼130 kDa (α₂δ-1 “uncleaved form”) and ∼105 kDa (α₂δ-1 “cleaved form”) (Fig. 1B, top right panel). As expected from the location of the HA epitope, in reduced deglycosylated samples, the HA
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C-terminal HA tag was affecting the properties of the protein, we also made a truncated α2δ-1ΔC-term construct with an internal HA tag (α2δ-1 mid-HA ΔC-term) (supplemental Fig. 1A). This construct was similarly expressed (supplemental Fig. 1B) and glycosylated (data not shown), compared with α2δ-1 mid-HA, and it was recognized by both α2δ-1 and HA Abs.

Truncated αδ-1ΔC-term Is Secreted into the Medium—In medium conditioned by tsA-201 cells expressing αδ-1ΔC-term-HA, we observed a band of ~165 kDa, which was immunoreactive to both α2δ-1 and HA Abs (Fig. 2A, lane 4), indicating that the anchorless construct was secreted. This band was absent from medium conditioned by untransfected cells or those expressing full-length WT α2δ-1 or α2δ-1 mid-HA (Fig. 2A, lanes 1–3).

The secreted anchorless protein was glycosylated, because PNGase-F treatment shifted the predominant band from ~165 to ~130 kDa, equivalent to the observed size of the unprocessed form of αδ-1ΔC-term-HA after glycosylation (Fig. 2B, lanes 1 and 2; see also Fig. 1B). Surprisingly, we barely detected any proteolytically processed αδ-1ΔC-term in the medium, as demonstrated by the absence of δ-1ΔC-term-HA peptide in Fig. 2A (lane 4).

Transfection with increasing amounts of αδ-1ΔC-term-HA cDNA (3, 6, or 10 μg) resulted in increased expression and secretion of αδ-1ΔC-term-HA at 72 h, as demonstrated by Western blot analysis (Fig. 2, C and D). We quantified the percentage of secreted αδ-1ΔC-term-HA, relative to the total amount expressed, by measuring the mean intensity of bands and taking into account the total volume of each fraction, as described under “Experimental Procedures.” For this calculation, we have summed the cleaved and uncleaved αδ-1 bands detectable in WCL and in the media to obtain the total expression. The proportion of αδ-1ΔC-term-HA secreted into the medium was 75.6 ± 2.8% of the total amount of αδ-1ΔC-term-HA expressed (n = 3; Fig. 2E). Truncation within the hydrophobic domain resulting in a longer αδ-1 construct was previously shown to result in a construct that was partially secreted into the medium (27). The truncated construct with an internal HA tag (α2δ-1 mid-HA ΔC-term) also showed secretion into the medium as the uncleaved protein (supplemental Fig. 1C).

Despite Removal of the Membrane Anchor, αδ-1ΔC-term Remains in Part Associated with the Plasma Membrane—We compared the distribution of full-length αδ-1 mid-HA to that of anchorless α2δ-1ΔC-term-HA, following heterologous expression in tsA-201 cells. Unexpectedly, we found that αδ-1ΔC-term-HA was also associated with the plasma membrane in nonpermeabilized cells (Fig. 3A, panel ii), to a similar extent to the robust cell surface staining observed for α2δ-1 mid-HA in nonpermeabilized cells (Fig. 3A, panel i) and to WT α2δ-1, as demonstrated by anti-α1 Ab staining (supplemental Fig. 2). In permeabilized cells, immunostaining was also observed intracellularly in both conditions (Fig. 3A, panels iii and iv). Association of αδ-1ΔC-term-HA with the plasma membrane was not affected by co-transfection with an α1 and β subunit (Cα2.2 and β1b; Fig. 3B; supplemental Fig. 2, compare A and B), indicating that αδ-1ΔC-term-HA does not require association with other calcium channel subunits for its membrane localization.

