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Calcium Channel $\alpha_2\delta$ Subunits: Differential Expression, Function, and Drug Binding

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Voltage-activated calcium channels are transmembrane proteins that act as transducers of electrical signals into numerous intracellular activities. On the basis of their electrophysiological properties they are classified as high- and low-voltage-activated calcium channels. High-voltage-activated calcium channels are heterooligomeric proteins consisting of a pore-forming α_1 subunit and auxiliary $\alpha_2\delta$, β, and—in some tissues—γ subunits. Auxiliary subunits support the membrane trafficking of the α_1 subunit and modulate the kinetic properties of the channel. In particular, the $\alpha_2\delta$ subunit has been shown to modify the biophysical and pharmacological properties of the α_1 subunit. The $\alpha_2\delta$ subunit is posttranslationally cleaved to form disulfide-linked α_2 and, δ proteins, both of which are heavily glycosylated. Recently it was shown that at least four genes encode for $\alpha_2\delta$ subunits which are expressed in a tissue-specific manner. Their biophysical properties were characterized in coexpression studies with high- and low-voltage-activated calcium channels. Mutations in the gene encoding $\alpha_2\delta$-2 have been found to underlie the ducky phenotype. This mouse mutant is a model for absence epilepsy and is characterized by spike wave seizures and cerebellar ataxia. $\alpha_2\delta$ subunits can also support pharmacological interactions with drugs that are used for the treatment of epilepsy and neuropathic pain.

KEY WORDS: Voltage-gated calcium channel; auxiliary subunit; Cacna2d; $\alpha_2\delta$; gabapentin; antiepileptics; ducky mouse.

INTRODUCTION

Calcium ions play a crucial role in the regulation of many biological processes. Variations in the intracellular Ca^{2+} concentration regulate muscle contraction, neurotransmitter and hormone release, electrical spiking behavior, cell differentiation, and the expression of genes. Intracellular Ca^{2+} levels can either be increased by release from the endoplasmic reticulum or by influx from the extracellular space. A primary route of Ca^{2+} influx occurs through voltage-activated calcium channels. These channels consist of the pore-forming α_1 and auxiliary β, $\alpha_2\delta$ and—in some tissues—γ subunits (for review, see Hofmann *et al.*, 1999). The α_1 subunit accounts not only for the ion channel pore but also contains the voltage sensor, selectivity filter, and the determinants for binding of drugs and toxins. The current through the α_1 subunit is modulated by interactions with different auxiliary subunits.

In the last years the number of genes encoding calcium channel subunits rapidly increased becuse of the availability of sequence databases. Today, 10 genes are known to encode for α_1, 4 genes for β, 4 genes for $\alpha_2\delta$, and up to 8 genes for γ subunits (for the recent nomenclature, see Ertel *et al.*, 2000). The diversity of calcium channels is further increased by alternative splicing and by the combination of an α_1 with a restricted number of auxiliary subunits. The coexpression of β and $\alpha_2\delta$ subunits enhances the level of expression and also confers to the gating properties of the channel. In particular, the $\alpha_2\delta$ subunit has been shown to modify not only the biophysical properties of the calcium channel but it has also been demonstrated that it is the target of antiepileptic drugs.

The major aspects of the structure, diversity, and function of calcium channel subunits are summarized in several excellent reviews (Catterall, 2000; Felix, 1999;

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Hofmann *et al.*, 1999). This paper will mainly focus on the recently extended family of $\alpha_2\delta$ proteins with a short summary on voltage-activated calcium channels.

GENERAL PROPERTIES OF VOLTAGE-ACTIVATED CALCIUM CHANNELS

Voltage-activated calcium channels have been initially characterized in terms of their electrophysiological and pharmacological properties. They have been denoted as L, N, P/Q, R, and T type. However, since the molecular cloning of these proteins, the diversity among calcium channel subunits increased dramatically. Today, calcium channels are classified in three Ca_v families (Ertel *et al.*, 2000): The four members of the Ca_v1 family ($Ca_v1.1$–1.4) are L-type calcium channels that are blocked by dihydropyridines (DHP). These channels present a wide distribution in heart, smooth muscle, brain, skeletal muscle, and some endocrine tissues. The calcium channels of the Ca_v1 family are regulated primarily by voltage and modulated by protein phosphorylation through second-messenger-driven kinase pathways. The calcium channels of the Ca_v2 ($Ca_v2.1$–2.3) family are DHP insensitive (non-L-type) and are predominantely expressed in the nervous system. They include the N, P/Q, and R-type channels. The three members of this family are regulated by G proteins and direct binding of SNARE proteins. The Ca_v1 and Ca_v2 families can be combined as high-voltage-activated (HVA) channels. HVA channels are activated at relatively depolarized membrane potentials, inactivate slowly during depolarization, and have large conductances.

The Ca_v3 calcium channels ($Ca_v3.1$–3.3) have been named as T-type or low-voltage-activated (LVA) channels. These channels are activated at more negative potentials and inactivate rapidly. They exhibit small conductances and show slow deactivation rates. The regulation of this family is not well studied, but there are reports that show effects of protein phosphorylation and G protein regulation.

