The novel product of a five-exon stargazin-related gene abolishes Ca\textsubscript{v}2.2 calcium channel expression

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We have cloned and characterized a new member of the voltage-dependent Ca\textsuperscript{2+} channel \(\gamma\) subunit family, with a novel gene structure and striking properties. Unlike the genes of other potential \(\gamma\) subunits identified by their homology to the stargazin gene, CACNG7 is a five- and not four-exon gene whose mRNA encodes a protein we have designated \(\gamma_7\). Expression of human \(\gamma_7\) has been localized specifically to brain. N-type current through Ca\textsubscript{v}2.2 channels was almost abolished when co-expressed transiently with \(\gamma_7\) in either Xenopus oocytes or COS-7 cells. Furthermore, immunocytochemistry and western blots show that \(\gamma_7\) has this effect by causing a large reduction in expression of Ca\textsubscript{v}2.2 rather than by interfering with trafficking or biophysical properties of the channel. No effect of transiently expressed \(\gamma_7\) was observed on pre-existing endogenous N-type calcium channels in sympathetic neurons. Low homology to the stargazin-like \(\gamma\) subunits, different gene structure and the unique functional properties of \(\gamma_7\) imply that it represents a distinct subdivision of the family of proteins identified by their structural and sequence homology to stargazin.

**Keywords:** calcium channel/expression/\(\gamma\) subunit/stargazin/suppression

**Introduction**

Voltage-dependent calcium channels (VDCCs) play a fundamental role in the coupling of membrane depolarization to many cellular processes by regulating cytoplasmic Ca\textsuperscript{2+} concentration in excitable cells. They are hetero-multimers consisting of a pore-forming \(\alpha_1\) subunit assembled with auxiliary \(\beta\), \(\alpha_2\delta\) and possibly \(\gamma\) subunits. These subunits can be encoded by several different genes with alternative splice variants and are expressed in a tissue-specific manner. Much work has concentrated on the characterization of functional properties of the \(\alpha_1\), \(\beta\) and \(\alpha_2\delta\) subunits (Birnbaumer et al., 1998; Dolphin, 1998; Jones, 1998; Perez-Reyes, 1998; Catterall, 2000). However, research concerning the role of the \(\gamma\) subunit has not been as extensive.

Until recent years, only a single gene, exclusively expressed in skeletal muscle, was believed to encode a VDCC \(\gamma\) subunit (Jay et al., 1990; Powers et al., 1993). Recordings of Ca\textsuperscript{2+} currents from dihydropyridine receptors (DHPRs) of skeletal myotubes from mice lacking this \(\gamma_1\) subunit suggest that its role is to limit calcium entry through these channels, increase the rate at which the channels inactivate and hyperpolarize the half-maximal potential for the voltage dependence of steady-state inactivation (Freise et al., 2000; Ahern et al., 2001). Subsequently, a second putative VDCC \(\gamma\) subunit, \(\gamma_2\), was identified based on its structural similarity to \(\gamma_1\) despite having only weak protein sequence identity (25%) (Letts et al., 1998). Mutations in the \(\gamma_2\) gene, cacng2, were found to underlie the absence epilepsy phenotype of the allelic stargazer (stg) and waggler (wag) mutant mice. Subsequent studies have identified six further putative \(\gamma\) subunits (\(\gamma_3\)–\(\gamma_6\)), not all of which have been cloned and expressed (Black and Lennon, 1999; Burgess et al., 1999, 2001; Klugbauer et al., 2000).