Ab revealed bands associated with uncleaved αδ-1ΔC-term-HA (~130 kDa) and δ-1ΔC-term-HA peptide (~19 kDa) (Fig. 1B, lane 8). Note that the αδ-1ΔC-term-HA showed increased HA immunoreactivity, compared with full-length αδ-1 mid-HA (Fig. 1B, middle panels). This is likely due to better accessibility of the C-terminally located HA epitope tag, rather than to increased expression levels of αδ-1ΔC-term-HA, as the corresponding bands revealed by the α2δ-1 Ab were of similar intensities (Fig. 1B, top panel). To examine whether the

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**FIGURE 1.** Biochemical properties of αδ-1ΔC-term-HA expressed in tsA-201 cells. A, scheme of αδ-1 constructs used. The site of truncation and the position of the HA epitope (light gray box) are marked as follows: construct i, wild-type (WT) αδ-1; construct ii, full-length αδ-1 with an internal HA epitope (αδ-1 mid-HA); construct iii, truncated αδ-1 with C-terminal HA (αδ-1ΔC-term-HA). The amino acid sequence of the C terminus of rat αδ-1, with the site of truncation at the GPI anchor attachment site predicted in our previous study (15) (ω-site; C in CCG underlined), and the C-terminal hydrophobic sequence (underlined) are shown at top. B, WCL from untransfected tsA-201 cells (lanes 1 and 5) or cells transfected with WT αδ-1 (lanes 2 and 6), αδ-1 mid-HA (lanes 3 and 7), or αδ-1ΔC-term-HA (lanes 4 and 8), either untreated (left panel) or treated with PNGase-F (right panel). Bands were visualized with the indicated Abs, either against αδ-1 (top panel) or against the HA epitope (middle two panels). The arrows on the right indicate bands corresponding to the deglycosylated proteins shown in the scheme in A, either uncleaved αδ-1 (upper band), cleaved αδ-1 (lower band), or free δ-1. The lower part of the same membrane was blotted with anti-GAPDH Ab as a loading control (bottom panel). WB, Western blot.
tion. Furthermore, the fact that the HA epitope is exposed in nonpermeabilized C-term-HA-transfected cells indicates that it has not utilized another hydrophobic region in the protein as a transmembrane domain, in which case the C-terminal HA tag would be intracellular. We also observed that, when expressed by nucleofection in DRG neurons, C-term-HA was associated with the cell surface, both in the cell bodies and in neurites, to a similar extent to mid-HA (Fig. 3, C and D).

To rule out that the C-terminal HA tag was in some way artifically mediating noncovalent membrane association, we also used the αδ-1 mid-HA ΔC-term construct. We found that this construct behaved similarly to αδ-1ΔC-term-HA, being associated with the plasma membrane in nonpermeabilized cells (supplemental Fig. 1D), as well as being secreted (supplemental Fig. 1C).

αδ-1ΔC-term-HA Retains Partial Functionality, in Terms of Enhancing Calcium Currents—First, we showed that the full-length WT αδ-1 mid-HA construct used in this study retained full functionality in comparison with untagged full-length CaV2.1/β1b alone (Fig. 4A). We then examined the ability of αδ-1ΔC-term-HA to enhance CaV2.1/β1b calcium currents, and surprisingly, we found that it retained substantial ability to

This figure shows the secretion of C-term-HA into the medium, as well as the effects of deglycosylation on its expression and the ability to enhance calcium currents. The secretion is visualized by Western blots (WB) and the ability to enhance calcium currents is demonstrated by changes in the cell surface localization of the channel.
cause an increase in these currents, relative to no $\alpha_2 \delta$ co-expression (Fig. 4, B and C), although the enhancement was smaller than that observed with WT $\alpha_2 \delta$-1 (Fig. 4, B and C). In the absence of $\alpha_2 \delta$-1, peak $I_{\text{ca}}$ at +5 mV (from Fig. 4B) was 9.0 ± 2.7% of that in the presence of WT $\alpha_2 \delta$-1, whereas for $\alpha_2 \delta$-1ΔC-term-HA it was 38.5 ± 7.6%, representing a 3.8-fold increase over no $\alpha_2 \delta$ ($p < 0.001$ compared with no $\alpha_2 \delta$-1, analysis of variance, and Dunnet’s post hoc test).

As observed previously (28), WT $\alpha_2 \delta$-1 increased the inactivation rate of the peak CaV2.1/β1b $I_{\text{ca}}$ (Fig. 4, D and E), $\tau_{\text{inact}}$ being reduced from 177 ms in the absence of $\alpha_2 \delta$-1 to 120 ms when WT $\alpha_2 \delta$-1 was co-expressed. The truncated $\alpha_2 \delta$-1ΔC-term-HA had a less marked effect on inactivation, with $\tau_{\text{inact}}$ being 145 ms (Fig. 4, D and E).