HVA calcium channels are heterooligomeric proteins consisting of up to five subunits. The putative structure of the pore-forming α_1 subunit together with auxiliary $\alpha_2\delta$, β, and γ subunits is shown in Fig. 1. The main α_1 subunit is organized in four repeated domains, each of which contains six transmembrane segments (S1 through S6) and a membrane-associated loop between the fifth and sixth transmembrane segment. The fourth segment of each domain serves as the voltage sensor for activation, moving outward and rotating under the influence of depolarization and initiating a conformational change that opens the pore. The region including S5–S6 is of particular interest

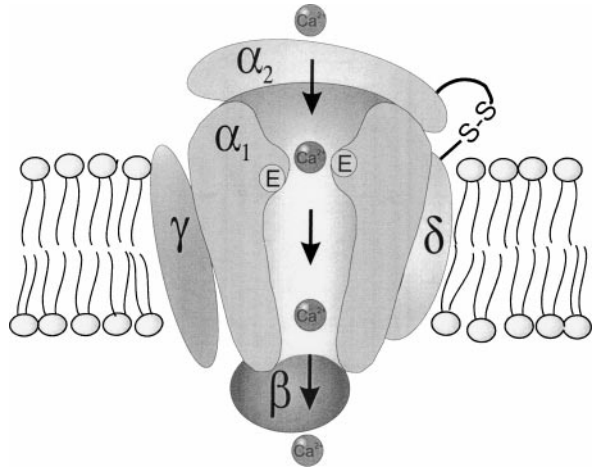

Fig. 1. Putative subunit arrangement of a high-voltage-activated calcium channel complex. The pore-forming α_1 subunit contains the selectivity filter, the voltage sensor, and the binding sites of the calcium channel blockers. Calcium ions (red circles) within the pore are positioned by four glutamic acid residues (E) which are arranged in an asymmetric configuration. Two of these residues are shown (yellow circles). The other proteins are auxiliary subunits which modulate the biophysical properties of the α_1 subunit. The $\alpha_2\delta$ subunit is encoded by a single gene and is formed by posttranslational processing. The α_2 protein is located outside of the cell and is linked via disulfide bridges to the δ protein. The β subunit has an intracellular location. The skeletal muscle calcium channel copurifies with a γ subunit. Recently identified similar proteins (γ2-8) could be part of some neuronal calcium channels or could be involved in trafficking of AMPA receptors to postsynaptic membranes.

since this structure forms the ion-conducting pore of the channel. In each domain the connecting loop between S5 and S6 contains a glutamic acid residue that is required for Ca^{2+} selectivity. The α_1 subunit also contains the interaction sites for the β subunits, the $\beta\gamma$ subunits of G proteins, the $\alpha_2\delta$ subunit, and the calcium channel blockers and activators.

The β subunits are intracellularly located hydrophilic proteins ranging from 50 to 78 kDa. A sequence comparison of all four β subunits revealed that all share a common central core, whereas their N- and C-termini and part of the central region differ significantly. The various β subunit isoforms cause different shifts in the kinetics and voltage dependence of gating. Therefore the association with different β subunits can substantially alter the physiological function of an α_1 subunit.

The γ subunit was cloned first from skeletal muscle and it was shown that it is an integral membrane protein consisting of 222 residues with a predicted molecular mass of 25 kDa (Bosse *et al.*, 1990; Jay *et al.*, 1990). Coexpression studies revealed a modulatory function of the γ subunit on L-type calcium channels; these include changes in

peak current and in the activation and inactivation kinetics (Eberst *et al.*, 1997; Singer *et al.*, 1991). These results were confirmed by the deletion of its gene, which showed that in the absence of the γ subunit, the peak Ca^{2+} current in skeletal muscle myotubes was increased and the steady-state inactivation was shifted to more positive potentials (Freise *et al.*, 2000). There is an increasing evidence for the existence of further γ subunits since the identification of a mutation in a related gene which causes epilepsy in stargazer mice (Letts *et al.*, 1998). This $\gamma 2$ subunit could be part of the P/Q-type calcium chnnel. Subsequent studies have identified six additional putative γ subunits, not all of which have been characterized in detail (Burgess *et al.*, 2001; Klugbauer *et al.*, 2000).

The $\alpha_2\delta$ subunit is a highly glycosylated protein that is encoded by a single gene. It is translated as a precursor polypeptide and is posttranslationally cleaved (De Jongh *et al.*, 1990; Jay *et al.*, 1991). The transmembrane δ part anchors the α_2 protein to the membrane via a single putative transmembrane segment. This association is mediated by disulfide bridges that are formed between numerous cysteine residues found in both proteins. The membrane topology was further refined using anti-α_2 antibodies and C-terminal deletion mutants (Brickley *et al.*, 1995; Wiser *et al.*, 1996). Structural studies have shown that the extracellular α_2 domain provides the crucial elements required for channel stimulation (Gurnett *et al.*, 1996) and that the δ domain harbors the regions important for the shift in voltage-dependent activation, steady-state inactivation, and the modulation of the inactivation kinetics (Felix *et al.*, 1997).

Amino acid sequence alignments revealed notable structural differences between LVA and HVA calcium channels. For instance, LVA calcium channels do not contain the consensus sequence in the I–II loop which was shown to be crucial for binding of a β subunit (Walker and De Waard, 1998). A consensus site on the HVA α_1 subunits to which the $\alpha_2\delta$ subunit binds has also not been identified. Gurnett *et al.* reported an interaction of repeat III of $Ca_v1.1$ with $\alpha_2\delta$-1 after extensive trypsinization (Gurnett *et al.*, 1997). However, a reconstitution of this interaction in expression systems failed.

