Review

The α2δ subunits of voltage-gated calcium channels ☆

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ABSTRACT

Voltage-gated calcium channels consist of the main pore-forming α_1 subunit, together, except in the case of the T-type channels, with β and $\alpha_2\delta$ and sometimes γ subunits, which are collectively termed auxiliary or accessory subunits. This review will concentrate on the properties and role of the $\alpha_2\delta$ subunits of these channels. These proteins are largely extracellular, membrane-associated proteins which influence the trafficking, localization, and biophysical properties of the channels. This article is part of a Special Issue entitled: Calcium channels.

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1. Introduction

Voltage-gated calcium (Ca$_V$) channels are required for many key functions in excitable cells, including transmitter release and muscle contraction [1]. The calcium channel complex in skeletal muscles is localized in skeletal muscle transverse tubules. Purification shows it to contain five protein bands: α$_1$ (~170 kDa), α$_2$ (~150 kDa), β (~52 kDa), δ (~17–25 kDa) and γ (~32 kDa) in approximately stoichiometric amounts [2,3]. The α$_1$ subunit was found to bind the calcium channel blockers 1,4-dihydropyridines (DHPs), and was therefore identified to be the pore-forming subunit. The β and α$_2$δ subunits were then termed auxiliary or accessory subunits. Here I will focus on the role of the α$_2$δ subunits, updating and building on several previous reviews on the subject [4–10]. I will concentrate on their role in the calcium channel complex, although recent evidence suggests they may have additional roles in cell function [9,11,12].

2. α$_2$δ subunit genes

Four α$_2$δ subunit genes have been cloned. *CACNA2D1* encodes α$_2$δ-1, the α$_2$δ subunit, which was first identified in skeletal muscle. It has fairly ubiquitous distribution, being present in cardiac and smooth muscle as well as brain, while *CACNA2D2* and *CACNA2D3*, encoding α$_2$δ-2 and α$_2$δ-3, are differentially expressed in neurons and some other tissues [13,14] (Table 1). *CACNA2D4*, encoding α$_2$δ-4, shows expression which is mainly non-neuronal, although it is present in retinal neurons [15,16]. The gene structure is similar for all α$_2$δ subunit genes, for example *CACNA2D2* has 39 exons. Several other similar genes have been identified by bioinformatic means [17], but they have not been shown to function as calcium channel α$_2$δ subunits.

2.1. α$_2$δ subunit splice variants

The first evidence for the presence of splice variants in α$_2$δ subunits came from the fact that the cDNA sequence of the main α$_2$δ-1 subunit isoform expressed in rat brain showed regions of divergence compared to the skeletal muscle transcript [18]. Multiple sequence alignments allowed three regions, termed A, B and C, to be identified as resulting from alternative splicing. Five different transcripts were found in mouse brain, skeletal muscle, cardiac and smooth muscle [19]. Alternative splicing of the other α$_2$δ subunits has also been described [13,15,20]. In preliminary studies we have recently found changes in alternative splicing of α$_2$δ-1 in rat dorsal root ganglion

(DRG) neurons have been observed following spinal nerve ligation (SNL) [21].

3. Determination of α$_2$δ subunit topological features

The α$_2$δ proteins have complex topological features, which were initially difficult to unravel. Following disulfide bond reduction, the molecular weight of the skeletal muscle α$_2$ subunit was ~150 kDa, and the δ subunit was between 17 and 25 kDa [22]. In contrast, without reduction the α$_2$ and δ subunits behaved as a single protein of ~175 kDa. The interpretation of this result is that α$_2$ and δ are disulfide-bonded under native conditions (Fig. 1A, B). Following the partial sequencing of the α$_2$ and δ proteins, and the cloning of a single gene [23], it then became clear that α$_2$ and δ are encoded by the same gene, with δ making up the C terminal end of a pre-protein that is then subject to proteolytic cleavage, post-translationally. N terminal sequencing of the δ protein bands indicated they all had the same proteolytic cleavage site [22], and the different sizes of the δ peptides were found to represent several glycosylation states [22]. Both α$_2$ and δ are heavily glycosylated, as their molecular weights are reduced by glycosidase enzymes [22,24] (Fig. 1B).

There are also multiple cysteines in both α$_2$ and δ, making it likely there are both intra- and inter-subunit disulfide bonds. The process of disulfide-linking and proteolytic cleavage of α$_2$ and δ must occur during trafficking of the α$_2$δ protein. Disulfide bond formation normally occurs co-translationally in the lumen of the endoplasmic reticulum (ER), and most proteolytic processing occurs later in the maturation of proteins, particularly in the trans-Golgi network. Thus it is likely that a loop formed by disulfide bonding in α$_2$δ is then proteolytically cleaved by a so far unidentified protease, at an unknown subcellular site (probably post-ER), leaving the two subunits disulfide-bonded. The residues have recently been identified that are involved in disulfide bond formation between α$_2$ and δ in α$_2$δ-1 [25].

