Targeting of Voltage-Gated Calcium Channel α₂δ-1 Subunit to Lipid Rafts Is Independent from a GPI-Anchoring Motif

Philip Robinson, Sarah Etheridge, Lele Song, Riddhi Shah, Elizabeth M. Fitzgerald*, Owen T. Jones*

Faculty of Life Sciences, University of Manchester, Core Technology Facility, Manchester, United Kingdom

Abstract

Voltage-gated calcium channels (Caᵥs) exist as heteromultimers comprising a pore-forming γ₂ subunit with accessory β and α₂δ subunits which modify channel trafficking and function. We previously showed that α₂δ-1 (and likely the other mammalian α₂δ isoforms - α₂δ-2, 3 and 4) is required for targeting Caᵥs to lipid rafts, although the mechanism remains unclear. Whilst originally understood to have a classical type I transmembrane (TM) topology, recent evidence suggests the α₂δ subunit contains a glycosylphosphatidylinositol (GPI)-anchor that mediates its association with lipid rafts. To test this notion, we have used a strategy based on the expression of chimera, where the reported GPI-anchoring sequences in the galanin-receptor-sensitive α₂δ-1 subunit have been substituted with those of a functionally inert Type I TM-spanning protein - PIN-G. Using imaging, electrophysiology and biochemical assays, we find that lipid raft association of PIN-α₂δ is unaffected by substitution of the GPI motif with the TM domain of PIN-G. Moreover, the presence of the GPI motif alone is not sufficient for raft localisation, suggesting that upstream residues are required. GPI-anchoring is susceptible to phosphatidylinositol-phospholipase C (PI-PLC) cleavage. However, whilst raft localisation of PIN-α₂δ is disrupted by PI-PLC treatment, this is assay-dependent and non-specific effects of PI-PLC are observed on the distribution of the endogenous raft marker, caveolin, but not flotillin. Taken together, these data are most consistent with a model where α₂δ-1 retains its type I transmembrane topology and its targeting to lipid rafts is governed by sequences upstream of the putative GPI anchor, that promote protein-protein, rather than lipid-lipid interactions.

Introduction

Voltage-gated calcium channels (Caᵥs) represent the primary means by which changes in membrane potential are coupled to the influx of second messenger calcium ions [1]. As such, Caᵥs play a major role in orchestrating diverse excitability cell functions, ranging from rapid events such as neurotransmitter release in nerves and excitation-contraction coupling in muscle, to longer lasting events such as synaptic plasticity. While it is well established that disruption of Caᵥs is involved in diverse pathologies, including neuropathic pain [2] and cardiac arrhythmia [3], much less is known about how Caᵥ function is modulated, physiologically, at the cellular level [4].

Biochemical and reconstitution studies show that Caᵥs comprise an α₂ subunit (~200 kDa) containing the voltage-sensing, gating and pore machineries [1], [5]. In high voltage-activated Caᵥ1 and Caᵥ2 family channels, α₂ is complexed in a 1:1 stoichiometry with a cytoplasmic auxiliary β subunit. These channels are also complexed with a second auxiliary (~125 kDa) subunit termed α₂δ/β, which, like β subunits, enhances cell surface expression and modulates the biophysical properties of channel heteromers [1], [6], [7]. Since multiple genes encode each type of Caᵥ subunit and their transcripts undergo RNA splicing, Caᵥs manifest a considerable potential for diversity not only in terms of biophysical function, but also in their modulation and cellular expression patterns [1], [7].

Irrespective of their location, emerging data has shown that Caᵥs are organised into large heterogeneous macromolecular assemblies containing a plethora of signal transduction proteins with which they interact and co-operate to meet local and global functional demands [4], [8], [9], [10]. Defining the mechanisms by which such assemblies are constructed and distributed is therefore crucial to understanding and manipulating Caᵥ function [10], [11], [12]. In this regard, an important step forward has been the observation that Caᵥ proteins co-localise with components of specialised cholesterol-rich membrane signalling domains termed lipid rafts [13], [14], in both heterogeneous expression systems and native tissues [15–21]. While alterations in Caᵥ currents seen with cholesterol-depleting agents argue that raft-association is physiologically significant, the precise effects appear to be subtype and/or tissue specific [16], [18–21]. Although different Caᵥs may associate with rafts using alternate modalities [18], [22], there is now compelling evidence for a major involvement of the α₂δ subunit [18], [20], [21]. Thus, α₂δ subunits co-localise with the lipid raft marker proteins caveolin and flotillin when expressed alone [18], [20], [21] and are also...
necessary and sufficient for the targeting of Ca_{2.2} complexes to rafts [21].

Until recently, how the α_{2δ}|δ subunit might mediate Ca_{α} raft targeting was unclear. Structurally, the α_{2δ}|δ subunit has been viewed as a type I transmembrane (TM) spanning protein (Fig. 1A) composed of a large exofacial α_{2δ} head region linked via disulfide bonds to a smaller membrane associated δ subunit [1], [7], [23], [24], [25]. Owing to the presence of features such as Von Willebrand factor A (VWA) and Cache domains, commonly found in integrins and other cell surface proteins, the α_{2δ} region is thought to have a modular structure [6], [7], [26] affording interactions with extracellular matrix proteins such as thrombospondin [27]. Structure-function analysis has also shown that the δ region mediates those interactions with Ca_{α},s that support current enhancement and the biophysical effects seen upon co-expression of α_{2δ}|δ subunits with α_{1β} complexes [28], [29]. In contrast, the δ polypeptide, while affecting the voltage-dependence of Ca_{α}s [28], has been viewed as primarily providing a means for attaching the δ polypeptide to the cell surface via its hydrophobic putative TM-spanning domain located proximal to the short, intracellular, carboxy terminus [1], [7], [20],[23]-[25]. However, a recent study has challenged this structural model and offered a new mechanism for Ca_{α} raft localisation by suggesting the α_{2δ}|δ subunit associates with the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor attached to the δ polypeptide [20]. In common with other GPI anchored proteins, GPI attachment is envisaged to occur through the action of an ER-resident GPI-transamidase which recognises, cleaves and modifies a motif located at the distal site/residue, to which the GPI moiety is amide-linked, b) two adjacent residues (ω1|2) with small side chains (typically G, A and S), c) a spacer sequence of >6 hydrophilic residues, commencing at the ω6|3 position and d) a stretch of hydrophobic residues (particularly L) capable of spanning the membrane [30], [31].

Since GPI-anchored proteins are highly concentrated in lipid rafts [14], [33], [34], the revised model of α_{2δ}|δ subunit structure has then been used to rationalise Ca_{α} raft targeting [20], [35] and the apparent weakness of the α_{2δ}|δ subunit-α_{1β} complex interaction [10], [36]. However, while seeming attractive in offering GPI attachment as a further regulatory locus [35], such a model requires that lipid-lipid interactions between a single δ subunit GPI anchor and liquid-ordered (L_{q}) raft lipids [37] can specify the raft association of Ca_{α},α (αβ), a large, multispanning, membrane protein complex, predicted to partition into liquid-disordered (L_{o}) bulk phase lipid [14], [38], [39]. Moreover, of the four mammalian α_{2δ}|δ subunits, only α_{2δ}|δ-3 shows a significant potential for GPI anchoring when analysed by predictive algorithms (Table 1). Recent evidence also indicates that the co-localisation of raft markers and α_{2δ}|δ-1 subunits (when expressed alone or with α_{1β} complexes) in cell surface aggregates demands an intact actin-based cytoskeleton [21]. However, while this is consistent with a role for actin in shaping the distribution and dynamics of GPI-anchored proteins [40], [41], [42], such observations are equally consistent with the hypothesis that α_{2δ}|δ-1 subunits reside in rafts, and/or higher order raft assembles, via organising principles based upon protein-protein [21], [42], [43], [44] and/or specialised lipid-protein [14], [45], [46], [47] interactions.