CLONING AND STRUCTURE OF NOVEL $\alpha_2\delta$ SUBUNITS

Since the identification of the first $\alpha_2\delta$ subunit (Ellis *et al.*, 1988) it took more than 10 years until further members of this protein family, $\alpha_2\delta$-2 and $\alpha_2\delta$-3 were described (Klugbauer *et al.*, 1999). A fourth human $\alpha_2\delta$ subunit was recently identified (Qin *et al.*, 2002).

The discovery of novel genes was based on database searches. The low sequence identity of 56% ($\alpha_2\delta$-2) and 30% ($\alpha_2\delta$-3) with the first subunit prevented their identification by molecular biological techniques. Despite this low sequence homology, all three sequences share important structural features. Fourteen cysteine residues are present in all of the three $\alpha_2\delta$ genes, further strengthening the postulate that these subunits are disulfide-linked proteins with similar higher order structures. Furthermore, all three $\alpha_2\delta$ subunits are highly glycosylated which can be seen by the numerous putative glycosylation sites. The incubation of brain membrane preparations with N-glycosidase F led to a shift in the protein mobility on Western blots. Deglycosylation resulted in the loss of approximately 30 kDa for all $\alpha_2\delta$ subunits (Marais *et al.*, 2001). Because glycosylation is essential for current stimulation by $\alpha_2\delta$-1 of $Ca_v2.1$ (Gurnett *et al.*, 1996), this is likely to be the case for $\alpha_2\delta$-2 and $\alpha_2\delta$-3 as well. Hydrophobicity profiles also indicated that all three subunits have a similar distribution of hydrophilic and hydrophobic residues and a comparable membrane topology. These structural implications were confirmed by Western blots which indicated that all subunits have a similar protein pattern either under nonreducing or reducing conditions. $\alpha_2\delta$-1 has a molecular weight of about 200 kDa under nonreducing conditions and 140 kDa in a reducing environment. The corresponding molecular weights of the $\alpha_2\delta$-2 and $\alpha_2\delta$-3 subunits are 190 and 166 kDa under nonreducing and 138 and 131 kDa under reducing conditions, respectively (Marais *et al.*, 2001). The cleavage site of $\alpha_2\delta$-1 is located between A934 and A935 (De Jongh *et al.*, 1990). The alanine at position 934 is conserved in $\alpha_2\delta$-2 and $\alpha_2\delta$-3, although the rest of the sequence in the region diverges. The approximate sizes of the δ proteins and the conservation of the alanine residue suggest that the cleavage sites of $\alpha_2\delta$-2 and $\alpha_2\delta$-3 could be the same as in $\alpha_2\delta$-1. In summary, these results indicated that all $\alpha_2\delta$ subunits consist of glycosylated, separate, disulfide-linked α_2 and δ proteins.

SPLICE VARIANTS OF $\alpha_2\delta$ SUBUNITS

Figure 2 indicates the sites of alternative splicing in all three $\alpha_2\delta$ subunits. Several splice variants of $\alpha_2\delta$-1 have been detected which arise from various combinations of three alternatively spliced regions. The combination of these regions results in five isoforms that are expressed in a tissue-specific manner (Angelotti and Hofmann, 1996). Skeletal muscle and brain expressed the single isoforms $\alpha_2\delta$-1a and $\alpha_2\delta$-1b, respectively. In the cardiovascular system all five splice variants could be detected. Heart