The α$_2$ subunit of α$_2$δ-1 has an N terminal signal motif, clearly showing that the N terminus is extracellular (Fig. 1A). It was initially suggested, based on hydrophobicity plots, that there were three hydrophobic and potential transmembrane regions [22,23], but this model located several of the predicted N-glycosylation motifs intracellularly, making it unlikely. It has also been shown that α$_2$ can be released from membranes by disulfide bond reduction, indicating that it is entirely extracellular [22]. In contrast, the δ protein is not released, showing it to be an integral membrane protein [22]. In agreement with these findings, site-directed topology mapping using anti-peptide antibodies [26] and studies using truncated α$_2$δ-1 constructs also showed that α$_2$ is extracellular [24,27,28].

Table 1
Summary of α$_2$δ subunits.

Gene name	Accession number[a] (human)	Protein name	Main tissue expression	Pathologies associated with gene disruption
CACNA2D1	NM_000722	α$_2$δ-1	Skeletal, cardiac and smooth muscles. CNS, PNS, endocrine tissues [22,55,117]	Cardiac dysfunction [104], disruption of neuropathic pain [105].
CACNA2D2	NM_001005505	α$_2$δ-2	CNS, especially cerebellum [14,20,55]	Epilepsy, cerebellar ataxia [14,112,118].
CACNA2D3	NM_018398	α$_2$δ-3	CNS, PNS [55]	Central processing of pain [115]
CACNA2D4	NM_172364	α$_2$δ-4	Retina, endocrine tissue [16].	Retinal dystrophy, night blindness [16,60]

CNS, central nervous system; PNS, peripheral nervous system.
[a] The accession number given is one of several available in Genbank, since sequences the various splice variants, and partial sequences are also available.
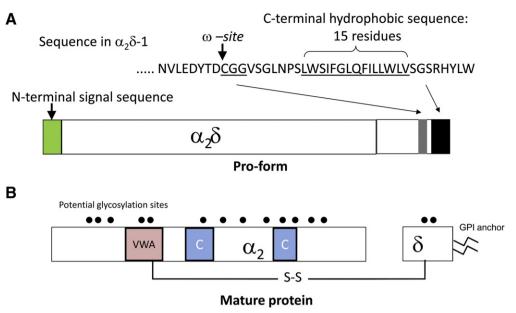
Fig. 1. Topological features of $\alpha_2\delta$ subunits, illustrated for $\alpha_2\delta$-1. A: $\alpha_2\delta$-1 pro-protein, with notable features. The sequence is taken from rat $\alpha_2\delta$-1 (NM_012919). B: mature $\alpha_2\delta$-1 protein, cleaved into α_2 and δ. The approximate positions of the VWA domain and the two bacterial chemosensory domains (C) are given. The mature form has multiple intrasubunit disulfide bonds, only the intersubunit disulfide bond between α_2 and δ is shown. It also has multiple identified glycosylation sites (●) in both α_2 and δ. About 35 kDa of carbohydrate residues can be removed with Endoglycosidase F [35], corresponding to utilization of between ~9 and ~12 glycosylation sites. There are 15 predicted N-glycosylation sites in $\alpha_2\delta$-1, two of which are in δ-1. $\alpha_2\delta$-1 is shown as a GPI-anchored protein; it is nevertheless possible that transmembrane forms exist.

The general topology of the $\alpha_2\delta$ protein is likely to be very similar for all $\alpha_2\delta$ subunits. They are all predicted to have N terminal signal sequences, although for $\alpha_2\delta$-2, the N terminal signal motif is longer than average for such sequences [29], but is nevertheless absent from the mature cell-surface-expressed protein [30]. When expressed, the signal sequence is co-translationally cleaved and the α_2 moiety is inserted in the lumen of the ER, and becomes completely extracellular [22,29]. After cleavage of the N-terminal signal sequence, there are 19 cysteines in the mature rat $\alpha_2\delta$-1, 19 in the mouse $\alpha_2\delta$-2 and 20 in rat $\alpha_2\delta$-3 sequences, of which 8 are in δ-1, 9 in δ-2 and 8 in δ-3. The $\alpha_2\delta$ subunits all show reduced molecular weights following by disulfide bond reduction [22,31,32], indicating that α_2 and δ are linked by one or more disulfide bonds. The pair of cysteines responsible for the disulfide bond between α_2 and δ-1 has been identified [25] (Fig. 1B).

4. Membrane anchoring of $\alpha_2\delta$ proteins

The $\alpha_2\delta$ subunits have only short predicted intracellular sequences distal to the C-terminal hydrophobic stretch of residues that forms a potential transmembrane domain [7]. The C-terminal sequence of rat $\alpha_2\delta$-1 is given in Fig. 1A. The C terminal sequence of rat $\alpha_2\delta$-3 is similar, being …..HPEENARE**CGGAS**SLQAQV<u>ALLLLPLVSSL</u>FSR. There are two hydrophilic residues at the extreme C-terminus, preceded by 13 hydrophobic residues (underlined), which is likely to be too short for a plasma membrane-spanning α-helix. This sequence is also split by a helix breaking proline. The predicted GPI-anchoring motif is CGG or GAS (bold). Similarly, the C-terminal sequence in mouse $\alpha_2\delta$-4 is … HPEENAQD**CGGAS**DTLPSS<u>PLLLLSLGAWLLPP</u>QLLW, with only one aromatic residue after the 17 residue C-terminal hydrophobic sequence, which contains two prolines. For these and other reasons, in various proteomic prediction programs some of these $\alpha_2\delta$ proteins are predicted to be glycosyl-phosphatidylinositol (GPI)-anchored [33,34], particularly $\alpha_2\delta$-3 and $\alpha_2\delta$-4. We have now obtained a large amount of biochemical, imaging and electrophysiological evidence in agreement with the hypothesis that both heterologously expressed and endogenous $\alpha_2\delta$ proteins can form GPI-anchored proteins [31] (Fig. 1A, B).