The \(\gamma_2\), \(\gamma_3\) and \(\gamma_4\) subunits form a subfamily exclusively localized to the central nervous system (CNS) (Letts et al., 1998; Klugbauer et al., 2000) whose interaction with VDCCs has been investigated in several studies (Letts et al., 1998; Klugbauer et al., 2000; Kang et al., 2001; Sharp et al., 2001). The \(\gamma_5\) and \(\gamma_6\) subunits (Burgess et al., 1999, 2001) are predicted to represent another subfamily of stargazin-related proteins, with extremely low sequence identity to \(\gamma_1\) and \(\sim 25\%\) identity to \(\gamma_2\). These subunits, like other members of the putative \(\gamma\) subunit superfAMILY, are proteins predicted to have four transmembrane segments with intracellular N- and C-termini, and were reported to be encoded by a gene assembled from four exons (Burgess et al., 2001). However, assembly of the full-length \(\gamma_5\) and \(\gamma_6\) cDNAs has not been described and there are no functional data for either of these \(\gamma\) subunits.

In the present study, we report the identification, cloning and functional characterization of a novel protein we have named the \(\gamma_7\) subunit. The first four exons of the gene encoding this protein are identical to those encoding the predicted \(\gamma_7\) subunit gene previously described by Burgess et al. (2001), but the transcription and translation of a final fifth exon results in the \(\gamma_7\) described in the present study having a very different and much longer C-terminus. Our results show that the co-expression of the \(\gamma_7\) subunit almost abolishes the functional expression and markedly suppresses the level of Ca\textsubscript{v}2.2 subunit protein. We also report the identification of the \(\gamma_5\) subunit which, like \(\gamma_7\), is predicted to be encoded by a five-exon gene.
Results

Cloning of the γ7 and γ7 genes

The full-length mouse stargazin sequence (Letts et al., 1998) was used as a query sequence to search the DNA databases. A short 487 bp sequence was assembled from three expressed sequence tags (ESTs) and was found to contain an open reading frame (ORF) with 26% identity to stargazin, although it contained no in-frame start or stop codons.

A 487 bp fragment corresponding to this in silico sequence was amplified from human whole brain cDNA, and sequence analysis confirmed the computer predictions. Primers specific for each end of this fragment were used to amplify the missing parts of the ORF using 5′ and 3′ RACE. A 330 bp fragment containing the missing 5′ sequence and start ATG codon, and a 400 bp band containing the missing 3′ sequence and stop codon were obtained. These three DNA fragments were then used to assemble the full-length 828 bp stargazin-like ORF in a single ‘splice-overlap’ PCR.

When this full-length sequence was used to search the human high-throughput genomic sequences (HTGS) using the BLASTn algorithm, a bacterial artificial chromosome (BAC), clone AC008440, derived from human chromosome 19 was identified. Analysis of this BAC using the gene prediction program Genscan (Burge and Karlin, 1997) predicts that the 828 bp ORF is assembled from five exons and encodes a 275 amino acid protein.

When compared with all of the previously published γ subunits, this 828 bp cDNA clone exhibited 100% identity to the previously predicted human γ7 subunit over the first four exons (Burgess et al., 2001). However, the sequence of the cDNA clone described here diverges from the γ7 sequence (Burgess et al., 2001), due to the presence of the fifth exon (Figure 1A and B). It would therefore appear that CACNG7 is in fact a five-exon gene that encodes the γ7 subunit, and the previously predicted four-exon γ7 sequence results from read-through into intron four (Figure 1B).

BLAST searches of mouse HTGS identified cacng7, the mouse orthologue of the γ7 gene within BAC AC079557, which, like its human counterpart, contained five exons. Confirmation that the mouse γ7 mRNA is expressed was obtained by amplifying the complete cDNA in a single RT–PCR from mouse cerebellar total RNA. The mouse orthologue possesses 70% identity to the human γ7 at the nucleic acid level and, remarkably, 100% identity at the protein level.

BLASTn searches and Genscan analysis of human chromosome 16 BAC AC079424 identified another related gene encoding a 275 amino acid protein. This gene also possessed five exons and predicted a protein with 70.5% amino acid identity compared with the human γ7 and 27% identity compared with human γ5. It exhibited 100% identity to the previously described human γ5 subunit (Burgess et al., 1999) over its first 190 amino acids but, like the γ5 subunit, the additional fifth exon encodes an alternative C-terminus. Unlike the previously predicted four-exon γ5 and γ7, the C-termini of the five-exon γ5 and γ7 share considerable identity (80%, Figure 1A). We therefore named this subunit γ5 (gene CACNG5) and subsequently identified the mouse orthologue on mouse chromosome 16 BAC AC079424. The mouse orthologue (cacng5) exhibited 89.5% identity at the nucleotide level and 97% identity at the protein level to the human sequence.