Does Intercellular Transfer Occur from Secreted Anchorless $\alpha_2 \delta$-1?—To examine whether transcellular transfer of secreted $\alpha_2 \delta$-1ΔC-term-HA might occur via the medium to neighboring cells and contribute to plasma membrane association or calcium current enhancement, tsA-201 cells expressing either $\alpha_2 \delta$-1 mid-HA or $\alpha_2 \delta$-1ΔC-term-HA were co-cultured with cells expressing CaV2.1/β1b/GFP. The cells were transfected separately and then washed and mixed after 5 h in culture. After a further 48 h in culture, cell surface $\alpha_2 \delta$-1 was examined by immunocytochemistry in fixed nonpermeabilized cells, for both $\alpha_2 \delta$-1ΔC-term-HA and $\alpha_2 \delta$-1 mid-HA (Fig. 5A, panels i and ii, red staining, white arrows), whereas GFP-positive cells were rarely found to be associated with any red staining. We found a very small amount of evidence of possible transfer of $\alpha_2 \delta$-1ΔC-term-HA to areas of the plasma membrane of CaV2.1/β1b/GFP-transfected cells (indicated by a yellow arrow in Fig. 5A, panel i). No evidence of transfer of full-length $\alpha_2 \delta$-1 mid-HA was observed (Fig. 5A, panel ii). These results suggest that attachment of $\alpha_2 \delta$-1ΔC-term-HA to cell surface components occurs mainly during the secretory process, rather than via secretion into the medium and reattachment.
Furthermore, co-culture of cells expressing CaV2.1/α2δ-1/H9252 for 24–36 h directly with cells transfected with α2δ-1mid-HA, with α2δ-1 mid-HA, or with empty vector as a control did not cause any increase in $I_{\text{Ba}}$ recorded from these cells (Fig. 5B).

Similarly, culture of tsA-201 cells expressing CaV2.1/α2δ-1/H9252 for 5 h with medium conditioned by cells expressing α2δ-1mid-HA did not cause any increase in $I_{\text{Ba}}$ compared with cells incubated with unconditioned medium (data not shown).

These results indicate that the enhancement of calcium currents only occurs after co-expression of α2δ-1mid-HA with the calcium channel α1 and β subunits, and it is likely to involve an interaction of this construct with CaV2.1/α2δ-1 channel complexes intracellularly, rather than once the α1/β complex has reached the plasma membrane.

Anchorless α2δ-1ΔC-term-HA at the Plasma Membrane Is Proteolytically Processed to α2 and δ to a Greater Extent than
We next examined the properties of the \(\alpha_2\delta-1\)C-term-HA on the cell surface by cell surface biotinylation (Fig. 6A). Interestingly, we observed that \(\alpha_2\delta-1\)C-term-HA in the cell surface biotinylated fractions was proteolytically cleaved to form \(\alpha_2-1\) to a greater extent, compared with the WCL (Fig. 6A, compare lanes 3 and 6). The proteolytic cleavage, quantified from three independent experiments, revealed an almost 2-fold increase of processing for \(\alpha_2\delta-1\)C-term-HA from the cell surface biotinylated fraction (80 ± 4.7% cleavage) compared with the corresponding WCL (43 ± 6%) (Fig. 6B). In contrast, there was a smaller increase from 38 ± 4.5 to 50 ± 3.3%, respectively, for full-length \(\alpha_2\delta-1\) mid-HA (Fig. 6). Therefore, the anchorless \(\alpha_2\delta-1\)C-term-HA that remained attached to the cell surface by an as yet unknown mechanism was mainly processed to \(\alpha_2-1\) and \(\delta\). In contrast, the secreted form of \(\alpha_2\delta-1\)C-term-HA was predominantly unprocessed, as demonstrated by the absence of \(\delta\)-HA in the medium (Fig. 2A).