To resolve the above hypotheses we have re-visited the raft localisation of the α_{2δ}|δ subunit using an established strategy [48], [49], [50] based on the expression of chimera, where the reported GPI-anchoring sequences in α_{2δ}|δ-1 have been swapped with those from a known Type I TM-spanning protein – PIN-G (Fig. 1B) [51]. Like its α_{2δ}|δ-2 and α_{2δ}|δ-3 counterparts, α_{2δ}|δ-1 has been described as a GPI-anchored protein [20]. However, unlike α_{2δ}|δ-2 and α_{2δ}|δ-3, the consequences of mutating the presumptive GPI-anchoring motif on α_{2δ}|δ-1 raft localisation or Ca_{α} currents have not been reported. Using imaging, electrophysiological and biochemical assays that we recently employed to analyse α_{2δ}|δ-1 in rafts [21], we now show that the raft localisation of α_{2δ}|δ-1 is preserved even after replacement of the reported GPI anchoring motif with the TM domain of PIN-G. Conversely, the GPI-anchoring motif is not sufficient to target PIN-G to lipid rafts. While the localisation of a PIN construct containing α_{2δ}|δ-1, and its GPI motif, to lipid rafts shows susceptibility to GPI-cleavage using phosphoinositide-specific phospholipase C (PI-PLC), this effect is assay-dependent and seems to lack specificity as it also disrupts the raft localisation of caveolin, but, interestingly, not flotillin. Our data therefore support a model where the raft localisation of α_{2δ}|δ-1 depends upon exofacial sequences upstream and independent of the putative GPI-anchoring motif.

**Results**

**Construction and GPI-anchoring potential of α_{2δ}|δ-1/PIN-G chimera**

To dissect the role of GPI anchoring in localising α_{2δ}|δ subunits to lipid rafts, a series of chimeras were prepared between rat α_{2δ}|δ-1 and PIN-G, a functionally inert Type I TM-spanning domain proximal to the short, intracellular, carboxy terminus [1], [7], [20],[23]-[25]. While such anchoring motifs do not have a strict consensus sequence, they contain common elements including a) an amino acid with a small side chain (notably G, C, D, A, N or S) known as the ω site/residue, to which the GPI moiety is amide-linked, b) two adjacent residues (ω1|2) with small side chains (typically G, A and S), c) a spacer sequence of >6 hydrophilic residues, commencing at the ω6|3 position and d) a stretch of hydrophobic residues (particularly L) capable of spanning the membrane [30], [31].

Since GPI-anchored proteins are highly concentrated in lipid rafts [14], [33], [34], the revised model of α_{2δ}|δ subunit structure has then been used to rationalise Ca_{α} raft targeting [20], [35] and the apparent weakness of the α_{2δ}|δ subunit-α_{1β} complex interaction [10], [36]. However, while seeming attractive in offering GPI attachment as a further regulatory locus [35], such a model requires that lipid-lipid interactions between a single δ subunit GPI anchor and liquid-ordered (L_{q}) raft lipids [37] can specify the raft association of Ca_{α},α (αβ), a large, multispanning, membrane protein complex, predicted to partition into liquid-disordered (L_{o}) bulk phase lipid [14], [38], [39]. Moreover, of the four mammalian α_{2δ}|δ subunits, only α_{2δ}|δ-3 shows a significant potential for GPI anchoring when analysed by predictive algorithms (Table 1). Recent evidence also indicates that the co-localisation of raft markers and α_{2δ}|δ-1 subunits (when expressed alone or with α_{1β} complexes) in cell surface aggregates demands an intact actin-based cytoskeleton [21]. However, while this is consistent with a role for actin in shaping the distribution and dynamics of GPI-anchored proteins [40], [41], [42], such observations are equally consistent with the hypothesis that α_{2δ}|δ-1 subunits reside in rafts, and/or higher order raft assembles, via organising principles based upon protein-protein [21], [42], [43], [44] and/or specialised lipid-protein [14], [45], [46], [47] interactions.

To resolve the above hypotheses we have re-visited the raft localisation of the α_{2δ}|δ subunit using an established strategy [48], [49], [50] based on the expression of chimera, where the reported GPI-anchoring sequences in α_{2δ}|δ-1 have been swapped with those from a known Type I TM-spanning protein – PIN-G (Fig. 1B) [51]. Like its α_{2δ}|δ-2 and α_{2δ}|δ-3 counterparts, α_{2δ}|δ-1 has been described as a GPI-anchored protein [20]. However, unlike α_{2δ}|δ-2 and α_{2δ}|δ-3, the consequences of mutating the presumptive GPI-anchoring motif on α_{2δ}|δ-1 raft localisation or Ca_{α} currents have not been reported. Using imaging, electrophysiological and biochemical assays that we recently employed to analyse α_{2δ}|δ-1 in rafts [21], we now show that the raft localisation of α_{2δ}|δ-1 is preserved even after replacement of the reported GPI anchoring motif with the TM domain of PIN-G. Conversely, the GPI-anchoring motif is not sufficient to target PIN-G to lipid rafts. While the localisation of a PIN construct containing α_{2δ}|δ-1, and its GPI motif, to lipid rafts shows susceptibility to GPI-cleavage using phosphoinositide-specific phospholipase C (PI-PLC), this effect is assay-dependent and seems to lack specificity as it also disrupts the raft localisation of caveolin, but, interestingly, not flotillin. Our data therefore support a model where the raft localisation of α_{2δ}|δ-1 depends upon exofacial sequences upstream and independent of the putative GPI-anchoring motif.

The biophysical properties of PIN-α_{2δ}|δ are retained following substitution of the GPI-anchoring motif with the transmembrane and intracellular sequence of PIN-G.

In order to confirm that PIN-α_{2δ}|δ was fully functional we compared its effects on the electrophysiological properties of Ca_{2.2}/B_{1β} channels, with those of WT α_{2δ}|δ-1. Preliminary experiments indicated that the presence of the GFP-tag on PIN-α_{2δ}|δ caused a marked hyperpolarisation of the V_{th} for activation and a slowing of both current activation and inactivation (Fig. S1).
These effects are consistent with previous reports on the biophysical effects of amino-terminal modifications of the α2δ subunit [55]. As a result, all subsequent electrophysiological experiments were conducted using constructs that lacked the GFP tag (deGFP; Fig. S1). As shown in Fig. 2, co-expression of PIN-α2δ conferred on Ca$_{2.2}$/β$_{1b}$ currents the typical hallmarks associated with the presence of WT α2δ-1. Thus, compared with Ca$_{2.2}$/β$_{1b}$ in the absence of α2δ-1, the peak current density, $I_{\text{max}}$, was...
enhanced approximately 4-fold, the $V_{50}$ for activation was hyperpolarised by some 13 mV on average and the rate of current inactivation was enhanced (decreased $t_{max}$) upon co-expression of PIN-$\alpha_2$-3 (see also Table S1). We next examined the functional effects of disrupting the GPI anchoring motif within $\alpha_2$-3. Somewhat surprisingly, and in contrast to data for the $\alpha_2$-2 and $\alpha_2$-3 GPI-anchoring-deficient mutants [20], co-expression of PIN-$\alpha_2$-3-PIN$_{TM}$ with Ca$_{2+}$/b$_{1h}$ produced identical currents to those of channels containing either PIN-$\alpha_2$ or WT $\alpha_2$-1. In the absence of any $\alpha_2$ sequences there was no functional effect on Ca$_{2+}$/b$_{1h}$ channels (Table S1; PIN-$\delta$).