Hydropathy plots predict that, like all the stargazin-related proteins, γ7 and γ5 have four transmembrane-spanning α-helices with predicted intracellular N- and C-termini (Figure 1C). The final transmembrane-spanning α-helices of γ7 and γ5 are predicted to be six and eight amino acids longer, respectively, than their equivalents in the previously predicted four-exon γ7 or γ5, and the full-length γ7 has a much more substantial cytosolic C-terminus than in the predicted four-exon γ7, in which only four intracellular amino acids are predicted after the final transmembrane segment.

The sequences have been deposited in the DDBJ/EMBL/GenBank database with the following accession Nos: human γ7 (AF458897), human γ5 (AF458898), mouse γ7 (AF458899) and mouse γ5 (AF458900).

Tissue distribution

The tissue distribution of the novel γ7 mRNA was analysed by northern blot. Figure 2 shows a human multiple tissue northern blot (Figure 2A) and two brain region blots (Figure 2B and C) hybridized with a probe corresponding to nucleotides 576–763 of the γ7 ORF. This region was chosen because it contains the least identity when compared with other γ-subunits (53% to human γ7), and is unique to γ7. Thus, this probe will not detect the predicted four-exon γ7, should this be expressed. This γ7-specific probe reveals two transcripts of ~2.4 and 3.0 kb, both of which are expressed only in brain. Both of these transcripts are expressed in all brain regions probed, although the shorter transcript is expressed at greater levels in several areas including cerebellum, amygdala, hippocampus and thalamus. These blots were stripped and re-probed using the same probe as that designed to detect the four-exon γ7 (Burgess et al., 2001). This probe, corresponding to nucleotides 80–482 of the γ7 ORF, would detect both the predicted four-exon γ7 and full-length γ7 transcripts. No additional transcripts were seen in any of the blots with this probe. Indeed, precisely the same expression profile was seen as with the full-length γ7-specific probe, including the same differential expression of the short and long transcripts seen in cerebellum, amygdala, hippocampus and thalamus (data not shown).

Influence of the γ7 subunit on heterologous expression of VDCCs and Kv3.1b

Having investigated the tissue distribution of the γ7 subunit in human brain, we next examined the effect of expression of this protein on Ba2+ currents recorded from neuronal VDCCs expressed in COS-7 cells. For comparison with immunocytochemistry data (see below), we transfected an N-terminal green fluorescent protein-tagged CaV2.2 construct (GFPCaV2.2), previously shown to have no significant differences in its biophysical properties compared with the non-tagged channel (Raghib et al., 2001). These experiments revealed an almost total abolition of whole-cell GFP-CaV2.2 Ba2+ current (Ibα) in cells co-transfected with γ7 (Figure 3A, upper trace). In 1 mM Ba2+, mean current density of cells expressing GFP-CaV2.2/β1b/α2δ1 was −13.5 ± 4.3 pA/pF at 0 mV (n = 20) (Figure 3B)
but, even when extracellular [Ba^{2+}] was increased to 10 mM, currents from GFP±CaV2.2/b₁b₂a₂d₂/g⁷-transfected COS-7 cells remained extremely small (±0.23 ± 0.08 pA/pF at 0 mV, n = 20, P < 0.01) (Figure 3A, lower trace, and B). In a recent report, it was stated that inhibition of CaV2.2 currents by the g⁷ subunit was dependent upon co-expression of an a²d subunit (Kang et al., 2001). To investigate if the same is true of the much more robust suppressive effect of g⁷, recordings were made from cells in the absence of co-transfected a²d subunit. The histograms in Figure 3B show that the influence of the g⁷ subunit on CaV2.2/b₁b₂ currents was independent of co-expression of an a²d subunit (CaV2.2/b₁b₂, ±14.9 ± 2.7 pA/pF at 0 mV in 1 mM Ba^{2+}; n = 16; CaV2.2/b₁b₂/g⁷, ±0.02 ± 0.03 pA/pF at 0 mV in 10 mM Ba^{2+}; n = 18, P < 0.01).