We have recently examined the behavior of an $\alpha_2\delta$-1 construct truncated at the predicted GPI-anchor site [35]. Similar to a previous study using a truncated Prion protein construct [36], the majority of C-terminally truncated $\alpha_2\delta$-1 is soluble and secreted into the medium. Unexpectedly, some of the truncated $\alpha_2\delta$-1 protein remains associated with detergent-resistant membranes (DRMs), also termed lipid rafts, and is extrinsically bound to the plasma membrane, by an as yet unknown mechanism [35]. Identification of the binding partners of $\alpha_2\delta$-1 responsible for this membrane tethering will provide an important insight into its function.

5. Structural and biochemical studies on $\alpha_2\delta$ subunits

5.1. Interaction of $\alpha_2\delta$ with $\alpha1$ subunits

After $\alpha_2\delta$ subunits were identified as components of the calcium channels in skeletal muscle, $\alpha_2\delta$ proteins were also found to be present in native cardiac (L type) [37] and brain N-type and P/Q-type channels [38,39]. It is likely that all native Ca_V1 and Ca_V2 calcium channels can associate with $\alpha_2\delta$ subunits, but it is still unclear whether these α_1 subunits might membrane $\alpha_2\delta$ subunits. Within different tissues and brain regions the calcium channel complexes formed are likely to depend on cellular expression [40].

Purification experiments using native T-type calcium channels are hampered by the lack of suitable selective drugs and antibodies to aid purification of the channels. Since the expression of cloned channels is substantial in the absence of $\alpha_2\delta$ subunits, it is assumed that native T-type channels normally exist without associated $\alpha_2\delta$ proteins in the plasma membrane [41,42].

5.2. Structural studies of calcium channel complexes using electron microscopy

Low resolution structures of calcium channel complexes purified from skeletal and cardiac muscle have been obtained using single particle averaging of electron microscopic (EM) images [43–45]. Tentative identification of the $\alpha_2\delta$ and β subunits within the particles has been possible using antibody labeling. Lectin labeling also identifies the position of $\alpha_2\delta$ which is the only glycosylated species in the cardiac calcium channel complex, since it lacks γ subunits [45]. The

density associated with $\alpha_2\delta$-1 subunit within the cardiac calcium channel complex was also identified by subtraction of the $Ca_V3.2$ single particle EM density, as this represented an α_1 subunit alone [45]. The density assigned to $\alpha_2\delta$ surrounded, but did not cover, the presumed pore region of the α_1 subunit [45]. EM studies in skeletal muscle have identified particles thought to correspond to DHPR complexes [46]. However, following extensive knock-down of $\alpha_2\delta$-1, by viral infection of short interfering RNA into myotubes, the size and pattern of the tetradic calcium channel particles was unaltered, indicating that they probably represent the α_1S ($Ca_V1.1$) subunit [47].

5.3. The domain structure of $\alpha_2\delta$ proteins

Bioinformatic analysis of $\alpha_2\delta$ sequences shows that all $\alpha_2\delta$ subunits contain certain domains, including a Von Willebrand Factor A (VWF-A or VWA) domain [17]. This domain was defined in von Willebrand Factor, where it is involved in binding to a number of cell adhesion and extracellular matrix proteins. VWA-like domains are usually about 200 residues long, and also found in some integrin subunits, collagens and laminin [48]. The VWA domain represents a dinucleotide binding fold with a metal ion adhesion (MIDAS) motif, which participates in divalent cation-dependent interactions. In general, VWA domains are involved in protein–protein interactions, via their MIDAS motif [17], which co-ordinates a divalent cation, most often Ca^{2+} or Mg^{2+} [17]. The $\alpha_2\delta$-1 and $\alpha_2\delta$-2 proteins contain a "perfect" MIDAS motif, in which all 5 co-coordinating amino acids are present. This has been predicted to indicate that a structural alteration of the protein complex will occur following divalent cation-binding and subsequent complex formation with another protein ligand [17]. The structure of $\alpha_2\delta$ VWA domains has been modeled by homology with other VWA domains present in the structure database [9,49]. A key component of the MIDAS motif is a 5 residue motif containing three of the co-ordinating residues ($D \times S \times S$), near the N-terminal end of the VWA domain, which is also present in $\alpha_2\delta$-3 and $\alpha_2\delta$-4. The functional relevance of the VWA domain is discussed below in Section 8.1.