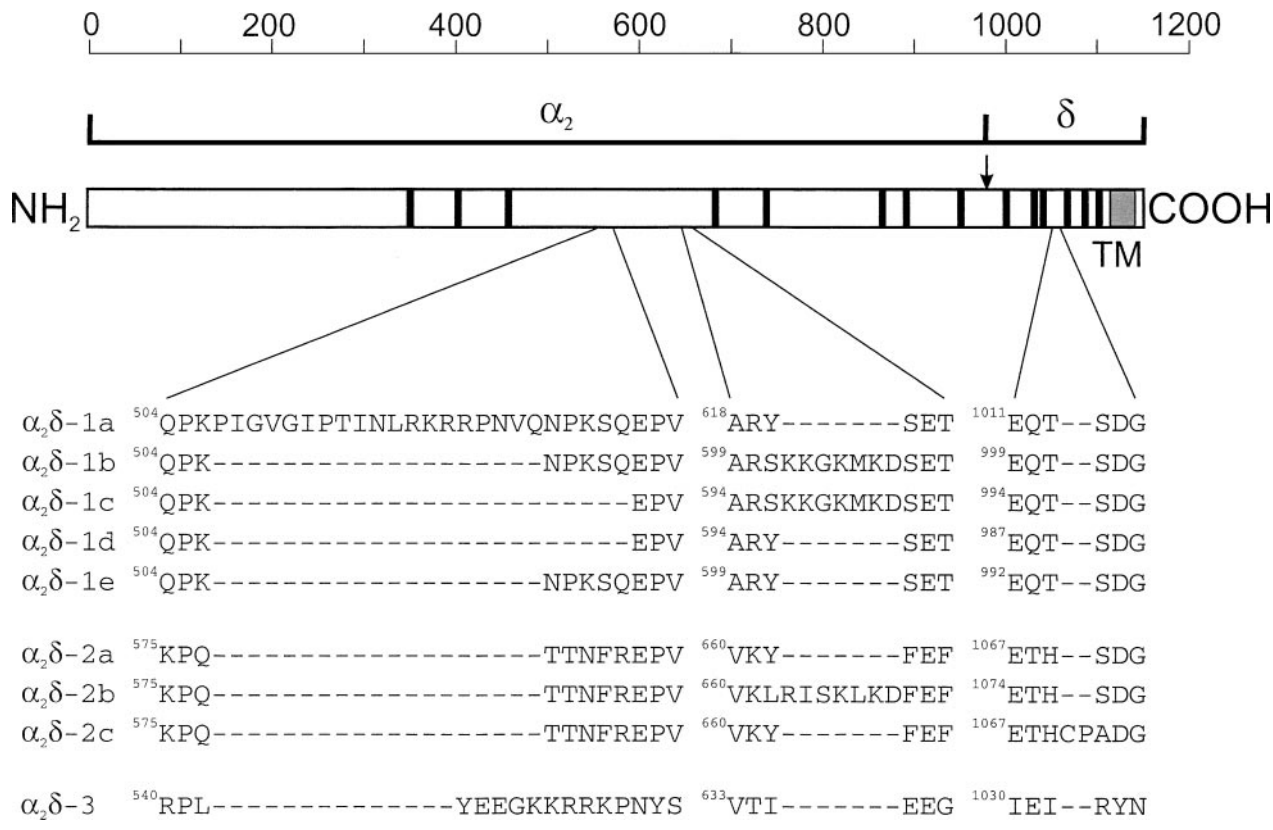

Fig. 2. Schematic diagram of the $\alpha_2\delta$ subunit protein structure and regions of alternative splicing. NH_2 and COOH indicate the amino and carboxy terminus of the protein. TM denotes the location of the transmembrane segment of the δ protein. The black bars show the position of 14 cysteine residues that are present in all three $\alpha_2\delta$ subunits. The arrow indicates the cleavage site between the α_2 and δ subunit. The amino acid sequences of the three regions of alternative splicing are shown below. Sequence data of murine $\alpha_2\delta$-la to $\alpha_2\delta$-le are from Angelotti and Hofmann (1996) (accession numbers U73483, U73484, U73485, U73486, U73487). The amino acid sequences of human $\alpha_2\delta$-2a to $\alpha_2\delta$-2c are from Hobom *et al.* (2000) (accession numbers AJ251367, AJ251368, and AF042792) and of murine $\alpha_2\delta$-3 are from Klugbauer *et al.* (1999) (accession number AJ010949).

expressed mainly isoforms $\alpha_2\delta$-1c and $\alpha_2\delta$-1d. Smooth muscle tissues expressed $\alpha_2\delta$-1d and $\alpha_2\delta$-1e.

For the $\alpha_2\delta$-2 subunit, two regions of alternative splicing were identified. The first is located in the α_2 protein (between residues 661/663) and is characterized by the addition of eight amino acid residues. The second is located in the δ protein (between residues 1069/1071) and includes three different residues (Hobom *et al.*, 2000). In the human heart only the $\alpha_2\delta$-2a splice variant was detected, whereas in hMTC (human medullary thyroid) cells all three isoforms, $\alpha_2\delta$-2a, $\alpha_2\delta$-2b, and $\alpha_2\delta$-2c, were identified at about the same levels. hMTC cells are a valuable tool to analyze the composition of T-type calcium channels since these cells predominantely express LVA calcium channels. The composition of T-type calcium channels is still a matter of debate because T-type channels would be unique if they only consist of a single α_1 subunit without auxiliary subunits.

cDNA cloning of the $\alpha_2\delta$-3 subunit did not uncover additional splice variants. However, for the human $\alpha_2\delta$-3 gene a possible splice variant carrying a 62 bp frame shift deletion was described which would result in a premature truncation of the protein (Hanke *et al.*, 2001). An expression analysis and functional characterization of this truncated protein is still missing.

TISSUE DISTRIBUTION OF $\alpha_2\delta$ SUBUNITS

The tissue distribution of $\alpha_2\delta$ subunits has been analyzed in many publications (Barclay *et al.*, 2001; Gong *et al.*, 2001; Hobom *et al.*, 2000; Klugbauer *et al.*, 1999; Marais *et al.*, 2001). The expression of $\alpha_2\delta$ subunits was investigated either on the mRNA level by Northern blot hybridization, RT-PCR amplification, in-situ hybridization or on the protein level by Western blots. The data are

Table I. Expression of $\alpha_2\delta$ Subunits in Murine and Human Tissues

	Brain		Heart		Lung		Spleen		Kidney		Liver		Testis		Skel. m.	
m$\alpha_2\delta$-1	+	+	+	+	+	+	+	+	+	+	+	+	+	n.d.	+	+
h$\alpha_2\delta$-1	+	+	+	n.d.	−	−	+	+	+	+	−	n.d.	n.d.	+	+	n.d.
m$\alpha_2\delta$-2	+	+	+	+	+	+	−	−	±	±	±	+	+	n.d.	−	−
h$\alpha_2\delta$-2	+	+	+	n.d.	+	−	−	−	±	−	±	n.d.	+	+	+	n.d.
m$\alpha_2\delta$-3	+	+	−	−	−	−	−	−	−	−	−	−	−	n.d.	−	−
h$\alpha_2\delta$-3	+	+	±	n.d.	−	−	−	−	−	−	−	n.d.	n.d.	−	±	n.d.