Fig. 1. Protein sequences, proposed splicing mechanism and hydropathy plots of a family of low homology stargazin-related genes. (A) Alignment of the five-exon g⁵ and g⁷ subunit sequences with the previously predicted four-exon g⁵ and g⁷ subunits using the Clustal_W algorithm. Dotted lines indicate consensus N-glycosylation sites and solid triangles beneath residues mark consensus sites for phosphorylation by cAMP- and cGMP-dependent protein kinase, protein kinase C, casein kinase II or tyrosine kinase. The exon–intron boundaries are marked by solid dots above the residue whose codon is interrupted by the adjacent intron. Note that the sequence identity between g⁵ and g⁷ is 80% conserved throughout the additional fifth exon, whereas the predicted four-exon g⁵ and g⁷ subunits differ greatly in sequence identities and length. The two large open triangles designate a pair of cysteine residues that are conserved amongst the putative VDCC γ subunits and may be involved in the formation of disulfide bridges. The transmembrane-spanning segments, as predicted by the TMpred program (Hofmann and Stoffel, 1993), are indicated by solid underlining. The residues highlighted in bold in the C-terminus of g⁷ identify the epitope for the anti-g⁷ antibody. (B) A schematic diagram of the g⁵ and g⁷ gene structure. The full-length genes are encoded by five exons interrupted by four introns, I₁–I₄. If the previously predicted four-exon g⁵ and g⁷ subunits were expressed, the extent of predicted read-through into intron 4 is displayed above the gene structure. The start codon, terminal amino acids of exon 4, and both normally spliced and intron-retained g⁵ or g⁷ are shown below the gene structure. (C) Hydropathy plots of the five-exon g⁵ and g⁷ subunits predicted by the TMpred program (Hofmann and Stoffel, 1993) compared with the previously predicted four-exon g⁵ and g⁷. Amino acid position is shown on the x-axis, and positive TMpred values indicate putative membrane-spanning regions; g⁵ and g⁷ are predicted to have four transmembrane-spanning α-helices with intracellular N- and C-termini.
To examine whether these effects were peculiar to transfection in mammalian expression systems, we next looked at the effect of γ7 on CaV2.2 currents expressed in *Xenopus* oocytes, where there cannot be any question as to whether all cDNAs are present in each cell. Figure 3C shows recordings made in 5 mM Ba2+ from *Xenopus* oocytes expressing CaV2.2/β1b, either with or without co-expression of γ7. The maximum conductance (Gmax), determined from the current–voltage (I–V) plots, was dramatically and significantly reduced when the human γ7 subunit was co-expressed compared with oocytes where it was not, with a corresponding 92.6% reduction in peak current amplitude at 0 mV (Figure 3C and Table I). The half-maximal value for the voltage dependence of activation (V50) was also shifted 3.4 mV more depolarized upon co-expression of γ7. An almost identical 94% inhibition of CaV2.2/β1b/α2δ2 currents was observed by γ7 in this system (data not shown). To examine whether the residual current represents the current induced by auxiliary subunits in *Xenopus* oocytes (Lacerda et al., 1994), we examined currents from oocytes transfected with only the auxiliary subunit β1b. However, we observed that these currents were also reduced from ~28.1 ± 9.6 nA (n = 10) to ~8.3 ± 5.3 nA (n = 9) upon co-expression of γ7. We next investigated the effects of γ7 on other CaV1.2 subtypes. The γ7 subunit also significantly reduced the Gmax of CaV2.1/β1b and CaV1.2/β1b VDCCs by 48 and 52%, respectively.