Formation of $\alpha_2$-$\delta$ puncta is independent of the GPI-anchoring motif

Upon expression in COS-7 cells and surface anti-HA immunostaining, PIN-$\alpha_2$-$\delta$ exhibited a labelling pattern (Fig. 3A) characterised by the appearance of numerous small puncta, spread randomly over the cell surface, and matching that of WT $\alpha_2$-$\delta$-1

![Figure 2](image-url)
Figure 3. Surface and total cellular distribution of PIN-α₂δ chimera expressed in COS-7 cells. A. PIN-α₂δ. B. PIN-α₂δ-PINTMI. C. PIN-δC. D. PIN-δC-PINTMI. Cells were labelled with anti-HA and Cy5 secondary antibodies using a surface-labelling specific protocol (Methods) and the distribution of surface (red) and total (green, GFP) PIN construct expression determined by fluorescence imaging. Note strong labelling at cell margins for PIN-δC and PIN-δC-PINTMI and highly punctate labelling for PIN-α₂δ and PIN-α₂δ-PINTMI. Scale bar 15 μm.

doi:10.1371/journal.pone.0019802.g003

Figure 4. Distribution profile of PIN-α₂δ chimera in detergent-resistant membranes is not affected by disruption of the putative GPI anchoring motif. COS-7 cells were transfected with the corresponding PIN chimera and the membranes analysed via immunoblotting of fractions from sucrose density gradients containing 1% Triton-X-100, using antibodies to caveolin (endogenous) (Panel A) or anti-HA (Panel B) for PIN chimera. Representative blots in panels A and B correspond to cells transfected with PIN-α₂δ, PIN-α₂δ-PINTMI, PIN-δC, and PIN-δC-PINTMI. Note the absence of PIN-δC or PIN-δC-PINTMI in raft fractions (3–6) and the presence in raft fractions of both PIN-α₂δ and PIN-α₂δ-PINTMI (asterisk in B). Immunodetection loading controls are denoted by ‘T’.

doi:10.1371/journal.pone.0019802.g004
Raft localisation requires \( \alpha_2/\delta \) sequences upstream of the GPI-anchoring motif

Elsewhere, we have shown an intimate link between the formation of puncta and the co-localisation of \( \alpha_2/\delta \) with lipid raft proteins [21]. Consequently, the presence of puncta in constructs lacking the putative GPI anchoring motif (PIN-\( \delta \)-PIN\(_{TM1} \) and PIN-\( \alpha_2/\delta \)-PIN\(_{TM1} \)) prompted us to examine and compare their raft localisation more directly. To this end, we exploited the ability of lipid raft components, including \( \alpha_2/\delta \) subunits [18], [20], [21], to migrate into low density fractions upon equilibrium centrifugation of cell lysates in sucrose density gradients containing ice-cold non-ionic detergents [14], notably Triton-X-100 [13], [56], [57]. Following centrifugation of lysates prepared at 48 h post-transfection, gradients were fractionated and fractions immunoblotted using anti-HA antibodies (Fig. 4). To control for gradient fidelity, each fraction was also analysed for the presence of the raft marker caveolin. Irrespective of the transfection condition, endogenous caveolin (22 kDa isoform) was detected as a single peak in fractions corresponding to the 5%–30% sucrose interface (Fig. 4A). In cells transfected with PIN-\( \alpha_2/\delta \) (Fig. 4B blot i) approximately 20% of the anti-HA immunoreactivity was distributed at the 5%–30% interface in caveolin-positive fractions, with the remainder locating to fractions of higher density centred on the 30–45% sucrose interface. In contrast, PIN-\( \delta \) – which contains the putative GPI motif – was localised exclusively in the higher density non-raft fractions (Fig. 4B blot iii). Next we examined the distributions of constructs PIN-\( \alpha_2/\delta \)-PIN\(_{TM1} \) (Fig. 4B blot ii) and PIN-\( \delta \)-PIN\(_{TM1} \) (Fig. 4B blot iv) which lack the putative GPI-motif. In both cases raft/non-raft distributions of HA-immunoreactivity were the same as their parent constructs (PIN-\( \alpha_2/\delta \) raft + non-raft and PIN-\( \delta \), non-raft, respectively). Thus, the raft localisation of PIN-\( \alpha_2/\delta \) appears independent of the GPI motif. Conversely, the presence of the GPI motif in PIN-\( \delta \) is insufficient to support raft localisation, implying that upstream sequences are required.

The expression of PIN-\( \alpha_2/\delta \) cell surface puncta is resistant to PI-PLC treatment

Taken together, these data contradict the notion that the association of \( \alpha_2/\delta \)-1 with lipid rafts is specified by the proposed GPI-anchoring motif [20]. To examine this issue further we tested for the existence of a GPI anchor through its susceptibility to PI-PLC cleavage [20], [58], [59]. First, we followed the approach of Davies et al., [2010] [20] who used imaging to assay the effect of PI-PLC on the surface expression of \( \alpha_2/\delta \) constructs. For comparison we also examined the surface and total (surface + intracellular) distribution of GFP-GPI, a well-defined GPI-anchored green fluorescent protein [54]. As shown in Fig. 5A–D, GFP-GPI was found throughout the cell where it was localised in both tubulovesicular structures and at the cell surface. Although known to reside in lipid rafts like other GPI-anchored proteins [34], [39], [60], GFP-GPI surface labelling was not present in the well-defined puncta seen with PIN-\( \alpha_2/\delta \) (e.g. Fig. 3), but rather it was distributed over the cell surface in a pattern reminiscent of a very fine, granular, meshwork (Fig. 5C,D). Following treatment with PI-PLC, all GFP-GPI-transfected cells showed a qualitative decrease in surface (Cy5/anti-GFP) labelling intensity and distribution compared with non-PI-PLC-treated cells (Fig. 5G–J). More quantitative comparisons based on determining the ‘on cell’ signal to noise (‘off cell’ background) ratio (S/B) of raw (i.e. non-background subtracted) images, showed that PI-PLC caused a reduction in GFP-GPI surface labelling intensity to 23% of control (i.e. –PI-PLC) levels (S/B)\(^{-1} = 0.44 \pm 0.066\) n = 8 (–PI-PLC) vs (S/B)\(^{-1} = 0.10 \pm 0.021\) n = 8 (+PI-PLC). In parallel, we examined the action of PI-PLC on the surface expression of PIN-\( \alpha_2/\delta \). In contrast to GFP-GPI, and as noted above, PIN-\( \alpha_2/\delta \) showed a pattern of surface labelling comprised of numerous high intensity puncta, with little interstitial (inter-punctual) labelling (Fig. 5L–O). Significantly, however, pre-treatment of cells with PI-PLC had no apparent effect on the labelling intensity (S/B)\(^{-1} = 0.75 \pm 0.18\) n = 6 (–PI-PLC) vs 0.95 \pm 0.27 n = 8 (+PI-PLC), p = 0.56 (Fig. 5 R–V and Fig. S2). Equally important, using detailed particle analysis we found no effect on the dimensions or density of the PIN-\( \alpha_2/\delta \) puncta (Fig. S2). Neither the number of particles of given area (size distribution) (Fig. S2A), nor the particulate area fraction (a measure of changes in particle dimension) (Fig. S2B) were affected by PI-PLC treatment. Thus, we found no evidence for the effects predicted were PI-PLC treatment able to induce either ‘stripping’ (i.e. decreased particle size), disassembly (formation of smaller puncta) or both (Fig. S2C–F).

The raft distribution of both PIN-\( \alpha_2/\delta \) and caveolin in sucrose gradients is altered by PI-PLC treatment