Note. Left column: Expression of $\alpha_2\delta$ mRNA as revealed by RT-PCR amplification and Northern blot hybridization. Right column: Detection of $\alpha_2\delta$ protein by Western analysis. + indicates expression, ± very weak expression, − no expression, n.d. not determined, m: mouse, h: human. The data are summarized from the following publications: m$\alpha_2\delta$-1 (Barclay *et al.*, 2001; Gong *et al.*, 2001); h$\alpha_2\delta$-1 (Gong *et al.*, 2001); m$\alpha_2\delta$-2 (Barclay *et al.*, 2001; Gong *et al.*, 2001); h$\alpha_2\delta$-2 (Klugbauer *et al.*, 1999; Gao *et al.*, 2000; Gong *et al.*, 2001); m$\alpha_2\delta$-3 (Klugbauer *et al.*, 1999; Barclay *et al.*, 2001; Gong *et al.*, 2001); h$\alpha_2\delta$-3 (Gong *et al.*, 2001).

summarized in Tables I and II. Since the relative abundance of mRNA and protein of a specific gene is not always proportional, Table I includes data of both, mRNA and protein expression.

$\alpha_2\delta$-1 is found ubiquitously in any tissue analyzed so far. However, tissue preparations from lung and liver indicate that $\alpha_2\delta$-1 is detectable in murine, but not in human lung and liver. This species-specific expression is also found for $\alpha_2\delta$-2. $\alpha_2\delta$-2 is also expressed in various tissues with the highest levels in brain, heart, pancreas, and skeletal muscle. Lower levels of the mRNA were seen in other tissues. There are also significant differences in the expression levels between mouse and human. Whereas $\alpha_2\delta$-2 can be detected by Northern hybridization in human skeletal muscle it is not present in the mouse skeletal muscle. $\alpha_2\delta$-2 is moderately expressed in the murine lung as seen by Northern hybridization and Western blots. In human lung $\alpha_2\delta$-2 is detected only by Northern hybridization, but it is clearly absent on Western blots (Gong *et al.*, 2001). However, it is possible that certain lung tumor cell types express higher levels (Gao *et al.*, 2000).

$\alpha_2\delta$-3 expression was found to be restricted to brain (Klugbauer *et al.*, 1999; Marais *et al.*, 2001). Only a weak expression of $\alpha_2\delta$-3 in heart and skeletal muscle was detectable using human Northern blots, but this result could not be confirmed by protein data (Gong *et al.*, 2001). $\alpha_2\delta$-4 was found to be expressed in human heart and skeletal muscle (Qin *et al.*, 2002).

The $\alpha_2\delta$ mRNA distribution shows significant variations within the mouse brain. Strong expression of $\alpha_2\delta$-1 was seen in the pyramidal cell layer of Ammon's horn in the hippocampus (CA1–3) and in the granular cell layer of the dentate gyrus (Klugbauer *et al.*, 1999). The olfactory bulb also stained strongly, and expression in the mitral and glomerular cell regions was shown by photographic emulsion. Staining was also observed in the cerebellar cortex and to a lesser extent in thalamic nuclei. Expression of $\alpha_2\delta$-1 in the cerebellum was restricted to the granular layer.

Transcripts of $\alpha_2\delta$-2 could be detected in the cerebellum, reticular thalamic nuclei, septal nuclei, habenulae, and in the colliculus inferior. The $\alpha_2\delta$-2 mRNA expression was found to be highest in the Purkinje cell layer of the cerebellum, in the habenulae, and the septal nuclei. The highest density of $\alpha_2\delta$-3 mRNA was found in the caudate putamen, entorhinal complex, hippocampus, and cortex. The $\alpha_2\delta$-3-specific probe indicated a high expression in the pyramidal cell layer of the hippocampus (CA1–3) and granular cell layer of the dentate gyrus.

FUNCTIONAL ROLE OF $\alpha_2\delta$ SUBUNITS

Since the expression pattern of $\alpha_2\delta$ subunits did not suggest specific interactions with any of the known α_1 subunits, functional characterizations were carried out with members of the L-type, non-L-type, and T-type calcium channels. Coexpression of the $\alpha_2\delta$-1 subunit with various combinations of α_1 and β subunits results in an increase in the current densities and dihydropyridine binding sites (Bangalore *et al.*, 1996; De Waard and Campbell, 1995; Felix *et al.*, 1997; Gurnett *et al.*, 1996; Jones *et al.*, 1998; Parent *et al.*, 1997; Shistik *et al.*, 1995; Singer *et al.*, 1991; Welling *et al.*, 1993), acceleration of current activation and inactivation (Bangalore *et al.*, 1996; De Waard and Campbell, 1995; Qin *et al.*, 1998; Shirokov *et al.*, 1998; Singer *et al.*, 1991), and a shift of the current–voltage curve in a hyperpolarizing direction (Felix *et al.*, 1997; Singer *et al.*, 1991).

The coexpression of $\alpha_2\delta$-2 together with $Ca_v1.2/\beta2a$, $Ca_v2.3/\beta3$, or $Ca_v2.1/\beta2a$ increased the current density and shifted the I–V curve to more negative potentials (Hobom *et al.*, 2000). These effects were observed with both splice variants, $\alpha_2\delta$-2a and $\alpha_2\delta$-2b. The time constant

of activation was significantly decreased when $\alpha_2\delta$-2 was coexpressed with $Ca_v1.2/\beta2a$. The same acceleration in activation and shift of the $I-V$ curve was observed when $\alpha_2\delta$-1 was used. All $\alpha_2\delta$ subunits shifted the steady-state inactivation towards more negative potentials and accelerated the time constant of inactivation of $Ca_v1.2/\beta2a$ in the presence of 10 or 30 mM Ba^{2+} as charge carrier.