![Table I. Influence of the γ7 subunit on the activation properties of VDCCs expressed in Xenopus oocytes](image)

Data are expressed as mean ± SEM of the number of replicates, n. *P < 0.05, **P < 0.01 and ***P < 0.001 according to an unpaired Student’s t-test. The peak Ima was at +10 mV for CaV2.1 and CaV1.2 and at 0 mV for CaV2.2.
regulates other heterologously expressed ion channels by so striking, we investigated the possibility that it down-
N-type and 10% L-type current (Plummer et al., 1989). Ca2+ currents recorded from cultured sympathetic neurones of the rat superior cervical ganglion (SCG). These neurones express 80±90% N-type channels and 10% L-type current (Plummer et al., 1989); Delmas et al., 1998) and provided a vehicle in which we could determine if the γ7 subunit could exert its influence upon pre-existing N-type VDCCs in these neurones, and that γ7 does not co-exist endogenously in SCG neurones with N-type channels.

**Effect of heterologous expression of γ7 in cultured sympathetic neurones.** (A) Left: example traces from sympathetic neurones transiently transfected with GFP or GFP and the γ7 subunit, elicited by a 100 ms step depolarization to +10 mV from a holding potential of −80 mV in medium containing 10 mM Ba2+. Right: histogram of mean current densities measured from sympathetic neurones at +10 mV. The number of experiments (n) for each condition is given in parentheses above the columns. (B) Upper row: a GFP-transfected sympathetic neurone (left panel), showing lack of immunostaining for endogenous γ7 (right panel). Lower row: a GFP- plus γ7-transfected sympathetic neurone (left panel), showing immunostaining for γ7 (middle panel). Data are representative of three transfections. Scale bars = 15 μm.

The subcellular distribution of the expressed γ7 subunit was transiently transfected into COS-7 cells or *Xenopus* oocytes was so striking, we investigated the possibility that it down-regulates other heterologously expressed ion channels by co-expressing it with the Shaw-like voltage-dependent potassium channel, KV3.1b. Figure 4C shows representative traces and the peak I–V relationship of KV3.1b expressed in *Xenopus* oocytes. Holding potential was −100 mV, and steps were between −30 and +100 mV for 100 ms. The scale bar refers to both panels.

In *Xenopus* oocytes upon co-expression of the γ7 subunit suggests that it may be affecting the expression of these channels rather than altering their biophysical properties. However, since the effect of γ7 on the Ba2+ currents through CaV2.2 VDCCs expressed in COS-7 cells or *Xenopus* oocytes was so striking, we investigated the possibility that it down-regulates other heterologously expressed ion channels by co-expressing it with the Shaw-like voltage-dependent potassium channel, KV3.1b. Figure 4C shows representative traces and the peak I–V relationship of KV3.1b currents expressed in *Xenopus* oocytes alone and co-expressed with the γ7 subunit, which indicate that the γ7 subunit had no effect on the peak current amplitude of heterologously expressed KV3.1b channels.

**Influence of the γ7 subunit on endogenous Ca2+ currents recorded from cultured sympathetic neurones**

We investigated the electrophysiological consequence of the acute expression of γ7 upon the endogenous VDCC currents from sympathetic neurones of the rat superior cervical ganglion (SCG). These neurones express 80–90% N-type and 10% L-type current (Plummer et al., 1989; Delmas et al., 1998) and provided a vehicle in which we could determine if the γ7 subunit could exert its influence upon pre-existing Ca2+ channels. Whole-cell patch-clamp recordings, performed 36–48 h post-isolation and transfection from control sympathetic neurones transfected with the GFP marker, gave a mean IBa current density at 0 mV of −13.6 ± 2.6 pA/pF (n = 6). However, this was not significantly altered in neurones transfected with GFP and the γ7 subunit (−15.2 ± 3.5 pA/pF, n = 6) (Figure 5A).