There are also two bacterial chemosensory-like or Cache domains in $\alpha_2\delta$ subunits, situated downstream of the VWA domain [50]. These were identified by homology with an extracellular domain found in many bacterial chemotaxis receptors. In bacteria, these proteins are involved in sensing both potential sources of nutrients or molecules to avoid. Several such domains have been crystallized in the presence of bound heme which is a redox sensor [51]. Other bacterial chemosensors are involved in chemotaxis to dipeptides, ribose and galactose, and aspartate and repellants [52]. In plants the ethylene receptor has a similar domain [53]. The potential function of these domains in $\alpha_2\delta$ subunits is discussed in Section 8.1.

6. Cellular localization of the $\alpha_2\delta$ subunits

6.1. $\alpha_2\delta$-1

A number of studies have examined the cellular and subcellular localization of the different $\alpha_2\delta$ subunits at the level of both transcript and protein. The $\alpha_2\delta$-1 subunit is strongly expressed in skeletal muscle but is also fairly ubiquitously present in other tissues, mainly in excitable cells [23,32]. The $\alpha_2\delta$-1 isoform is also present in cardiac and smooth muscle. In these tissues it is the principal $\alpha_2\delta$ subunit associated with $Ca_V1.2$ [37,54]. Within the mouse brain, message for $\alpha_2\delta$-1 is present throughout the brain, particularly in cerebral cortex, hippocampus and cerebellum [13]. The $\alpha_2\delta$-1 mRNA is found in many neuronal cell types [55], and is also present in neurons of the peripheral nervous system, including DRG neurons [56,57]. Interestingly, in neurons the presence of $\alpha_2\delta$-1 transcript was partially correlated with excitatory neurons rather than inhibitory interneurons [55].

6.2. $\alpha_2\delta$-2

The $\alpha_2\delta$-2 protein is expressed in fewer tissues than is $\alpha_2\delta$-1. In both human and mouse tissues, $\alpha_2\delta$-2 is found in brain, using in situ hybridization, Northern blots and PCR based localization [14,58,59]. In the brain, $\alpha_2\delta$-2 is concentrated in cerebellum, where it is highly expressed in Purkinje cells [14,29,32]. $\alpha_2\delta$-2 is also found in other brain regions including striatum and hippocampus [14]. Within the central nervous system, the cellular distribution of $\alpha_2\delta$-2 mRNA was found to correlate partially with GABAergic neurons [14,55]. Message for $\alpha_2\delta$-2 was also found in human lung tissue, in two reports [32,59], although not in an earlier study [13]. However, the $\alpha_2\delta$-2 protein was hardly detectable [32]. The reason for the discrepancy between low protein expression, despite high transcript levels, is unclear. In contrast, $\alpha_2\delta$-2 mRNA was not found in mouse lung tissue [14].

6.3. $\alpha_2\delta$-3

In the mouse, $\alpha_2\delta$-3 mRNA and protein are solely expressed in brain, whereas in humans, the transcript is also found in skeletal muscle and heart [32]. Within mouse brain $\alpha_2\delta$-3 mRNA is found in many brain regions, including caudate-putamen, cerebral cortex and hippocampus [55].

6.4. $\alpha_2\delta$-4

The $\alpha_2\delta$-4 subunit was initially reported to show very restricted distribution of transcription in some endocrine tissues, and not present in brain [15]. However, another study has shown a more ubiquitous distribution of $\alpha_2\delta$-4 mRNA, albeit with low expression in brain and muscle [60]. It is also present in the retina [60], where genetic mutations result in a form of night blindness [16,60].

7. Subcellular localization of $\alpha_2\delta$ subunits

7.1. Skeletal muscle

Within skeletal muscle, $\alpha_2\delta$-1 is strongly concentrated in the skeletal muscle transverse (T) tubules in association with the DHPR complex. In the T-tubule-sarcoplasmic reticulum junction, the DHPRs are present in a tetrad structure, juxtaposed with the ryanodine receptors which are on the sarcoplasmic reticulum [61,62].

7.2. Presynaptic terminals

In brain and spinal cord, the $\alpha_2\delta$-1 protein is mainly found in the neuropil, and at a much lower level in somata [63]. In hippocampal cultures it co-localizes with presynaptic boutons [64]. It is also highly concentrated in synaptosomes together with the Ca_V2 calcium channels involved in transmitter release.

In primary afferent DRG neurons, its presence in their central terminals in the spinal cord has been confirmed electron microscopically [57]. Nevertheless it is not exclusively presynaptic as it was also present in dendritic structures [57]. We have also found that $\alpha_2\delta$-1 subunits are transported from the site of synthesis in the DRG cell bodies, down both their central and peripheral axons, and are found within trafficking vesicles [57]. The finding that $\alpha_2\delta$-1 is transported in peripheral axons opens the possibility that it might affect other processes, such as regeneration following nerve damage, as well as calcium channel trafficking.

There is little information on the subcellular distribution of mammalian $\alpha_2\delta$-2 or $\alpha_2\delta$-3 proteins, mainly because of the lack of appropriate antibodies. Evidence from *Drosophila* suggests that the $\alpha_2\delta$-3 homolog (*straitjacket*) interacts with *cacophony*, a calcium channel that is localized to active zones and involved in presynaptic release [65]. Furthermore, the *C. elegans* $\alpha_2\delta$ subunit (UNC36) is required for the presynaptic

localization of UNC2, which is a Ca$_V$2 calcium channel homolog [66]. These results all point to the likelihood that α$_2$δ subunits play a role in targeting calcium channels to specific presynaptic locations associated with active zones. In retinal rods and cones, α$_2$δ-4 has also been found to be presynaptically localized to the ribbon synapses of the salamander retina [67].