In contrast to the coexpression studies with $Ca_v1.2/\beta2a$, the effects of $\alpha_2\delta$-2 on $Ca_v2.3/\beta3$ were more prominent than those seen with $\alpha_2\delta$-1. $\alpha_2\delta$-2 shifted the the voltage dependence of activation to more hyperpolarized potentials. This shift was not observed with the $\alpha_2\delta$-1 subunit, but $\alpha_2\delta$-1 and $\alpha_2\delta$-2 shifted the steady-state inactivation curve towards more negative potentials. These effects on the $Ca_v2.3$ calcium channel were observed only in the presence of a γ subunit (Hobom *et al.*, 2000).

In situ hybridization also indicated a possible colocalization of the $\alpha_2\delta$-2 subunit with $Ca_v2.1$ in Purkinje cells. $\alpha_2\delta$-2, but not $\alpha_2\delta$-1, shifted the voltage dependence of activation to more negative potentials when coexpressed with $Ca_v2.1/\beta2a$. The magnitude of this shift was small and significant only with the $\alpha_2\delta$-2b splice variant. The time constant of activation and the steady-state inactivation curve were affected more by the $\alpha_2\delta$-1 than the $\alpha_2\delta$-2 subunit. None of the $\alpha_2\delta$ subunits changed the inactivation time constants (Hobom *et al.*, 2000).

Coexpression studies of $\alpha_2\delta$-3 were performed with $Ca_v1.2$ and $Ca_v2.3$ calcium channels (Klugbauer *et al.*, 1999). When coexpressed with $Ca_v1.2$ alone, the effects of $\alpha_2\delta$-3 on Ba^{2+} currents were less noticeable than when $\beta2a$ was coexpressed. $\alpha_2\delta$-3 did not affect the current density, time course of current inactivation, and voltage dependence of the steady-state inactivation of $Ca_v1.2$ alone. However, $\alpha_2\delta$-3 shifted the activation curve in the hyperpolarizing direction and slightly accelerated the time of current activation during the depolarizing pulse. In combination with $Ca_v1.2/\beta2a$, $\alpha_2\delta$-3 significantly increased the current density, shifted the voltage dependence of current activation in a hyperpolarizing direction, accelerated the current activation during the depolarizing pulse, accelerated the current inactivation at positive membrane potentials, shifted the steady-state inactivation curve in a hyperpolarizing direction, and significantly changed its slope (Klugbauer *et al.*, 1999). As with $Ca_v1.2$, $\alpha_2\delta$-3 affected most of the gating-related parameters except for the time constant of current activation during membrane depolarization when coexpressed with $Ca_v2.3/\beta3$. In contrast to $Ca_v1.2$, the effects of $\alpha_2\delta$-1 and $\alpha_2\delta$-3 on the voltage dependence of current activation and inactivation of $Ca_v2.3$ were significantly different. The $\alpha_2\delta$-1 subunit shifted both activation and steady-state inactivation curves in a hyperpolarizing direction, but the change in current activation was not statistically significant. In both curves, the shift evoked by $\alpha_2\delta$-3 was significantly larger.

The mechanism whereby $\alpha_2\delta$ modulates the conductances of α_1 is not well understood. The increase in current density and DHP-binding sites can be explained by an improved targeting of the α_1 subunit to the cell membrane and maturation of the channel complex (Shistik *et al.*, 1995), which leads to an increased amount of charge moved during channel activation (Bangalore *et al.*, 1996; Qin *et al.*, 1998). It was suggested that the increase in current requires the presence of an intact α_2 protein, whereas the shift of voltage-dependent activation and steady-state inactivation as well as the acceleration of the inactivation kinetics are caused by the transmembrane δ protein (Felix *et al.*, 1997). However, on the basis of the amino acid similarity of the three subunit forms, it seems more likely that α_2 harbors the relevant residues responsible for the observed effects on α_1 and that the δ domain functions only as a membrane anchor for α_2. This interpretation is further strengthened by the sequences of the δ proteins, which are not conserved.

POSSIBLE ASSOCIATION OF $\alpha_2\delta$ SUBUNITS WITH T-TYPE CALCIUM CHANNELS

Very little is known about the function of auxiliary subunits for the modulation of T-type calcium channels. Coexpression of $Ca_v3.1$ with either the $\alpha_2\delta$-1 or $\alpha_2\delta$-3 subunit did not reveal a significant effect of these auxiliary proteins on T-type current in HEK293 cells (Lacinova *et al.*, 1999). $\alpha_2\delta$-1 did not affect the voltage relations for peak and sustained current, kinetics of current activation and inactivation, voltage dependence of current inactivation, and time course of the recovery from inactivation.

So far only one report showed that $\alpha_2\delta$-1 significantly increased the current density of $Ca_v3.1$ (Dolphin *et al.*, 1999). Coexpression of $\alpha_2\delta$-1 increased the plasma membrane localization of $Ca_v3.1$ in COS-7 cells and conversely, the expression of $Ca_v3.1$ increased immunoreactivity for $\alpha_2\delta$-1, suggesting an interaction between these two subunits. As a consequence of this interaction the current through $Ca_v3.1$ was increased about twofold. These results suggest that $\alpha_2\delta$-1 interacts with $Ca_v3.1$ possibly by an improved targeting or stabilization of the calcium channel complex in some heterologous expression systems (Dolphin *et al.*, 1999).