**Some \(\alpha_2\delta-1\)C-term Is Associated with Lipid Rafts**—Because some \(\alpha_2\delta-1\)C-term-HA was associated with the plasma membrane, we also examined whether it was still associated with DRMs, also termed lipid rafts, as demonstrated for WT \(\alpha_2\delta-1\) and other \(\alpha_2\delta\) subunits (15, 26). We isolated DRMs from cells expressing WT \(\alpha_2\delta-1\), \(\alpha_2\delta-1\) mid-HA, or \(\alpha_2\delta-1\)C-term-HA by discontinuous sucrose gradient centrifugation as described previously (26). Untransfected tsA-201 cells express a small amount of endogenous \(\alpha_2\delta-1\) (Fig. 7A), which localizes in DRMs. In transiently transfected tsA-201 cells, 66 ± 5.7% of...
Anchorless $\alpha_2\delta$-1 Enhances Calcium Currents

A

|       | + PNGase-F |
|-------|------------|
| WCL   |            |
| UT    | $\alpha_2\delta$-1 mid-HA |
| $\alpha_2\delta$-1ΔC-term-HA |
| Biotinlated |            |
| UT    | $\alpha_2\delta$-1 mid-HA |
| $\alpha_2\delta$-1ΔC-term-HA |

WB: $\alpha_2\delta$-1

A

B

$\alpha_2\delta$-1 mid-HA

$\alpha_2\delta$-1ΔC-term-HA

% cleavage of $\alpha_2\delta$-1

WCL cell DRM

FIGURE 6. $\alpha_2\delta$-1ΔC-term-HA associated with the plasma membrane is highly processed to $\alpha_2\delta$-1 and $\delta$-1. A, samples of deglycosylated WCL (left panel) and precipitated cell surface-biotinylated proteins (right panel) from untransfected cells (UT, lanes 1 and 4) and cells transfected with $\alpha_2\delta$-1 mid-HA (lanes 2 and 5) or $\alpha_2\delta$-1ΔC-term-HA (lanes 3 and 6) were resolved on a 3–8% Tris acetate gel. Western blots (WB) were revealed with $\alpha_2\delta$-1 Ab. Lower panel, Western blotting with anti-Akt Ab (cytoplasmic protein) was used as a biotinylation control. Note the difference in relative proportions between the bands corresponding to $\alpha_2\delta$-1 and $\alpha_2\delta$-1 in WCL (lanes 2 and 3) and cell surface biotinylated fractions (lanes 5 and 6). B, proteolytic cleavage of $\alpha_2\delta$-1 to $\alpha_2\delta$-1 was calculated for different subcellular fractions for $\alpha_2\delta$-1 mid-HA (white bars) and $\alpha_2\delta$-1ΔC-term-HA (black bars) using blots revealed with $\alpha_2\delta$-1 Ab ($n = 3$ independent experiments $\pm$ S.E.). An example of one of the blots used for quantification of cleavage in the WCL and on the cell surface is shown in A, and for the DRM fraction is shown in Fig. 7, E and F. *p < 0.05 compared with $\alpha_2\delta$-1 mid HA, Student’s t test.

WT $\alpha_2\delta$-1 (Fig. 7B, fractions 5–7; $n = 3$) and 59.5 ± 5.7% of $\alpha_2\delta$-1 mid-HA (Fig. 7C, $n = 3$) were found in DRM fractions. The DRM localization of the endogenous marker flotillin-1 was quantified to be 85.4 ± 3.9%, whereas the transferrin receptor, which was used a marker for the soluble fractions, was essentially absent from DRMs ($n = 3$; Fig. 7, A--D). In contrast, we observed a large proportion of $\alpha_2\delta$-1ΔC-term HA (Fig. 7D) and $\alpha_2\delta$-1 mid-HA $\Delta$C-term (supplemental Fig. 1) to be in the soluble fractions (11–13), as judged by both $\alpha_2\delta$-1 and HA immunoreactivity. This distribution would be expected for a soluble protein in the process of being secreted. However, a significant fraction of both anchorless constructs (22.9 ± 4.7% of $\alpha_2\delta$-1 mid-HA $\Delta$C-term and 29.1 ± 2.1% of $\alpha_2\delta$-1ΔC-term-HA, $n = 3$) remained associated with the DRMs (Fig. 7D and supplemental Fig. 1E). This result suggests that the GPI anchor is not the only means by which the protein is retained in DRMs.