7.3. Membrane microdomain localization of α$_2$δ subunits

All the α$_2$δ proteins are strongly localized in detergent-resistant membrane (DRM) fractions, which are cholesterol-rich, and also called "lipid rafts". This localization is seen both following heterologous expression of α$_2$δ proteins, and when examining native α$_2$δ proteins in neurons [31,68]. This suggests they may be localized in specific microdomains in neuronal membranes. In agreement with this, experiments in retina have shown the mobility of α$_2$δ-4, and by assumption, also the L-type channels involved in transmitter release in rods and cones, is highly confined to synaptic regions, but increases transiently on transmitter release, and shows less restricted movement following lipid raft disruption [67].

8. Effects of α$_2$δ subunits on the pharmacological and biophysical properties associated with specific calcium channels

The α$_2$δ subunits affect the electrophysiological properties of Ca$_V$1 and Ca$_V$2 calcium channels, and some of these effects are dependent on the expression of a β subunit [69]. It is likely that β subunits enhance the movement of calcium channels out of the ER, by promoting correct folding and protecting the Ca$_V$1 and Ca$_V$2 channels from proteasomal degradation [70,71].

8.1. Effect of α$_2$δ on plasma membrane expression of calcium channels

For Ca$_V$1 and Ca$_V$2 calcium channels, β subunits are a key subunit allowing trafficking to the plasma membrane [72]. The specific effect of the α$_2$δ subunits alone is difficult to determine as several heterologous expression systems, including *Xenopus* oocytes, and HEK-293 cells contain endogenous β subunits [73,74]. When endogenous oocyte β3 is knocked down we observed a large reduction in functional expression of Ca$_V$2.2 [75]. Furthermore, expression systems may also contain a low level of endogenous α$_2$δ [76,77].

Several studies have found that α$_2$δ subunits increase the expression of various Ca$_V$ α$_1$ subunit/β subunit combinations, all α$_2$δ subunits having comparable effects, where comparisons have been made. For Ca$_V$1.2, α$_2$δ-1 co-expression in oocytes increased the amount of α$_1$ subunit protein associated with the plasma membrane [78]. The peak Ca$_V$1.2 current amplitude was also increased 3-fold by co-expression of α$_2$δ-1 [79]. In another study the α$_2$δ-2 subunit increased Ca$_V$1.2 currents 2-fold [80].

For Ca$_V$2.1, the α1/β4 current was increased 2–7-fold by co-expression of α$_2$δ-2 in different expression systems [14,29,49,68]. The Ca$_V$2.1/β4/α$_2$δ-2 calcium channel combination is likely to exist in cerebellar Purkinje cells, in which these subunits are highly expressed [14,29]. However, α$_2$δ-2 had no effect on the single channel conductance of Ca$_V$2.1/β4, or any of the other single channel properties measured, indicating that the increase in whole cell current is a result of an increased number of functional channels inserted in the plasma membrane [14,29].

For Ca$_V$2.2 one study found the α$_2$δ-2 subunit to increase Ca$_V$2.2 currents, by 9-fold [80]. In our experiments, we found the α$_2$δ-1, α$_2$δ-2 and α$_2$δ-3 subunits to all increase peak Ca$_V$2.2/β1b currents to a similar extent, by about 5-fold [31,49,64,81]. Furthermore for single channel currents, α$_2$δ-1 subunits were also found to reduce the number of null traces recorded, indicating that the Ca$_V$2.2 channels in the plasma membrane are more likely to be in an activatable state [82].

For Ca$_V$2.3 channels, it was reported that α$_2$δ-1 subunits do not increase the current amplitude when co-expressed in *Xenopus* oocytes [83]. A different result was obtained in HEK-293 cells, where the maximum conductance for Ca$_V$2.3 was increased 2-fold by α$_2$δ-1 alone, although the α$_2$δ subunit gave no additional increase over that produced by β subunits [84]. It is therefore possible that Ca$_V$2.3 may be less influenced by α$_2$δ subunits.

T-type calcium channels do not require accessory β or α$_2$δ subunits for expression. Nevertheless, both α$_2$δ-1 and α$_2$δ-2 increased Ca$_V$3.1 currents almost 2-fold [77,80], and thus it is possible that T type channels might associate with α$_2$δ subunits. However, in other studies α$_2$δ-1 and α$_2$δ-3 produced minor effects on Ca$_V$3.1 current, whereas α$_2$δ-2 increased Ca$_V$3.1 current density [13,58,85].