A more straightforward way to analyze the function of $\alpha_2\delta$ subunits on T-type currents is to analyze the expression of calcium channel subunits in a single cell line. hMTC cells predominantely express T-type calcium channels and therefore should be a good tool to identify

endogenous auxiliary $\alpha_2\delta$'s. Molecular cloning revealed that $\alpha_2\delta$-2 is found in this cell line (Hobom *et al.*, 2000). Hobom and coworkers found some effects of $\alpha_2\delta$-2 on T-type current through $Ca_v3.1$ channels. In HEK293 cells $\alpha_2\delta$-2a significantly increased the current density of the $Ca_v3.1$ channel. The steady-state inactivation was significantly shifted by both splice variants $\alpha_2\delta$-2a and $\alpha_2\delta$-2b in the depolarizing direction. The kinetics of current activation were not significantly altered, but the time course of inactivation was significantly accelerated in the presence of $\alpha_2\delta$-2.

However, since all of these effects were small, it is difficult at the moment to judge whether the $\alpha_2\delta$-2 subunit simply facilitates membrane targeting of T-type channels or has a clear modulatory effect on T-type channels.

THE DUCKY MOUSE PHENOTYPE IS ASSOCIATED WITH MUTATIONS IN THE $\alpha_2\delta$-2 GENE

In mice five spontaneous autosomal recessive mutations have been described that show a phenotype similar to those occuring in human idiopathic generalized epilepsy (Puranam and McNamara, 1999). These mutations include the tottering, slow-wave epilepsy, lethargic, stargazer, and ducky phenotype and affected animals exhibit bilaterally synchronous spikewave discharges on cortical electroencephalogram recordings. Most of these phenotypes are caused by mutations in calcium channel genes, such as tottering ($Ca_v2.1$), lethargic ($\beta4$), stargazer ($\gamma2$), and finally ducky ($\alpha_2\delta$-2). The mouse mutant ducky is also a model for absence epilepsy and is characterized by spike-wave seizures and cerebellar ataxia (Snell, 1955). These mice display a reduced size, have abnormalities in the Purkinje cell dendritic tree (Brodbeck *et al.*, 2002) and are not able to breed or survive beyond day 35. The *du* locus was localized to mouse chromosome 9 and it was shown that only homozygous mice feature the obvious phenotype.

Recently, Barclay *et al.* (2001) showed that mutations in the Cacna2d2 gene were found to underlie the phenotype in the original *du* and a second du^{2J} strain. In each of the strains the mutation of the Cacna2d2 gene results in the loss of the full-length $\alpha_2\delta$ protein. It was found that in *du/du* mice a head to tail gene duplication of exons 2–39 occurred (Barclay *et al.*, 2001). As a consequence from this large genomic rearrangement, two different transcripts were found to be expressed in these mice. Transcript 1 encodes exons 1–3 of $\alpha_2\delta$-2 which were spliced to a novel region X and contains the first 414 bp of Cacna2d2, 24 bp of region X, and a stop codon. Transcript 2 only contains exons 2–39 of $\alpha_2\delta$-2. Both transcripts were detected only at low levels in *du/du* mice. No full-length Cacna2d2 transcript could be identified by Northern blots in *du/du* mice. The absence of full-length Cacna2d2 message in *du/du* Purkinje cells was also shown by in situ hybridization (Barclay *et al.*, 2001; Brodbeck *et al.*, 2002). Southern blot hybridizations indicated that more than 150 kb of genomic DNA was duplicated including exons 2 and 3 and the unknown region X.

In du^{2J}/du^{2J} mice a two base pair deletion (T and G) in exon 9 was found to be the reason for a frameshift in the Cacna2d2 transcript. This frameshift leads to a truncation of the $\alpha_2\delta$-2 protein thereby loosing more than 800 amino acids. This mouse line is characterized by infrequent bilateral spike-wave discharges of high amplitude and 5–7 Hz. These spontaneous discharges were accompanied by behavioral arrest.

In vitro studies have shown that $\alpha_2\delta$-2 increases current amplitude and alters the kinetics of activation and inactivation for various types of calcium channels (Hobom *et al.*, 2000). Therefore, the lack of full-length $\alpha_2\delta$-2 should result in a reduction of calcium current in Purkinje cells. And in fact, Purkinje cells from *du/du* mice showed a 35% decrease in the peak P-type calcium current when compared with $+/+$ animals (Barclay *et al.*, 2001). This effect was not observed in cerebellar granule cells, which in contrast to Purkinje cells do not express $\alpha_2\delta$-2 in high amounts (Hobom *et al.*, 2000). However, there was no effect on voltage dependence of activation or on the kinetics of activation or inactivation in Purkinje cells from *du/du* mice. Furthermore, the coexpression of the truncated $\alpha_2\delta$-2 subunit (transcript 1) with $Ca_v2.1/\beta4$ in COS-7 cells produced a consistent reduction in the Ba^{2+} current (Brodbeck *et al.*, 2002). This result indicates that besides the loss of functional $\alpha_2\delta$-2 as an important factor for the ducky phenotype, also the expression of a truncated $\alpha_2\delta$-2 subunit possibly contributes to a reduced calcium current in Purkinje cells.