As observed previously, proteolytic cleavage of $\alpha_2\delta$-1 to $\alpha_2$ and $\delta$ was more pronounced in isolated DRMs than in WCL (15, 26). However, that increase was greater for $\alpha_2\delta$-1ΔC-term-HA (Fig. 7E) than for full-length $\alpha_2\delta$-1 mid-HA (Fig. 7F, quantification included in Fig. 6B). Therefore, similarly to the cell surface biotinylated $\alpha_2\delta$-1ΔC-term-HA, isolated DRM fractions also contained more processed $\alpha_2\delta$-1ΔC-term-HA (Fig. 6B).

How Is $\alpha_2\delta$-1ΔC-term Associated with the Plasma Membrane—

We examined a number of possibilities that could be responsible for the extrinsic interaction of $\alpha_2\delta$-1ΔC-term with the cell surface. The fact that the HA epitope at the C terminus is accessible in nonpermeabilized cells strongly suggested that the truncated construct does not adopt a transmembrane configuration. To rule out the possibility that $\alpha_2\delta$-1ΔC-term-HA formed an integral membrane protein, we treated DRMs isolated from cells expressing $\alpha_2\delta$-1ΔC-term-HA or $\alpha_2\delta$-1 mid-HA with neutral (pH 7.4) or basic carbonate (pH 11.5) buffers. This method has been used previously to examine whether proteins are extrinsically associated with the membrane (16, 21, 29). We found that a high pH wash could release more $\alpha_2\delta$-1ΔC-term-HA from DRMs into the supernatant, which was not the case for $\alpha_2\delta$-1 mid-HA. Less $\alpha_2\delta$-1ΔC-term-HA was released by neutral pH washes (Fig. 8). This indicates that $\alpha_2\delta$-1ΔC-term-HA is not an integral membrane protein, rather the interaction involves electrostatic association.

To test whether, similar to prion protein, the interaction of $\alpha_2\delta$-1ΔC-term-HA with DRMs and cell surface involved interaction with glypicans, we treated cells with heparin (100 μg/ml), which should interfere with any interaction with heparan sulfate proteoglycans. Incubation of either isolated DRMs or transfected cells with heparin had no effect on the association of $\alpha_2\delta$-1ΔC-term-HA with DRMs or the plasma membrane (data not shown). We also found that mutation of the metal ion-dependent adhesion motif in the von Willebrand factor-A domain (6) of $\alpha_2\delta$-1ΔC-term-HA did not prevent the protein from interacting with the plasma membrane (data not shown), indicating that the interaction does not require this site.

DISCUSSION

The recent discovery that $\alpha_2\delta$ subunits can be anchored to the membrane via a GPI moiety rather than a transmembrane protein domain has provided a novel point of view concerning some of their previously investigated properties as key modulators of CaVs currents (10, 11). However, it has also opened new questions related to the role of membrane anchoring for the physiological function of $\alpha_2\delta$ proteins. The initial aim of this
**FIGURE 7.** α₂δ-1ΔC-term-HA is partially associated with DRMs. A–D, WCL from untransfected tsA-201 cells (A) or those expressing WT α₂δ-1 (B), α₂δ-1 mid-HA (C), or α₂δ-1 ΔC-term-HA (D) were subjected to sucrose gradient fractionation to isolate DRMs (fractions 5–7). Fractions were examined using α₂δ-1 (top panels) and HA (middle panels) Abs. In each case, the distributions of the endogenous DRM marker, flotillin-1, and non-raft marker, transferrin receptor (TfR) were also examined (bottom two panels). Data are representative of at least three experiments. Quantification of % of material present in DRMs is given in the “Results.” E and F, isolated DRM fractions of α₂δ-1ΔC-term-HA (E) and α₂δ-1 mid-HA (F)-transfected cells were deglycosylated with PNGase-F (lane 2 compared with lane 1) to show the presence of α₂δ-1 (E and F) and δ-1-HA (E). The % cleavage of α₂δ-1 into α₂δ-1 was quantified for three independent experiments and included in the graph shown in Fig. 6B. Note that proteolytic cleavage of the protein into α₂δ-1 and δ-1 in DRMs is increased relative to WCL, as also shown in quantification on Fig. 6B.
## Anchorless α₂δ-1 Enhances Calcium Currents