Expression of γ7 protein in γ7-transfected neurones was verified by immunocytochemistry (Figure 5B), which also demonstrated the presence of only a very low level of immunoreactivity for endogenous γ7 in neurones transfected with GFP alone (Figure 5B). Although the morphology of sympathetic neurones cultured for 36–48 h is quite variable, in a preliminary observation, we also noted an alteration in neurite morphology in sympathetic neurones transfected with γ7 (Figure 5B), but this will require further detailed examination in a future study. It would therefore appear that the γ7 subunit is unable to acutely influence the properties of pre-existing N-type VDCCs in these neurones, and that γ7 does not co-exist endogenously in SCG neurones with N-type channels.

**Immunocytochemical analysis of the effects of the γ7 subunit**

The subcellular distribution of the expressed γ7 subunit and its effects upon the expression of CaV2.2 subunits were determined using immunocytochemistry and confocal laser scanning microscopy. When the γ7 subunit alone was transiently transfected into COS-7 cells (Figure 6A), expression of γ7 protein was observed throughout the cytoplasm and not specifically associated with the plasma membrane (delineated by Oregon Green–phalloidin). Figure 6B shows the typical fluores-
The fluorescence pattern of a cell transiently transfected with GFP–CaV2.2/β1b. Figure 6C shows two cells where the γ7 subunit has also been co-transfected. A striking reduction in the fluorescence of GFP–CaV2.2 was observed upon co-expression of the γ7 subunit, whilst the γ7 levels remained comparable with those in cells transfected with the γ7 subunit alone.

Immunocytochemical methods also confirmed the earlier electrophysiological findings that the γ7 subunit does not adversely affect the expression of the Kv3.1b subunit. Figure 6D displays the expression of a γ7-myc/his fusion protein expressed alone in a COS-7 cell. The expression pattern of this construct is similar to that of the untagged γ7 (Figure 6A). Shown in Figure 6E is the expression of Kv3.1b when transfected alone. The distribution and the expression level of Kv3.1b were unaltered by the co-expression of the γ7-myc/his fusion protein (Figure 6F).

In transient transfections, co-transfected subunits are not always co-expressed in every cell. We therefore sought to quantify the extent of the reduction in observed GFP–CaV2.2 fluorescence caused by γ7 subunit co-expression. This was measured by counting the number of GFP-positive cells in both the GFP–CaV2.2/β1b and GFP–CaV2.2/β1b/γ7 transfected cultures. The mean percentage of the total cells per 16 mm² field of view with GFP–CaV2.2 fluorescence, which entirely co-localizes with β1b expression, was reduced from 16.4 ± 3.4% in GFP–CaV2.2/β1b-transfected cells to 3.3 ± 0.7% in cells additionally transfected with the γ7 subunit (Figure 7A). The same histogram also shows that almost all cells that exhibited GFP–CaV2.2 fluorescence in the CaV2.2/β1b/γ7 transfections were also labelled for γ7 (3.0 ± 0.7%), implying that there is some residual GFP–CaV2.2 fluorescence in cells expressing the γ7 subunit. The
that the CaV2.2 protein levels II±III loop of CaV2.2 (band at ~240 kDa). When the untagged CaV2.2, the intensity of the CaV2.2 band was greatly reduced, but expression of the protein was not interfered with the correct partial synthesis protein. Similar results were obtained by western blotting where it was observed to label a major weight bands were observed that might represent degradation products of the GFP±CaV2.2 or untagged CaV2.2 expressed in COS-7 cells. (A) Western blotting and immunodetection using anti-GFP or anti-rabbit brain CaV2.2 II±III loop Ab. Lane 1, untransfected COS-7 cells; lane 2, GFP±CaV2.2; lane 3, CaV2.2 Δ3′UTR; lane 4, CaV2.2 Δ3′UTR and γ7. The positions of molecular weight markers are shown on the left. Blots are representative of three similar experiments. (B) Western blotting and immunodetection with anti-β1b Ab. Lane 1, untransfected COS-7 cells; lane 2, GFP±CaV2.2/β1b; lane 3, GFP±CaV2.2/β1b/γ7; lane 4, GFP±CaV2.2/β1b/γ7-myc/his.