Another conclusion which could be drawn from above results is based on the fact that mice with the ducky phenotype are similar to animals with mutations in either the $Ca_v2.1$ or the $\beta4$ subunit. In line with the predominant Purkinje cell expression of these subunits, the data strongly suggest that $\alpha_2\delta$-2 is part of the P/Q type calcium channel.

THE $\alpha_2\delta$ SUBUNIT AS TARGET FOR GABAPENTIN

Gabapentin is an antiepileptic drug that has found recently also application in the treatment of neuropathic pain

and anxiolytic disorders (Beydoun *et al.*, 1995; Rosenberg *et al.*, 1997). It was found that gabapentin binds specifically to the $\alpha_2\delta$-1 subunit of voltage-gated calcium channels with a high affinity and it has been postulated that gabapentin reduces calcium current by indirectly modulating the α_1 subunit. This specific binding of gabapentin to $\alpha_2\delta$-1 was the first described interaction between a regulatory subunit of the calcium channel and a drug. The K_d of porcine brain $\alpha_2\delta$-1 was reported as 9.4 nM (Brown *et al.*, 1998) but as 37.5 nM for porcine $\alpha_2\delta$-1 expressed in COS-7 cells (Brown and Gee, 1998), 16 nM and 59 nM for rabbit $\alpha_2\delta$-1 in COS-7 cells (Gee *et al.*, 1996; Marais *et al.*, 2001). Gabapentin binding is dependent on the presence of both α_2 and δ subunits, which do not have to be translated as a single precursor protein (Wang *et al.*, 1999). However, neither α_2 nor δ bind the drug when expressed alone, indicating that the interaction of both subunits is necessary. Cleavage of the precursor protein is also not required for binding (Brown and Gee, 1998).

Recently the binding of gabapentin to $\alpha_2\delta$-2 and $\alpha_2\delta$-3 subunits was investigated (Gong *et al.*, 2001; Marais *et al.*, 2001). $\alpha_2\delta$-2 was found to bind gabapentin with a K_d of 153 nM (Marais *et al.*, 2001) or 156 nM (Gong *et al.*, 2001), whereas $\alpha_2\delta$-3 and $\alpha_2\delta$-4 did not show any binding. Inhibition of gabapentin binding by selected amino acids to the membranes from porcine $\alpha_2\delta$-1 and human $\alpha_2\delta$-2 overproducing cell lines resulted in a similar binding profile (K_i values are 72 nM for $\alpha_2\delta$-1 and 190 nM for $\alpha_2\delta$-2) (Gong *et al.*, 2001).

Despite this detailed knowledge of gabapentin-binding the function of gabapentin for the activity of calcium channels is not clearly understood. In patch-clamp studies with hippocampal granule cells, no effect of gabapentin was reported (Schumacher *et al.*, 1998). However, a reduction in calcium current in isolated neurons and in rat neocortical slices has been described following application of gabapentin (Fink *et al.*, 2000; Stefani *et al.*, 1998). Calabresi and coworkers found gabapentin to reduce most excitatory properties of striatal spiny neurons, which could account for the anticonvulsant effect of the drug (Calabresi *et al.*, 1999). Others have shown that gabapentin and a related compound, pregabalin, reduced the release of norepinephrine when stimulated by potassium and electrical pulses (Dooley *et al.*, 2000). This result was recently confirmed by Fink *et al.* (2002) using human neocortical synaptosomes.

The calcium channels affected by gabapentin are not known, but candidates are L-type (Stefani *et al.*, 1998) and P/Q-type (Fink *et al.*, 2000, 2002) calcium channels. However, no consistent effect of gabapentin on $Ca_v1.2$, $Ca_v2.1$, and $Ca_v3.2$ currents in heterologous HEK293 expression systems was observed (Marais *et al.*, 2001). The complexity of the interaction between gabapentin and $\alpha_2\delta$ is further illustrated by a temperature-dependent influence of ruthenium red, $MgCl_2$, and spermine on gabapentin binding (Taylor and Bonhaus, 2000). Initial studies on gabapentin binding with tissue homogenates showed strong binding in rat skeletal muscle and brain, where $\alpha_2\delta$-1 is most highly expressed (Gee *et al.*, 1996). A lower binding of gabapentin was seen in liver and kidney, which express considerable levels of $\alpha_2\delta$-1. A possible explanation for these conflicting results is that the affinity of gabapentin to $\alpha_2\delta$ is modulated by other subunits. One can speculate that effects of gabapentin depend on the composition and environment of the channel. The lack of clinical side effects of the drug on skeletal muscle or other $\alpha_2\delta$-1 expressing tissues supports this view (Beydoun *et al.*, 1995).

Gabapentin has also been reported to be an agonist at $GABA_B$ receptors (Ng *et al.*, 2001). Gabapentin significantly activated Kir3.1/3.2 channels through the gb1a–gb2 heterodimer, but not through other combinations of the $GABA_B$ receptor subunits. This interaction may imply a postsynaptic mechanism that is independent of a presynaptic inhibition of P/Q-type calcium channels. Gabapentin could enhance the inhibitory GABAergic input via somadendritic $GABA_B$ receptors on pyramidal neurons. This pathway could lead to a decrease of glutamate release from nerve terminals possibly in addition to the inhibition of calcium influx through presynaptic calcium channels on the same terminals (Fink *et al.*, 2002). Another possible explanation would be that the agonist effect of gabapentin on $GABA_B$ receptors is specific to hippocampal CA1 pyramidal neurons whereas gabapentin inhibits calcium influx in the neocortex by its action on voltage gated calcium channels.

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