### DRM + PNGase-F

| α₂δ-1 mid-HA | α₂δ-1 ΔC-term-HA | α₂δ-1 mid-HA | α₂δ-1 ΔC-term-HA |
|--------------|-----------------|--------------|-----------------|
| **pellets**  |                 |              |                 |
| ph 7.4       | α₂δ-1           | ph 11.5      | α₂δ-1           |
| ph 11.5      |                 | ph 11.5      |                 |
| **supernatants** |                |              |                 |
| ph 7.4       | α₂δ-1           | ph 11.5      | α₂δ-1           |
| ph 11.5      |                 | ph 11.5      |                 |

**WB:** α₂δ-1

**HA**

FIGURE 8. α₂δ-1ΔC-term-HA, but not α₂δ-1 mid-HA, is released from membranes by alkaline carbonate treatment. Isolated DRM fractions from tsA-201 cells transfected with α₂δ-1 mid-HA (lanes 1, 2, 5, and 6) or α₂δ-1ΔC-term-HA (lanes 3, 4, 7, and 8) were treated with buffer composed of either 0.1 M Tris, pH 7.4, or 0.1 M K₂CO₃, pH 11.5, as indicated, to dissociate extrinsically associated proteins. After centrifugation, the pellets (left panel) and supernatants (right panel) were deglycosylated with PNGase-F followed by Western blotting (top panel α₂δ-1 Ab; bottom panel anti-HA Ab to reveal the δ-1-HA peptide).

Our study was to address some of those issues with respect to membrane anchoring of α₂δ-1.

Previous in vitro studies have shown that α₂δ-1, α₂δ-2, and α₂δ-3 subunits all increase the maximum conductance of whole cell calcium channel currents arising from α₁β subunit combinations for the Ca₉.1 and Ca₉.2 classes, in several different expression systems (30–34). For α₂δ-1, it was previously shown that expression of the α₂-1 or δ-1 alone did not enhance calcium currents through Ca₉.2.1 channels (10). Furthermore, these authors also found that expression of α₂δ-1 with the transmembrane segment from another protein (adhalin) did not enhance calcium currents, which is now unsurprising in the light of our recent finding that the α₂δ subunits can form GPI-anchored proteins (15). Replacing the transmembrane segment with an unrelated sequence might interfere with the cleavage of GPI-anchoring signal sequence and the subsequent attachment of the GPI moiety. It has been found that uncleaved GPI precursor proteins show aggregation in the endoplasmic reticulum (35).

It was initially suggested that the transmembrane segment of δ was required for calcium current stimulation, and the entire extracellular portion of α₂δ-1 was implicated in subunit interaction with Ca₉.2.1 (10). Moreover, co-expression of Ca₉.2.1 channels with δ-1 alone affected the biophysical properties of the currents but did not enhance their amplitudes (26). We have now revisited this issue with respect to our recent findings that α₂δ subunits can form GPI-anchored proteins.

We have created an anchorless α₂δ-1 (α₂δ-1ΔC-term) by adding a stop codon immediately prior to the predicted site of attachment of the GPI moiety (15, 36). This approach has previously been employed successfully to study the role of GPI anchoring on the behavior of the prion protein (16). By this means, we obtained a soluble protein deprived of hydrophobic membrane anchoring but containing α₂δ-1 and all the extracellular parts of δ-1, both of which were both previously shown to be of major importance for channel regulation and interaction (11, 26). In our study we found the surprising result that the C-terminal membrane anchoring is not the only determinant of the ability of α₂δ-1 to enhance calcium channel currents, because α₂δ-1ΔC-term still produced a substantial increase of calcium currents when co-expressed with Ca₉.2.1 and β1b. Interestingly, we did not observe such effects upon external application of secreted α₂δ-1ΔC-term to cells previously transfected with Ca₉.2.1 and β1b, suggesting that an intracellular interaction with other subunits is required for the functionality of α₂δ-1 in the calcium channel complex. Moreover, this result implies that other factors than membrane anchoring are likely to be involved in the current-potentiating effects of α₂δ-1.

As expected, a large proportion of α₂δ-1ΔC-term is secreted into the medium, when it is expressed in tsA-201 cells. However, we also found, using both immunocytochemistry and cell surface biotinylation, that α₂δ-1ΔC-term constructs, despite the lack of a C-terminal membrane anchor, remain partially associated with the plasma membrane. However, α₂δ-1ΔC-term-HA does not utilize another hydrophobic region as a transmembrane anchor, because both the α₂δ-1 and HA Abs can access their epitopes in nonpermeabilized cells.