As a further test for the presence of a GPI anchor in PIN-\( \alpha_2/\delta \), we examined the effect of PI-PLC on the partitioning of PIN-\( \alpha_2/\delta \) in lipid raft fractions obtained using equilibrium centrifugation in sucrose gradients containing ice-cold Triton-X-100. As shown in Fig. 6, gradient analysis of lysates from cells expressing GFP-GPI (Fig. 6A, blot i) showed anti-GFP immunoreactivity exclusively in lipid raft fractions at the 5–30% sucrose interface. In contrast, lysates from cells pre-treated with PI-PLC (Fig. 6B, blot i) showed anti-GFP immunoreactivity was detected in both the raft and non-raft fractions (Fig. 6A, blot i). As before (Fig. 4), anti-HA immunoreactivity was detected in both the raft and non-raft fractions (Fig. 6A, blot ii). However, following pre-treatment of cells with PI-PLC all the anti-HA immunoreactivity appeared in the higher density, non-raft fractions (Fig. 6B, blot ii). While these data supported the contention that PIN-\( \alpha_2/\delta \) is GPI-anchored [20], it was also possible that PI-PLC might have a more globally disruptive effect on lipid raft integrity, particularly given the lack of effect of molecular disruption of the GPI anchoring motif. To examine such a possibility we, therefore, examined the effect of PI-PLC on the gradient distribution of both caveolin (Fig. 6A,B, blot iii) and flotillin (Fig. 6A,B, blot iv) - two endogenous raft markers with separate and independent modes of raft association [61], [62], which both co-localise in puncta containing \( \alpha_2/\delta \) [21]. As anticipated, both caveolin (Fig. 6A, blot iii) and flotillin (Fig. 6A, blot iv) were concentrated in raft fractions in the absence of PI-PLC pre-treatment. However, following PI-PLC pre-treatment, the distribution of caveolin (Fig. 6B, blot iii), but not flotillin (Fig. 6B, blot iv), shifted such that it was found primarily in the higher density non-raft fractions. Thus, PI-PLC appears to have a generally disruptive effect on the integrity of lipid rafts, whose detection depends upon whether caveolin or flotillin is used as a marker.
Figure 5. Effect of PI-PLC cell pre-treatment on the cell surface distribution of GFP-GPI (control) and PIN-α2δ expressed in COS-7 cells. Panels A–I correspond to GFP-GPI fluorescence in the absence (A–C) and presence (G–I) of PI-PLC cell treatment. For clarity, panels A and G depict just the surface (red channel, anti-GFP) labelling corresponding to the merged (red (surface) and green (GFP, surface + intracellular)) images shown in B and H. Panels C and I correspond to high magnification views of the boxed areas shown in A and G, respectively. Note strong surface labelling and evidence of clustering of GFP-GPI, in the absence of PI-PLC and diminution of surface cluster and interstitial fluorescence after PI-PLC treatment. Since contiguity between GFP-GPI clusters precluded standard particle analysis, the effect of PI-PLC on GFP-GPI clustering was analysed further by generating contour maps (panels D and J) (level scale (0–255) shown to right) of the labelling seen in panels C and I, respectively. Line scans based on the contour maps were then constructed to show differences in fluorescence intensity in the absence (white and yellow in D and F) or presence (red and orange in D and F) of PI-PLC cell treatment. Panel K shows the effect of PI-PLC cell pre-treatment on the signal to background fluorescence for raw images (n>8) collected using identical imaging conditions. *** denotes statistically significant difference (P<0.001); Student's t-test. Panels L–T correspond to images from cells transfected with PIN-α2δ in the absence (L–N) and presence (R–T) of PI-PLC. Panels L and M (−PI-PLC) and R and S (+PI-PLC) show merged images for total (surface + intracellular) (green, GFP) and surface (red, anti-GFP) for separate cells. Panels N and T correspond to high magnification views of the boxed areas shown in L and R (red, (surface) channel only). Note the presence of extensive PIN-α2δ clustering irrespective of whether or not the cells had been treated with PI-PLC. Panels O and U correspond to contour maps (above) of the labelling seen in panels N and T, respectively (level scale (0–255) shown to right). Line scans corresponding to the contour maps were then constructed to show differences in fluorescence intensity in the absence (white and yellow in P and Q) or presence (red and orange in P and Q) of PI-
PLC cell treatment. Panel V shows the effect of PI-PLC cell pre-treatment on the signal to background fluorescence for raw images (n=8) collected using identical imaging conditions. Note lack of effect of PI-PLC on PIN-α2δ/δ distribution (O and U) or intensity (V). All images are representative examples from data sets comprised of >8 images (>2 experiments). Scale bars are as follows: panels A, B, G, H, L, M, R and S, 20 μm; panels C, I, N and T, 4 μm.
doi:10.1371/journal.pone.0019802.g005

Treatment with PI-PLC alters the cellular distribution of caveolin but not flotillin

To obtain further evidence for a generalised effect of PI-PLC on raft integrity, we examined the cellular distribution of caveolin and flotillin before and after PI-PLC treatment, using imaging assays (Fig. 7). As documented elsewhere [21], both of these raft marker proteins localise to puncta and large aggregates throughout permeabilised, non-PI-PLC-treated, COS-7 cells (Fig. 7A (caveolin), 7D (flotillin)). However, following pre-treatment of cells with PI-PLC there was a marked alteration in caveolin labelling to patterns consisting of patches of intense labelling proximal to the cell nucleus and the appearance of more diffuse labelling over the cell surface [Fig. 7B]. In contrast, pre-treatment of cells with PI-PLC had no effect on the distribution of flotillin (Fig. 7E) which remained punctate throughout. These data are therefore consistent with those from the sucrose-density gradient experiments and support the notion that PI-PLC – a primary tool for defining raft localisation (O and U) or intensity (V). All images are representative examples from data sets comprised of >8 images (>2 experiments). All images are representative examples from data sets comprised of >8 images (>2 experiments). Scale bars are as follows: panels A, B, G, H, L, M, R and S, 20 μm; panels C, I, N and T, 4 μm.
doi:10.1371/journal.pone.0019802.g005

Discussion

In this study we have tested the notion that the Cav2.2/δ-1 subunit is a GPI anchored protein, by substitution of the putative GPI-anchoring motif, including the downstream sequence for-...
localisation must depend upon additional determinants upstream of the δ site rather than merely the number of residues upstream of the ω site. Although it is conceivable that determinants upstream of δ somehow promote GPI-anchor attachment, our observation that raft localisation is conserved in both PIN-α2/δ and the anchor-deficient PIN-α2/δ-PIN₁₂₃, argues strongly against any involvement of the putative GPI-anchor motif reported by Davies et al (2010) [20]. While we cannot rule out the possibility of cryptic (i.e. internal) GPI-anchor motifs these are very rare and are thought to resemble the classic carboxy terminal anchoring motifs in structure [64], [65]. Indeed, using predictive algorithms to assess the GPI-modification potential for sequentially truncated α2/δ-1 constructs, we have been unable to detect any additional regions within α2/δ-1 that could serve as obvious GPI-anchoring motifs (Fig. S3).

Notwithstanding the above, our data do not exclude the possibility that GPI-anchoring plays an indirect role in α2/δ raft localisation. Indeed, upon treatment with PI-PLC, PIN-α2/δ was no longer associated with lipid rafts when assessed by sucrose gradient analysis. While this effect has been interpreted as arising via the release of α2 and regions of δ-1 up to the ω site [20] (Table 2), it appears to be non-specific since PI-PLC also prevented the raft-association of caveolin which, in contrast to GPI-anchored proteins, is localised to the inner membrane leaflet [61]. Significantly, depletion of caveolin has been reported to redistribute Type I TM proteins from raft to non-raft fractions [66] which may explain the data reported by Davies et al., [20] where flotillin was the primary raft marker (Table 2). In support of this, our images showing that PI-PLC causes partial dispersal of caveolin, are highly reminiscent of those obtained from COS-7 cells treated with the cholesterol-depleting agent, methyl-β-cyclodextrin (M-β-CD) [21]. However, while M-β-CD also disperses flotillin and prevents its co-localisation in lipid raft fractions, PI-PLC does not. Thus, PI-PLC treatment can disrupt raft integrity, but not completely. To our knowledge, potentially disruptive effects of PI-PLC on raft structure have not been examined, although phospholipase C activity and low concentrations of its end product - diacylglycerol, are known to destabilise model membranes including those containing raft lipids [67]. Quite why caveolin and flotillin should show differential raft partitioning after PI-PLC treatment is also unclear, but likely reflects their differing modes of membrane association. While both proteins are acylated, only caveolin has a transmembrane domain [61,62,68]. Irrespective of the mechanisms, a differential effect of PI-PLC on caveolin and flotillin raft localisation, clearly, warrants caution when using these markers alone to assess raft integrity.

Taken together, our chimera studies show that Cavα2, α2/δ-1 raft localisation is independent of the putative GPI-anchoring motif and that this motif does not localise chimera to rafts. By inference, our data do not support the revised model for the topology, membrane association (i.e. GPI anchoring) or ability of α2/δ-1 subunits to target Cavα₂ to lipid rafts. Rather, raft association – at least for α2/δ-1 - appears to require sequences upstream of the ω site.