**Effect of co-expression of the γ7 subunit on the CaV2.2 protein levels**

GFP–CaV2.2 or untagged CaV2.2 expressed in COS-7 cells are detectable by western blot using an Ab against the II–III loop of CaV2.2 (band at ~240 kDa). When the γ7 subunit was co-expressed with either the GFP–CaV2.2 or untagged CaV2.2, the intensity of the CaV2.2 band was greatly reduced, but expression of the protein was not completely abolished (Figure 7B). No smaller molecular weight bands were observed that might represent degradation products of the GFP–CaV2.2 or untagged CaV2.2 or partially synthesized protein. Similar results were obtained both in the absence and presence of the auxiliary β1b subunit, and also using the anti-GFP Ab (data not shown). Additionally, the co-expression of γ7 or γ7-myc/his with CaV2.2/β1b did not reduce the expression of the β1b subunit (Figure 7C). These data extend the findings from confocal imaging and electrophysiology experiments by revealing that the γ7 subunit suppresses the expression of GFP–CaV2.2 protein rather than interfering with the correct conformational folding or trafficking of the GFP-labelled protein that could result in reduced fluorescence or recorded current densities.

**Discussion**

We have described the identification of two genes that encode putative VDCC subunits γ7 and γ5, by their sequence and structural homology to the mouse stargazin gene (cacng2), and have cloned and expressed the cDNA for both human and mouse γ7. The γ7 subunit is predicted to be a 275 amino acid, 31 kDa protein possessing four transmembrane-spanning domains with intracellular N- and C-termini. The in silico sequence for γ5 also predicts a 275 amino acid, 31 kDa protein with identical transmembrane topology to γ7. Their overall size is much closer to that predicted for the skeletal muscle γ1 subunit than for the neuronal γ2, γ3 and γ4 subunits. Their sequence homology with the γ1 subunit is extremely low, but both the γ7 and γ5 subunits possess ~25% identity to the human γ2 subunit, with higher degrees of conservation occurring in the predicted transmembrane regions (up to 36%). It must also be noted that the C-termini of the γ7 and γ5 subunits differ from those of γ2, γ3 and γ4 subunits, as they are much shorter and lack the classical consensus motif for interaction with PDZ domains (Kornau et al., 1997; Tomita et al., 2001). This C-terminal motif of γ2 (also present in γ1 and γ4 subunits) has been shown to play a crucial role in trafficking AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptor subunits to their correct location in the postsynaptic density (Chen et al., 2000). This difference suggests that γ7 and γ5 may have a different function from that of the γ2, γ3 and γ4 subunits.

The γ7 and γ5 subunits are, however, 70.5% identical to one another and, unlike all other putative γ subunits, are assembled from five exons, the first four of which are identical to exons 1–4 of the genes encoding the previously predicted γ7 and γ5 subunits (Burgess et al., 1999, 2001). Sequence analysis of the BAC clones in which these genes were found suggests that the previously predicted four-exon γ7 and γ5 subunit sequences result from inclusion of intronic sequences at the exon 4–intron 4 boundary of CACNG7 and CACNG5 (Figure 1B). Inspection of the sequence of intron 4 of CACNG7 and CACNG5 reveals that in-frame stop codons are found 25 and 19 nucleotides, respectively, downstream of the exon 4–intron 4 boundary. The final eight C-terminal amino acids of the predicted four-exon γ7 and γ5 subunit sequences result from inclusion of intronic sequences at the exon 4–intron 4 boundary. The C-terminal 106 amino acids of the predicted four-exon γ6 would be encoded by the entirety of intron 4, resulting in a C-terminal γ6 sequence which has extremely low sequence homology to the other γ subunits.