One of the main mechanisms for the effect of α$_2$δ subunits on HVA channel current density is likely to be an increase in the plasma membrane expression of the Ca$_V$1 and Ca$_V$2 α$_1$ subunits and decrease of their turnover [49,86], although how this occurs is still unclear. As a step in understanding this process, we have found the MIDAS motif in the VWA domain of α$_2$δ-1 and α$_2$δ-2 subunits is essential for this process [49,64]. Mutation of this motif virtually abolished the ability of α$_2$δ-1 [64] and α$_2$δ-2 [49] subunits to increase calcium currents in expression systems. We also found that the MIDAS mutant of α$_2$δ-2 caused α$_1$ subunits to be retained in intracellular compartments [49]. Since VWA domains are involved in protein–protein interactions, via their MIDAS motif [17], it is possible that the α$_2$δ VWA domains are interacting either with a trafficking protein, involved in trafficking either of α$_2$δ alone, or of the entire calcium channel complex to the plasma membrane. Alternatively, or in addition, the VWA domains may be involved in the interaction with the calcium channel α$_1$ subunit, leading indirectly to increased trafficking of the complex.

It is possible that in α$_2$δ subunits the chemosensory-like domains are also involved in the trafficking function of α$_2$δ subunits. One might speculate that they are implicated in binding to gabapentinoid drugs (See Section 11.1), and in binding to the putative endogenous ligand(s) that have been found to compete for gabapentin binding, whose identity is unknown [87,88]. The role of the endogenous ligand(s) in α$_2$δ function is unclear but they might be important for full function. One piece of evidence that supports this hypothesis is that mutation of the RRR motifs involved in gabapentin binding within α$_2$δ-1 and α$_2$δ-2, to RRA which markedly reduces the gabapentin binding affinity, also significantly impedes the ability of α$_2$δ-1 and α$_2$δ-2 to enhance calcium currents [81,89]. Presumably these mutations would also inhibit the binding of the endogenous ligand(s).

Although the exact site at which the α$_2$δ subunits intervene in the calcium channel trafficking process remains to be established, it is assumed that they interact with one or more exofacial domains of the α$_1$ subunit. For example, it has been described that the α$_2$ subunit of α$_2$δ-1 binds to domain III of Ca$_V$1.1 [28]. We have also studied the trafficking of α$_2$δ subunits [31,49,68,81,90], and our evidence indicates that they must interact with intracellular trafficking proteins. Our finding that a proportion of the α$_2$δ-1 truncated at the predicted C-terminal GPI-anchor site is still in part extrinsically-associated with the plasma membrane provides support for this proposition [35]. Furthermore, this truncated α$_2$δ-1 construct is still able to enhance calcium channel currents, although to a smaller extent than full-length α$_2$δ-1, indicating that intrinsic membrane-anchoring is not essential for this process [35].

8.2. Effects of α$_2$δ subunits on single channel properties

There is very little information concerning the effect of α$_2$δ subunits on single channel properties of calcium channels. The α$_2$δ subunits have not been found to affect the single channel conductance of native [14] or expressed Ca$_V$2.1 [29] or Ca$_V$2.2 [82] calcium channels. However, surprisingly, the co-expression of α$_2$δ-1 was found

to reduce single channel open times measured for the Ca$_V$2.2/β1b combination [82].

8.3. Effects of α$_2$δ subunits on voltage-dependence of activation and inactivation of calcium channel currents

The presence of low concentrations of endogenous β [74,75], and indeed α$_2$δ subunits [35], in various different expression systems make it difficult to accurately assess the effects of the individual subunits separately. Since β subunits also influence inactivation, the effects of α$_2$δ subunits on the voltage-dependent and kinetic properties of calcium channels may also depend on which β subunit is expressed.

The α$_2$δ-1 subunit was found to have little effect on the voltage-dependence of activation of Ca$_V$1.2 [78,91–93], or on the voltage-dependence of charge movement [94]. In contrast, another study found that α$_2$δ-1 hyperpolarized the voltage-dependence of activation of Ca$_V$1.2/β4 by about 10 mV [79]. This effect was also found with a "δ" construct, although δ did not enhance the current amplitude. A further study found that the activation of the Ca$_V$1.2/β3 combination was shifted to more negative potentials by α$_2$δ-1, using the cut-open oocyte technique [95]. They also found that α$_2$δ-1 did not affect the voltage-dependence of charge movement. These results point towards α$_2$δ subunits increasing the coupling efficiency between voltage sensor movement and channel opening, and also suggest there are more channels in the membrane [95]. The α$_2$δ-2 subunit also had little effect on the activation voltage-dependence, when co-expressed with Ca$_V$2.1 and β4 in Cos-7 cells [29]. However, α$_2$δ-1 was found to shift the activation of Ca$_V$2.3 to more depolarized potentials, both in the absence of β subunits and in the presence of either β1b or β2a [83]. In contrast in HEK-293 cells α$_2$δ-1 had no effect on the voltage-dependence of activation gating for Ca$_V$2.3 [84]. Thus some effects of α$_2$δ subunits on activation appear to depend on the particular α1 subunit with which it is expressed.

In most cases α$_2$δ-1 and α$_2$δ-2 hyperpolarized the steady-state inactivation of Ca$_V$1.2, Ca$_V$2.2 and Ca$_V$2.3 currents by a similar amount [58,79]. However α$_2$δ-1 was not found to affect the steady-state inactivation of Ca$_V$2.3, either in the absence or presence of β subunits [83].