Figure 7. Effect of PI-PLC cell pre-treatment on the distribution of endogenous caveolin and flotillin in COS-7 cells. Panels A and B correspond to caveolin labelling in the absence (A) and presence (B) of PI-PLC cell pre-treatment. Panel C depicts intensity profiles (averaged in y axis) corresponding to boxes shown in A and B (red and black lines corresponding to profiles with and without PI-PLC, respectively). By averaging the fluorescence intensity, such ‘box scans’ reduce the noisiness seen in individual line scans. Note aggregation of caveolin fluorescence proximal to the nucleus (B) and increase in intensity (C) in images from cells pre-treated with PI-PLC. Panels D and E depict flotillin labelling in the absence (D) and presence (E) of PI-PLC cell pre-treatment. The corresponding box scans are shown in F (red line: +PI-PLC; black line: − PI-PLC). Note similarity in flotillin distribution irrespective of cell pre-treatment with PI-PLC. Scale bars: 15 μm.
doi:10.1371/journal.pone.0019802.g007
site that most likely mediate protein-protein rather than lipid-lipid interactions, a scenario more consistent with emerging views of raft biogenesis and aggregation [14], [42], [69].

Materials and Methods

Chemicals

The construct encoding wild-type rat Cav α2/δ-1 (Neuronal splice variant; Genbank accession number: NM_012919.2) in pcDNA3.1 was supplied by T.P. Snutch (Univ. British Columbia, Canada). Rabbit Cav2.2 in pMT2 (D14157), rat Cavβ1b in pMT2 (X61394) and the mut-3 variant of GFP-pMT2 (U73901) were supplied by A.C. Dolphin (University College London, UK). The pcDNA3.1 plasmid was obtained from Invitrogen, UK. Primary antibodies were obtained from the following sources: anti-α2/δ-1 (Upstate/Millipore, UK), anti-flotillin-1, anti-clathrin, anti-GFP (Sigma-Aldrich, UK) and anti-HA (Covance, UK). Secondary antibodies were obtained as follows: FITC-conjugated anti-rabbit and anti-mouse IgGs (Jackson Immunoresearch, UK), Cy5-conjugated anti-mouse and anti-rabbit IgG (Jackson Immunoresearch, UK) and horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgGs (Dako, UK). All other reagents were obtained from Sigma-Aldrich, UK, unless stated otherwise.

Molecular biology

An α2/δ-1 construct bearing an HA epitope tag between amino acid residues I612 and K613, was generated using a three step

Table 2. Comparison of experimental approaches and conclusions in the present study and that of Davies et al., [20].

|                  | This study                                           | Davies et al. [20]                          |
|------------------|------------------------------------------------------|----------------------------------------------|
| A) Substrates    | HA α2/δ-1 (Rat), PIN α2/δ-1 chimera                  | No constructs or mutants employed            |
| Cell types used  | COS-7                                                | Rat DRG, Hippocampus, tsA-201 cells, cardiac muscle (data not shown) |
| Immunodetection | Anti-HA/Anti-GFP                                      | Anti- α2/δ-1                                 |
| B) Evidence for GPI-Anchoring motif in α2/δ-1 | Probable in only 1/3 algorithms                        | Not given                                   |
| Algorithms       | Not inferred (see introduction)                      | Inferred from α2/δ-2, α2/δ-3 data and partial homology. |
| C) Raft isolation| COS-7 cell lysates                                    | Hippocampal tissue lysates, tsA-201 cell lysates, cardiac muscle (data not shown) |
| Detergent        | Triton-X-100, 4°C                                     | Triton-X-100, 4°C                           |
| Raft markers     | Endogenous Caveolin and Flotillin-1                   | Endogenous Flotillin-1                      |
| Conclusions      | Localisation of α2/δ-1 in rafts                       | Localisation of WT α2/δ-1 in rafts          |
| D) Imaging       | COS-7                                                | Rat DRG                                     |
| α2/δ-1           | Transfected constructs                                | Endogenous                                  |
| Labelling method | Surface protocol                                     | Non-permeabilised*                          |
| Detection        | Immunofluorescence                                   | Immunofluorescence                          |
| Quantification   | Intensity and Particle analysis                       | Intensity                                   |
| Conclusions      | Formation of α2/δ-1 puncta independent of GPI anchoring motif but requires upstream sequences | N/A                                          |
| E) PI-PLC        | Concentration 4 U/ml, 1 h, 37°C                       | 4–8 U/ml, 1 h, 37°C                        |
| Treatment - rafts| Live COS-7 cells prior to lysis                       | Hippocampal tissue lysates                  |
| Conclusions      | Raft localisation of α2/δ-1 reduced                   | Raft localisation of α2/δ-1 reduced         |
| Treatment - imaging | COS-7 cells, surface protocol                       | ‘non-permeabilised* DRG cells               |
| Conclusions      | Formation of surface α2/δ-1 puncta resistant to PI-PLC | Surface expression of α2/δ-1 reduced by PI-PLC |
| F) Electrophysiology | CaV2.2/b1b +/- PIN- α2/δ-1 chimera, or, WT-α2/δ-1   | α2/δ-1 not tested.                          |
| Cells            | COS-7                                                | tsA-201                                     |
| Conclusions      | Current density unaffected by loss of GPI anchoring motif | Not tested (reduced current density in α2/δ-2/-3 on disruption of GPI anchoring motif) |

Key differences are our use of: a) both caveolin and flotillin as raft markers, b) a carefully controlled surface-labelling protocol, c) lysates from live cells treated with PI-PLC and d) the extensive use of chimera which ablate the purported GPI-anchoring motif. Asterisks denote the use of non-permeabilised cells without reference to controls. As we show elsewhere [21], fixative alone can cause significant cell permeabilisation.

doi:10.1371/journal.pone.0019802.t002
strategy as described in Robinson et al. (2010) [21]. All PIN constructs were prepared through the sequential insertion, deletion or substitution [70] of specified rat α2/δ-1 sequences into the PIN-G plasmid (Genbank: AY841887), using the QuickChange® II kit (Agilent Technologies, U.K.) and mutagenic megaprimers prepared by PCR. Construct fidelity was confirmed by in-house sequencing (see Fig. 1 and Fig. S8C for chimera junctions).

Cell culture and transient transfection

Culture and transient transfection of COS-7 cells (European Cell Culture Collection, Health Protection Agency, U.K.), were carried out as described in Robinson et al. (2010) [21]. Transient transfections were performed in serum-free Dulbecco’s modified Eagle’s medium (DMEM) at a cell confluency of 60–70% using FuGene 6 (Roche Diagnostics, U.K.); imaging and electrophysiology; or Turbofect (Fermentas, U.K.; biochemical experiments) at a total DNA:reagent ratio of 1:3 (w/v); (total DNA: 2 μg for 6-well plates/35 mm dishes, 12 μg DNA for 10 cm plates). Transfections with Ca2.2, Cav5, and Ca,α2/δ-1 used a ratio of 3:1:1 by mass of subunit cDNA. For transfections omitting α2/δ cDNA, the α2/δ cDNA was replaced with pcDNA3.1 to maintain the equivalent mass ratio. Cells were maintained at 37°C, 5% CO2 in complete medium for a total of 48 hours (including any re-plating step), after which cells were: a) fixed for microscopy (below), b) re-plated onto 22 mm square coverslips for electrophysiology, or c) lysed for biochemical experiments. For re-plating post-transfection, cells were detached using a non-enzymatic cell dissociation solution (Sigma Aldrich, U.K.) before re-seeding in fresh complete medium.