The interaction of α₂δ-1ΔC-term with membranes occurs via a noncovalent linkage, because alkaline carbonate treatment disrupted the DRM association. Furthermore, the interaction is not affected by the presence or absence of other calcium channel subunits. Our finding that secreted α₂δ-1ΔC-term does not re-attach to the plasma membrane following secretion further suggests that its association with the plasma membrane occurs during the maturation and trafficking of the protein.

There are 16 predicted N-linked glycosylation sites in the rat α₂δ-1 sequence. We have found that secreted α₂δ-1ΔC-term was heavily glycosylated, because PNGase-F removed all N-glycosylation (~35 kDa) producing a shift to an apparent mass of ~130 kDa, corresponding to unprocessed deglycosylated α₂δ-1ΔC-term (Fig. 2B).

We have shown in our previous studies that α₂δ subunits are strongly localized in DRMs, both in native tissue and following heterologous expression (15, 26). The GPI-anchoring of α₂δ subunits, as for other proteins, is likely to be an important determinant of their localization in these domains (15) but not the sole factor (16, 37). This is reinforced by the finding that α₂δ-1ΔC-term remains, in part, associated with DRMs. Much of the DRM fraction is derived from the cholesterol-rich plasma membrane, as determined by combined cell surface biotinylation and DRM studies (17). Thus, the partial association of the α₂δ-1ΔC-term constructs with DRMs is in agreement with our evidence that some α₂δ-1ΔC-term is associated with the plasma membrane.

Removal of the C-terminal GPI anchor signal sequence from prion protein did not completely prevent its lipid raft or membrane association (16), although the anchorless prion protein was mainly secreted. Furthermore, prion protein was found to interact with GPI-anchored heparan sulfate proteoglycans.
(glypicans), which play a role in retaining it in DRM fractions (17). In contrast, in this study heparin, which should disrupt such an interaction, did not prevent cell surface or DRM association of α2δ-1AΔC-term.

We have found previously that heterologously expressed α2δ proteins are only partially proteolytically processed into α2 and δ in many expression systems (15, 26, 38). This behavior of expressed α2δ subunits contrasts with the complete processing of native α2δ proteins, where no full-length α2δ is observed (15, 38). In this study, we found that α2δ-1AΔC-term is also incompletely processed, and in particular the secreted form shows very little proteolytic cleavage. In contrast, α2δ-1ΔC-term in both the DRM fraction and the cell surface-biotinylated fraction exhibits a much greater proportion of cleaved α2-1 and δ-1ΔC-term than the WCL or the secreted fraction. These results indicate that the protease in question is likely to be absent from the constitutive secretory pathway, but it is present in the biogenesis pathway for membrane components. The increased proteolytic cleavage of plasma membrane and DRM-associated α2δ-1ΔC-term, compared with full-length α2δ-1, may result from its greater flexibility and availability as a substrate. Furthermore, a number of proteases have also been localized to lipid rafts (39, 40), which may relate to the increased processing of α2δ in DRM fractions.

Because it has been found that the proteolytic cleavage of α2δ-1 is important for its function to enhance calcium channel currents (41), it is likely that the proteolytically cleaved α2δ-1ΔC-term associated with the plasma membrane is responsible for its function, but this remains to be conclusively demonstrated by using a protease-deficient mutant of α2δ-1ΔC-term.

The main physiological relevance of this study is that the truncation of α2δ-1 at its predicted GPI anchor site does not prevent the ability of this construct to affect calcium channel function. This indicates that intrinsic association of α2δ-1 to the plasma membrane is not essential for its function. Our finding that anchorless α2δ-1 is still in part extrinsically associated with the plasma membrane and DRMs suggests that α2δ-1ΔC-term may be processed by two alternative routes, a secretory pathway and a membrane biogenesis pathway, and in the latter pathway it becomes associated with one or more binding partners that determine its association with membranes. This now gives us an important means of identifying the physiological binding partner(s) of α2δ proteins involved in controlling their trafficking and cell surface localization.

Our future research will therefore be aimed at identifying the binding partner(s) with which α2δ-1ΔC-term is interacting during the trafficking process, and which may also serve to tether it to the plasma membrane. It will also be of great interest to determine whether this interaction is related to the surprising ability of α2δ-1ΔC-term to produce a partial enhancement of calcium channel currents.

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