From these results it appears that there are no absolute rules concerning the effects of α$_2$δ subunits on calcium channel voltage-dependent properties. In part, this may be because it is difficult to dissect out these biophysical effects from the fact that there are also more channels in the plasma membrane, as well as the fact that expression systems may have endogenous α$_2$δ subunits.

8.4. Effect of α$_2$δ subunits on calcium current kinetics

In several studies, the α$_2$δ-1 subunit increased the inactivation rate for both the Ca$_V$1.2 [79,96] and Ca$_V$2.1 [79]. In another study, both α$_2$δ-1 and α$_2$δ-2 increased inactivation of Ca$_V$1.2, and Ca$_V$2.3 (and surprisingly also Ca$_V$3.1) currents [58]. The α$_2$δ-1 subunit was also found increase the inactivation of Ca$_V$1.2 gating currents [94]. The increased inactivation may explain the reported ability of α$_2$δ-1 to increase the affinity for DHP antagonists, since these drugs show increased binding to inactivated channels [97]. It has also found α$_2$δ-1, α$_2$δ-2 and α$_2$δ-3 all increased inactivation for several different HVA channels [6,31,49]. In skeletal muscle the Ca$_V$1.1 currents are very slowly activating, and paradoxically, the activation rate is increased following knockdown of α$_2$δ-1 [47,98].

8.5. Effect of α$_2$δ subunits on pharmacological properties of calcium channels

Several indirect effects of α$_2$δ subunits have been observed on calcium channel pharmacology. It has been found that expression of α$_2$δ-1 subunits reduced the on-rate and affinity of block of N-type calcium channels by several ω-conotoxins, including ω-conotoxin-GVIA and ω-conotoxin-MVIIA [99]. This may reflect masking of the binding site near the channel pore by the α$_2$δ protein.

In other studies, both α$_2$δ-1 and the other auxiliary subunits have been found to contribute to increasing the affinity and B$_{max}$ of L-type channels (both Ca$_V$1.1 and Ca$_V$1.2) for DHP antagonists [79,97,100]. This result suggests that the α$_2$δ subunits may alter the channel conformation. As described in Section 8.4, the increased affinity for DHP antagonists may result from the increased inactivation seen with α$_2$δ-1, since DHP antagonists favor the inactivated channel state [79].

9. Differential association between specific α$_2$δ and α$_1$ subunits

Following the cloning of four different α$_2$δ subunit genes, it is of interest to ask whether there is any differential association between particular α$_1$ and α$_2$δ subunits. In heterologous expression systems no specificity has been reported, but in vivo there may be greater selectivity. For example in Purkinje cells α$_2$δ-2 is the main, if not the only α$_2$δ expressed, at least in mice [14], and it is likely to associate with Ca$_V$2.1, the main α$_1$ subunit in these cells. Expression profiling showed that Ca$_V$2.1, β4 and α$_2$δ-2 were the most abundant transcripts in cerebellum, whereas Ca$_V$2.3, β2, and α$_2$δ-1 were the most prevalent transcripts in hippocampus [40].

10. Effect of α$_2$δ subunits on transmitter release

The concentration of α$_2$δ-1 in presynaptic terminals [57], and their presence and function in calcium channel complexes (Ca$_V$2.1 and Ca$_V$2.2) that are key to transmitter release, indicates that these α$_2$δ subunits are likely to affect presynaptic function. It has recently been found that transient over-expression of α$_2$δ subunits in cultured hippocampal neurons leads to an enhancement of the presynaptic concentration both of the α$_2$δ protein and of endogenous Ca$_V$2.1 channels [64]. Whether the channels are on the cell surface in the presynaptic boutons was not tested in this study, and paradoxically the α$_2$δ subunits decreased the presynaptic Ca^{2+} elevation resulting from a single action potential. However, all the α$_2$δ subunits examined (α$_2$δ-1, α$_2$δ-2 and α$_2$δ-3) caused an increase in vesicular release in response to an action potential, which depended on an intact MIDAS motif [64].

11. Mutations and epigenetic regulation of *CACNA2D* genes indicate the involvement of α$_2$δ subunits in multiple pathologies

11.1. α$_2$δ-1

It is well-established that α$_2$δ-1 plays a role in the development of chronic pain associated with nerve injury (neuropathic pain) and its therapy (see [9] for recent review). Some of this work has come from the use of mouse mutants [89,101]. Experimental peripheral nerve injury results in an elevation in the level of α$_2$δ-1 mRNA in the damaged sensory neurons (trigeminal neurons and DRGs), as evidenced from in situ hybridization [56], microarray data [102] and quantitative PCR [57]. There is a corresponding augmentation of α$_2$δ-1 protein in DRGs and spinal cord, as shown by Western blotting [103] and immunohistochemistry [57]. In contrast, Ca$_V$2.2 mRNA and protein is not generally found to be up-regulated following sensory nerve damage [101,102]. This suggests that up-regulated α$_2$δ-1 enhances Ca$_V$2.2 trafficking and presynaptic function.