Western immunoblotting

At 48 h post-transfection, COS-7 cells were washed in PBS and lysed at 4°C in a radio-immunoprecipitation assay (RIPA) buffer with Complete MINI EDTA-free protease inhibitor cocktail (Roche, U.K.). The cell lysates were then passed through a 22-gauge syringe needle 10 times to shear genomic DNA, and centrifuged at 10,000 g for 20 min at 4°C. Supernatants were then incubated at 37°C for 15 min with Laemmli loading buffer containing 20 mM DTT and then heated to 95°C for 2 min. Sample proteins were resolved by SDS-PAGE on 10% Tris-HCl gels for 80 min at 160 V (Mini-Protein cell, BioRad, U.K.) and then transferred by electrophoresis (100 V for 2 h) onto nitrocellulose membranes (Whatman, U.K.). Air dried membranes were immersed overnight in blocking buffer (5% non-fat dry milk in Tris-buffered saline (TBS)) washed three times with TTBS and then incubated with the appropriate primary antibody in TTBS for 1 h at 20°C. The membranes were then re-washed with TTBS and incubated for 1 h at 20°C with the appropriate secondary HRP-conjugated antibody (1:1000) in TTBS. After further washing with TTBS, the membranes were treated with Western Lightning enhanced chemiluminescence reagent (Perkin Elmer, U.K.) and immunoreactive proteins detected by exposure to film (GE Life Sciences, U.K.).

Sucrose gradient fractionation

As we described recently [21], transiently transfected COS-7 cells were washed in PBS and lysed 48 h post-transfection with MBS (Mes-buffered saline: 25 mM Mes, pH 6.5, 150 mM NaCl) with 1% Triton-X-100 at 4°C. For a single experiment, 9×10 cm dishes were used and 150 μl of MBS/Triton-X-100 was added to lyse the cells. Cells were scraped off the dish, passed through a 22-gauge needle 10 times to shear genomic DNA and 450 μl of lysate was reserved for use as a control. The remaining 900 μl of lysate was mixed with 900 μl of 90% sucrose/MBS (w/v), placed in a 5 ml polypropylene centrifuge tube (Sorvall) and carefully overlaid with 1.5 ml of 30% sucrose/MBS, followed by 1.5 ml of 5% sucrose/MBS. Gradients were spun at 36,300 rpm (140,000 gav) in a Sorvall Discovery 100SE ultracentrifuge using an AH-650 rotor for 16 h at 4°C. Post-centrifugation, 15 fractions were taken from top to bottom of the tube and analysed in subsequent Western immunoblotting. To concentrate proteins, fractions were incubated with 25% trichloroacetic acid (final), at 4°C for 30 min. Samples were centrifuged at 14,000 rpm (13,000 gav) at 4°C for 20 min and the pellets washed twice with ice-cold acetone, ensuring not to disrupt the pellets. Pellets were dried at 42°C for 10 min before re-suspension in 50 μl of MBS and analysed by Western immunoblotting.

Immunocytochemistry

Cells for fluorescence microscopy were re-plated 24 hours post-transfection onto 13 mm coverslips coated with 0.01% poly-L-lysine. To preclude fixation artefacts, all imaging experiments of surface expression were performed using a two-step protocol [21]. Briefly, COS-7 cells (48 h post-transfection) were cooled on ice to 4°C and after 10 min, treated with primary antibody diluted in PBS. After 1 h at 4°C, coverslips were washed 3 times with PBS and the cells fixed with 4% (w/v) paraformaldehyde for 20 min at 20°C. Cells were then treated with the appropriate (Cy5 or FITC) fluorophore-conjugated secondary antibody for 1 h at 20°C. In order to detect intracellular epitope expression, cells were permeabilised post-fixation with 0.5% saponin for 10 min at 20°C, prior to incubation with primary antibody. Nuclear staining was performed with DAPI (4’,6-diamidino-2-phenylindole; 1 μg/ ml) for 2 min at 20°C, prior to mounting with Prolong Gold Anti-fade reagent (Invitrogen/Molecular Probes).

PI-PLC treatment

At 48 h post-transfection, COS-7 cells were washed with serum-free DMEM and incubated with PI-PLC (Invitrogen, U.K.; 4 Units/mL) for 1 h at 37°C. The cells were then washed in DMEM to remove PI-PLC, placed on ice and processed for imaging (above) or immunoblotting.

Fluorescence deconvolution microscopy and image analysis

Images of cells on coverslips were acquired on a Delta Vision RT (Applied Precision, Image Solutions, UK) restoration microscope using a ×60 objective lens and appropriate wavelength filters. The images were collected using a Coolscope HQ (Photometrics) camera with a Z optical spacing of 0.1 μm. Raw images were then deconvolved using Softworx software and displayed as maximum projections using NIH Image J (W.S. Rasband, NIH Bethesda, USA; Wright Cell Imaging facility bundle: http://www.uhnres.utoronto.ca/facilities/wcif.htm).

Whole-cell patch-clamp electrophysiology

As described previously [21], COS-7 cells were transiently transfected with Ca2.2, Cav5, and α2/δ-1:mut3-GFP-pMT2 cDNA in a 3:1:1:0.2 mass ratio and current recordings made 48 h post-transfection. Where α2/δ or mutant GFP was omitted, empty pcDNA3.1 vector was substituted to maintain the equivalent mass of DNA. Electrophysiologically recordings of barium currents were made from green fluorescent COS-7 cells, using the whole-cell configuration of the patch clamp technique and the following solutions [71]. The internal solution contained (mM): caesium aspartate 140.0; EGTA 5.0; MgCl2 2.0; CaCl2 0.1; Hepes 20.0; K2ATP 1.0; adjusted to pH 7.2 with CsOH and 310 mosm/l with sucrose. The external solution contained (mM): TEABr...
of pre-treatment with PI-PLC. **B.** Distribution of fractional coverage represented by PIN-\(\alpha_\delta\) particles. Inset: data re-plotted using expanded scale. Here and elsewhere [21], we define fractional coverage as the % of the total particulate area (\(C_p\)) within a region of interest (ROI), not the area of the ROI accounted for by particles of area \(A_P\) (i.e. \(N_P/A_P/C_p\), where \(C_p = \sum_{i=1}^{N_t} A_P A_P'\) and \(A_P'\) is the area of the largest particle in the data set). Using this representation it is possible to discriminate cases where coverage of the total particle area arises from many small particles or a lesser number of larger particles. For example, in the simple situation where there are 4 particles each of size 10 pixel\(^2\) and 1 particle of size 60 pixel\(^2\), then \(C_p = 100\), then for the smaller particles \(N_t/N_p = 0.8\) and the fractional coverage = 0.4, for the larger particle \(N_p/N_t = 0.2\) and fractional coverage = 0.6. In contrast, if the same total particulate area is comprised of 60 particles each of size 1 pixel\(^2\) and 4 particles each of size 10 pixel\(^2\), then \(N_t/N_p = 0.94\) and the fractional coverage = 0.6, for the larger particles \(N_p/N_t = 0.06\) and fractional coverage = 0.4. Note overlap of data, irrespective of pre-treatment with PI-PLC. Particle analysis was performed with Image J, using the adaptive thresholding plug-in, with thresholded images checked visually for accuracy. All data were extracted from 3 images from separate experiments. **C.** and **D.** Computer modelling of the effects of particle re-distribution on fractional coverage Fractional coverage graphs (D) were determined for the three particle size distributions shown in C. Note marked, and well-defined effect of particle re-distribution. For simplicity, the distribution curves in C were generated using equations based on a binomial distribution with terms \(p^n (q)^{1-n}\) (red) and \(q^n (p)^{1-n}\) (blue), where \(q = 1-p\), respectively. In each case, the number of particles \(N_t\) was adjusted to give an identical total particulate area, \(N_t = 1 \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \times \time...

"
GPI-modification for that and 2δ proteins shown in A inferred using Big-Fi predictor software [http://expasy.org/tools/]. Proteins with positive or negative GPI modification potential are shown in blue and red, respectively. Asterisks denote proteins where the 2 site differs from that inferred. Right panel detailed sequence comparison of inferred (red) and predicted (asterisk) sites. In most cases the inferred 2 site is very close (<2 residues) to that found experimentally.

C. Analysis of potential upstream GPI-anchoring motifs in the delta subunit of WT 2δ-1 (or PIN-2δ(blue)) and PIN-2δ-PINTMI (red). Here, the GPI anchoring potential was determined (using Big-Fi [29]) as a function of successive truncation (1 residue at a time) of the carboxy terminus. Note: based on the length of the GPI-anchoring motif, any site is predicted to lie 20–30 residues upstream of the 2 site.

Table S1 Biophysical properties of Ca v2.2/β1d channels co-expressed with WT 2δ-1, PIN-2δ-1, PIN-2δ-PINTMI and PIN-δ. Imax is the maximum peak current density. Individual current density-voltage plots were fitted with a Boltzmann function:

\[
I = \frac{(g(V - V_r)/k) + \exp(-(V - V_{50,act}))/k}{(1 + \exp(-(V - V_{50,act}))/k)}
\]

where \(V_r\) is the reversal potential, \(V_{50,act}\) is the voltage for half maximal activation of current, \(g\) is the conductance, and \(k\) is the slope factor. Statistical analysis used Students unpaired t-test. Asterisks denote statistically significant differences from \(-|\gamma|\delta-1\), as follows: * = p<0.05, ** = p<0.001. n is the number of cells tested per treatment.

Acknowledgments

We would like to thank Dr. Felix Elortza (CICbioGUNE, Derio, Spain) for advice regarding proteomics and predictive analysis of GPI-anchored proteins.

Author Contributions

Conceived and designed the experiments: OTJ EMF PR. Performed the experiments: PR LS SE RS. Analyzed the data: PR OTJ EMF LS. Contributed reagents/materials/analysis tools: OTJ EMF. Wrote the paper: OTJ EMF.

References

1. Catterall WA, Perea-Reyes E, Snutch TP, Striessnig J (2005) International Union of Pharmacology XLVIII Nomenclature and structure-function relationships of voltage-gated calcium channels. Pharmacol Rev 57: 411–425.
2. Perret D, Luo ZD (2009) Targeting voltage-gated calcium channels for neuropathic pain management. Neurotherapeutics 6: 679–692.
3. Saplwski I, Timothy KW, Decher N, Kumar P, Sachse FB, et al. (2005) Severe arthrythmia disorder caused by cardiac L-type calcium channel mutations. Proc Natl Acad Sci USA 102: 8089–8096.
4. Dai S, Hall DD, Hell JW (2009) Supramolecular assemblies and localized regulation of voltage-gated ion channels. Physiol Rev 89: 411–452.
5. Ertel EA, Campbell KP, Harpold MM, Hoffmann F, Mori Y, et al. (2000) Nomenclature of voltage-gated calcium channels. Neuron 25: 533–535.
6. Canti C, Nieto-Rostro M, Foucault I, Heblich F, Wratten J, et al. (2005) The metal-ion-dependent adhesion site in the Van Willebrand factor A-domain of 2β subunits is key to trafficking voltage-gated Ca channels. Proc Natl Acad Sci USA 102: 11230–11235.
7. Davies A, Hendrich J, Van Minh AT, Wratten J, Douglas L, et al. (2007) Functional biology of the β2 subunits of voltage-gated calcium channels. Trends Pharmacol Sci 28: 220–229.
8. Elliott EM, Malouf AT, Catterall WA (1995) Role of calcium channel subtypes in calcium transients in hippocampal CA3 neurons. J Neurosci 15: 6433–6444.
9. Pani B, Singh BB (2009) Lipid rafts/caveolae as microdomains of calcium signalling. Cell Calcium 45: 625–633.
10. Muller CS, Haupt A, Bild W, Schindler J, Knaus HG, et al. (2010) Quantitative proteomics of the Cav2 channel nano-environments in the mammalian brain. Proc Natl Acad Sci U S A 107: 14950–14957.
11. Striessnig J, Koschak J (2008) Exploring the function and pharmacotherapeutic potential of voltage-gated Ca2+ channels to a caveolar macromolecular signalling complex is required for β3-adrenergic regulation. Proc Natl Acad Sci USA 103: 7500–7505.
12. Antonis A, Douglas L, Hendrich J, Wratten J, Van Minh AT, et al. (2006) The calcium channel α2δ-1 subunit co-localizes with Ca2.1 in lipid rafts in cerebellum: Implications for localization and function. J Neurosci 26: 8748–8757.
13. Xia F, Leung YM, Gausso G, Gao X, Chen Y, et al. (2007) Targeting of voltage-gated Ca1.2 and Ca1.3 channels and soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins to cholesterol-rich lipid rafts in pancreatic alpha-cells: effects on glucagon stimulus-secretion coupling. Endocrinology 148: 2157–2167.
14. Davies A, Kasurin I, Alvarez-Laviada A, Douglas L, Nieto-Rostro M, et al. (2010) The α2β subunits of voltage-gated calcium channels form GPI-anchored proteins, a post-translational modification essential for function. Proc Natl Acad Sci U S A 107: 16534–16539.
15. Robinson P, Etheridge S, Song L, Armenise P, Jones OT, et al. (2010) Formation of N-type (Cav2.2) voltage-gated calcium channel membrane microdomains: Lipid raft association and clustering. Cell Calcium 48: 183–194.
16. Klappe PCI, Meier MM, Jarrard RE, Przybyla JA, Liu G, et al. (2009) The intracellular II-III loops of Ca1.2 and Ca1.3 uncouple L-type voltage-gated Ca2+ channels from glucagon-like peptide-1 potentiation of insulin secretion in INS-1 cells via displacement from lipid rafts. J Pharmacol Exp Ther 330: 283–293.
17. Balijepalli RC, Foell JD, Hall DD, Hell JW, Kump TJ (2006) Localization of cardiac L-type Ca2+ channels to a caveolar macromolecular signalling complex is required for β3-adrenergic regulation. Proc Natl Acad Sci USA 103: 7500–7505.
18. Perret D, Luo ZD (2009) Targeting voltage-gated calcium channels for neuropathic pain management. Neurotherapeutics 6: 679–692.
19. Davies A, Kasurin I, Alvarez-Laviada A, Douglas L, Nieto-Rostro M, et al. (2010) The α2β subunits of voltage-gated calcium channels form GPI-anchored proteins, a post-translational modification essential for function. Proc Natl Acad Sci U S A 107: 16534–16539.
20. Robinson P, Etheridge S, Song L, Armenise P, Jones OT, et al. (2010) Formation of N-type (Cav2.2) voltage-gated calcium channel membrane microdomains: Lipid raft association and clustering. Cell Calcium 48: 183–194.
21. Klappe PCI, Meier MM, Jarrard RE, Przybyla JA, Liu G, et al. (2009) The intracellular II-III loops of Ca1.2 and Ca1.3 uncouple L-type voltage-gated Ca2+ channels from glucagon-like peptide-1 potentiation of insulin secretion in INS-1 cells via displacement from lipid rafts. J Pharmacol Exp Ther 330: 283–293.
22. De Jongh KS, Warner C, Catterall WA (1990) Subunits of purified calcium channels. J Biol Chem 265: 283–293.
23. Wiser O, Trus M, Tohi D, Halevi S, Giladi E, et al. (1996) The α2δ subunit binds to a caveolar macromolecular signalling complex required for β3-adrenergic regulation. Proc Natl Acad Sci USA 103: 7500–7505.
24. Balijepalli RC, Foell JD, Hall DD, Hell JW, Kump TJ (2006) Localization of cardiac L-type Ca2+ channels to a caveolar macromolecular signalling complex is required for β3-adrenergic regulation. Proc Natl Acad Sci USA 103: 7500–7505.
29. Felix R, Garnett CA, DeWaard M, Campbell KP (1997) Dissection of functional domains of the voltage-dependent Ca\(^{2+}\) channel \(\alpha_{2}\delta\) subunit. J Neurosci 17: 6834–6891.

30. Eisenhaber B, Bork P, Eisenhaber F (1999) Prediction of potential GPI-modification sites in proprotein sequences. J Mol Biol 292: 741–758.

31. Zacks MA, Garg N (2006) Recent developments in the molecular, biochemical and functional characterization of GPI and the GPI-anchoring mechanism. Mol Membr Biol 23: 209–225.

32. Paulick MG, Bertozzi CR (2008) The glycosylphosphatidylinositol anchor: a complex membrane-anchoring structure for proteins. Biochemistry 47: 6991–7000.

33. Brown DA, Rose JK (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell 68: 533–544.

34. Levental I, Grzybek M, Simonx K (2010) Greasing their way: lipid modifications determine protein association with membrane rafts. Biochimica et Biophysica Acta 1806: 6303–6316.