The development of hypersensitivity resulting from peripheral nerve injury has been examined in a number of transgenic mouse lines. It is of great interest that α$_2$δ-1 over-expressing mice show a neuropathic phenotype of hyperalgesia and tactile allodynia compared to wild-type mice, under control conditions [101], indicating that α$_2$δ-1 is required to increase the excitability of DRG neurons. In contrast, α$_2$δ-1 knockout mice have a cardiac phenotype, but surprisingly no gross defects in skeletal muscle function [104]. We have

recently shown that they also have deficits in sensory perception and in the development of neuropathic hypersensitivity [105].

Gabapentin and pregabalin are anti-epileptic drugs that are also of therapeutic use in neuropathic pain (for review see [106]). They were identified from purification and ligand binding studies to bind to $\alpha_2\delta$-1 [107] and $\alpha_2\delta$-2 [32], which were found to represent their main binding proteins. A number of amino acids in $\alpha_2\delta$-1 were shown to be involved in the binding of gabapentinoid drugs, in particular, the third arginine (R) in an RRR motif, located N-terminal to the VWA domain [68,89]. This motif is also present in $\alpha_2\delta$-2 [68]. In a knockin mutant mouse bearing a mutation in this motif in $\alpha_2\delta$-1 (RRR mutated to RRA), these gabapentinoid or $\alpha_2\delta$ ligand drugs were no longer effective in the alleviation of chronic pain resulting from nerve injury [89]. This result therefore identified $\alpha_2\delta$-1 as the protein responsible for the therapeutic effects of these drugs in neuropathic pain. Although human mutations in *CACNA2D1* have been identified to be associated with several forms of cardiac dysfunction, including Brugada [108] and short QT [109] syndromes, as yet no human mutations or single nucleotide polymorphisms in this or other $\alpha_2\delta$ genes have been found to be associated with epilepsies [110].

11.2. $\alpha_2\delta$-2

The mouse strain Ducky (*du*), which is a spontaneously arising mutant, exhibits spike-wave epilepsy and cerebellar ataxia [14]. The mutation is in the $\alpha_2\delta$-2 subunit gene, *Cacna2d2*, which, as described above, is robustly expressed in cerebellar Purkinje cells. The mutation results in a loss of expression of full length $\alpha_2\delta$-2, and another allele (*du²J*) produces a similar phenotype [29]. The calcium currents in Purkinje cells of *du/du* mice are reduced at 6–8 days old [14], possibly due to the absence of $\alpha_2\delta$-2. Furthermore, there is a reduction of the Purkinje cell dendritic tree and markedly reduced spontaneous activity in Purkinje cells [111]. A third allelic mutant mouse, *Entla*, was identified which has generalized seizures, as do mice with a targeted deletion of *Cacna2d2* [112,113]. Despite these findings in mice, to date no human mutations in $\alpha_2\delta$ subunits have yet been reported to be associated with epileptic phenotypes. It is worth noting that all the mouse mutations are recessive, meaning the mice only have a significant phenotype as homozygotes, and the presence of one wild-type copy of the $\alpha_2\delta$-2 transcript is sufficient for normal function. It is likely that the same would be true in humans. Thus, if such recessive mutations are quite rare, they will only be observed as homozygotes in isolated populations, as a result of consanguineous marriage, like other rare recessive mutations, such as specific voltage-gated Na^+ channels [114].

11.3. $\alpha_2\delta$-3

A *Drosophila melanogaster* screen recently identified *straitjacket*, the Drosophila homolog of *CACNA2D3*, as a 'pain gene' [115]. This study showed that in both Drosophila and mice mutants lacking this gene there is an impairment in the avoidance of noxious heat. The authors found that this was a result of altered central processing. Furthermore, two intronic SNPs in *CACNA2D3* were associated with altered pain perception in humans, although the mechanism for this difference is unknown. In another study, a splice site mutation in *CACNA2D3* was also found to be one of a number of 'Likely Gene-Disrupting Mutations' in autism spectrum disorder [116].

11.4. $\alpha_2\delta$-4

Mutations in *CACNA2D4* (encoding $\alpha_2\delta$-4 subunits) have been shown to lead to dysfunction of photoreceptors, resulting in a recessive form of night blindness, and slowly progressing cone dystrophy. A spontaneous mouse mutation in this gene has also been identified, showing a similar phenotype of autosomal recessive cone dystrophy [16,60]. Both

mutations are truncating, and would lead to non-functional or very poorly functional $\alpha_2\delta$-4 proteins. Since $\alpha_2\delta$-4 is the main $\alpha_2\delta$ subunit in these cells, the loss of functional $\alpha_2\delta$-4 protein will be highly likely to result in reduced $Ca_V1.4$ calcium channel trafficking and function, culminating in less neurotransmitter release from the photoreceptor terminals. The cone dystrophy might be a direct result of reduced photoreceptor function, or the loss of full-length $\alpha_2\delta$-4 on the cell surface destabilizing synaptic structures, or an indirect effect of the translation of a non-functional truncated $\alpha_2\delta$-4 protein.

12. Conclusion

The $\alpha_2\delta$ subunits have marked effects on the properties of calcium channels, and also very clear links to disease processes. Whether they are present in all calcium channel complexes remains an open question, as does their mechanism of action. There is also evidence that $\alpha_2\delta$ subunits may have additional functions as well as being calcium channel subunits, which is covered more fully elsewhere [9].

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