35. Bauer CS, Tran-Van-Minh A, Kadurin I, Dolphin AC (2010) A new look at the glycosylphosphatidylinositol anchor: a complex membrane-anchoring structure for proteins. Biochemistry 47: 6991–7000.

36. Gurnett CA, Felix R, Campbell KP (1997) Extracellular interaction of the voltage-dependent Ca\(^{2+}\) channel \(\alpha_{2}\delta\) subunits. Curr Opin Neurobiol 20: 563–571.

37. Garnett CA, Felix R, Campbell KP (1997) Extracellular interaction of the voltage-dependent Ca\(^{2+}\) channel \(\alpha_{2}\delta\) and \(\tau_{1}\) subunits. J Biol Chem 272: 18506–18512.

38. Quin PJ (2010) A lipid matrix model of membrane raft structure. Prog Lipid Res 49: 390–406.

39. Lundback JA, Andersen OS, Werde T, Nielsen C (2003) Cholesterol-induced protein sorting: an analysis of energetic feasibility. Biophys J 84: 2000–2009.

40. Baumgart T, Hammond AT, Sengupta P, Hess ST, Holowka DA, et al. (2007) Large-scale fluid/liquid phase separation of proteins and lipids in giant plasma membrane vesicles. Proc Natl Acad Sci U S A 104: 3165–3170.

41. Chichili GR, Rodgers W (1996) Replacement of the glycoinositol phospholipid moiety of glycoproteins by the actin cytoskeleton. J Biol Chem 271: 36692–36691.

42. Goswami D, Gowerthunker D, Bilgrami S, Ghosh S, Raghupathy R, et al. (2008) Nanoclusters of GPI-anchored proteins are formed by cortical actin-driven activity. Cell 135: 1085–1097.

43. Chen Y, Veracini L, Benistant C, Jacobson K (2009) The transmembrane protein CBP plays a role in transiently anchoring small clusters of Thy-1, a GPI-anchored protein, to the cytoskeleton. J Cell Sci 122: 3966–3972.

44. Douglass AD, Vale RD (2005) Single-molecule microscopy reveals plasma membrane microdomains created by protein-protein networks that exclude or trap signaling molecules in T cells. Cell 121: 917–926.

45. Stetzkowski-Marden F, Gans K, Recouvreur M, Caraud A, Caraud J (2006) Agrin elicits membrane lipid condensation at sites of acetylcholine receptor clusters in C2C12 myotubes. J Lipid Res 47: 2121–2133.

46. Ge M, Gidwani A, Brown HA, Holowka D, Baird B, et al. (2003) Ordered and disordered phases coexist in plasma membrane vesicles of RBL-2H3 mast cells. An EMK study. Biophys J 85: 1278–1288.

47. Mayor S, Maxfield FR (1995) Insolubility and redistribution of GPI-anchored proteins at the cell surface after detergent treatment. Mol Biol Cell 6: 929–944.

48. Parton RG, Hanzl-Bayer M, Hancock JF (2006) Biogenesis of caveolae: a structural model for caveolin-induced domain formation. J Cell Sci 119: 787–796.

49. Wang J, Maziarz K, Ratnam M (1999) Recognition of the carboxyl-terminal signal for GPI modification requires translocation of its hydrophobic domain across the ER membrane. J Mol Biol 286: 1303–1310.

50. Watanabe K, Nagaoka T, Strizzi L, Mancino M, Gonzales M, et al. (2008) Caveolin-3 knockout mice develop a progressive cardiomyopathy and show hyperactivation of the p42/44 MAPK cascade. J Biol Chem 277: 38988–38997.

51. Mayor S, Maxfield FR (1995) Insolubility and redistribution of GPI-anchored proteins at the cell surface after detergent treatment. Mol Biol Cell 6: 929–944.

52. Mayor S, Maxfield FR (1995) Insolubility and redistribution of GPI-anchored proteins at the cell surface after detergent treatment. Mol Biol Cell 6: 929–944.

53. Pierleoni A, Martelli PL, Casadio R (2008) PredGPI: a GPI-anchor predictor. BMC Bioinformatics 9: 392.

54. Nichols BJ, Kemworthy AK, Polishchuk RS, Lodge R, Roberts TH, et al. (2001) Rapid cycling of lipid raft markers between the cell surface and Golgi complex. J Cell Biol 153: 529–541.

55. Sandalov A, Oxiedo N, Andrade A, Felix R (2004) Glycosylation of asparagines 136 and 184 is necessary for the \(\alpha_{2}\delta\) subunit-mediated regulation of voltage-gated Ca\(^{2+}\) channels. FEBS Lett 576: 21–26.

56. Schroeder R, London E, Brown D (1994) Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. Proc Natl Acad Sci U S A 91: 12130–12134.

57. Sengupta P, Hammond A, Holowka D, Baird B (2008) Structural determinants for partitioning of lipids and proteins between coexisting fluid phases in giant plasma membrane vesicles. Biochim Biophys Acta 1778: 20–32.

58. Ferguson MA, Low MG, Cross GA (1985) Glycosylphosphatidylinositol is covalently linked to Trypanosoma brucei variant surface glycoprotein. J Biol Chem 260: 14547–14553.

59. Elortza F, Nuñez TS, Foster LJ, Strndhall, A Pec, SC, et al. (2003) Proteomic analysis of glycosylphosphatidylinositol-anchored membrane proteins. Mol Cell Proteomics 2: 1261–1270.

60. Mayor S, Maxfield FR (1995) Insolubility and redistribution of GPI-anchored proteins at the cell surface after detergent treatment. Mol Biol Cell 6: 929–944.

61. Parton RG, Hanzl-Bayer M, Hancock JF (2006) Biogenesis of caveolae: a structural model for caveolin-induced domain formation. J Cell Sci 119: 787–796.

62. Rowan AJ, Hoegg MB, Robbins SM (2007) The SPH domain-containing proteins: more than lipid raft markers. Trends Cell Biol 17: 594–602.

63. Bernstein GM, Jones OT (2006) Kinetics of internalization and degradation of \(\alpha_{2}\delta\)-type voltage-gated calcium channels: Role of the \(\alpha_{2}\delta-1\) subunit. Cell Calcium 41: 27–40.

64. Su B, Bothwell AL (1989) Biosynthesis of a glycosylphosphatidylinositol-glycan-linked membrane protein: signals for posttranslational processing of the Ls-V6 antigen. Mol Cell Biol 9: 3369–3377.

65. Watanabe K, Nagaoka T, Strizzi L, Mancino M, Gonzales M, et al. (2008) Characterization of the glycosylphosphatidylinositol-anchor signal sequence of human CypC with a hydrophilic extension. Biochimica et Biophysica Acta 1778: 2671–2681.

66. Woodman SE, Park DS, Cohen AW, Cheung MW-C, Chandra M, et al. (2002) Caveolin-3 Knock-out Mice Develop a Progressive Cardiomyopathy and Show Hyperactivation of the p112/44 MAPK Cascade. J Biol Chem 277: 38988–38997.

67. Baezúrez M, Lopez DJ, Montes LR, Seti J, Vasil AI, et al. (2011) Imaging the early stages of phospholipase C/sphingomyelinase activity on vesicles containing coexisting ordered-disordered and gel-fluid domains. J Lipid Res 52: 635–645.

68. Stuermer CA (2011) Microdomain-forming proteins and the role of therapeutics/flotillins during axon regeneration in zebrafish. Biochim Biophys Acta 1812: 415–422.

69. Kusum A, Suzuki K (2005) Toward understanding the dynamics of membrane-raft-based molecular interactions. Biochim Biophys Acta 1746: 234–251.

70. Geiger M, Cizek R, Dreswillo D, Schmidt R (2001) Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligation. Biotechniques 31: 88–90, 92.

71. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 391: 83–100.

72. Bernstein GM, Mendonca A, Wadia J, Burnham WM, Jones OT (1999) Kindling induces a long-term enhancement in the density of N-type calcium channels in the rat hippocampus. Neuroscience 94: 1083–1109.