The sequence of the corresponding mouse orthologues of the predicted human four-exon γ7 and γ5 can be deduced by analysis of the relevant mouse BACs. However, the stop codons within the fourth intron of each mouse gene are not in the same or even similar positions to their human equivalents, and their C-terminal regions do not retain homology to their human counterparts. In contrast, the in silico prediction of the mouse five-exon γ5 sequence exhibits 89.5% identity to that of human γ5 at the nucleic acid level and 97% identity at the protein level. Our results strongly indicate that the previously predicted four-exon γ5 and γ6 subunits are not expressed in vivo.

The γ5 and γ6 subunits represent a distinct subdivision of the γ subunit family of proteins identified by structural and sequence homology to stargazin. In addition to the lower sequence homology to the neuronal γ2, γ3 and γ4 subunits, the identification of these gene products, assembled from five exons rather than four, as in the case for the stargazin...
gene and its closer homologues, is evidence for further evolutionary diversion from a common ancestor. This conclusion was also reached in a recent genomic analysis of the γ subunit superfamily (Chu et al., 2001).

Northern blot analysis of the γ subunit mRNA revealed it to be expressed exclusively in neuronal tissue. Two probes were used in different hybridizations, one specific for full-length γ and the other, used in RT–PCR-based expression analysis in the previous study (Burgess et al., 2001), which would detect mRNAs for both full-length γ and the putative four-exon γ. Both probes hybridized to ~2.4 and ~3 kb mRNAs, with identical expression patterns in all tissues in which they are expressed. Thus the two bands cannot represent separate transcripts for the previously predicted four-exon γ and the five-exon γ described here. It would appear highly unlikely, given these data, that the four-exon γ is expressed in vivo.

Co-expression of the γ subunit almost completely abolished Ba²⁺ current through N-type Cav2.2 VDCCs expressed in both Xenopus oocytes and COS-7 mammalian expression systems. It also significantly reduced maximum Ba²⁺ conductance in the non-N type Cav2.1 and Cav1.2 VDCCs, but the effects were not so pronounced. In contrast to the effects of the neuronal γ subunit, which has been reported in a previous study to inhibit N-type Cav2.2/β3/α2δ1 currents by ~37% (Kang et al., 2001), the influence of the γ subunit is not dependent upon co-expression of an exogenous α2δ subunit as part of the Ca²⁺ channel complex.

Several mechanisms could underlie the suppression of VDCC currents by the γ subunit, including: (i) interference with the functioning of the channel at the plasma membrane; (ii) inhibition of the delivery of the channel to the plasma membrane; (iii) prevention of the correct folding of the pore-forming subunit; (iv) more rapid protein degradation; and (v) suppression of channel protein synthesis.

Confocal imaging of COS-7 cells transiently expressing N-terminally tagged GFP–Cav2.2 Ca²⁺ channel complexes revealed that co-expression of the γ subunit, which itself is highly expressed throughout the cell, resulted in an almost complete loss of GFP fluorescence. This suggested reduced synthesis or stability of GFP–Cav2.2, or interference of the γ subunit with the correct folding of the GFP-tagged protein. Western blot analysis of the levels of both GFP–Cav2.2 and untagged Cav2.2 revealed that co-expression of γ results in a much reduced steady-state level of Cav2.2. This is likely to be due to a reduction in the level of Cav2.2 protein synthesized when γ is co-expressed, because no smaller molecular weight products of degradation were evident. It nevertheless is also possible that γ induces rapid and complete targeting of Cav2.2 to a degradation pathway. Whilst northern blot analysis is required to examine whether the Cav2.2 mRNA level is reduced in the presence of γ, we have noted in the present study that the reduction in Cav2.2 protein level still occurs following the removal of its 3′-untranslated region (UTR) (Figure 7B), which has been implicated in message stability (Schorge et al., 1999).

These results raised the possibility that the γ subunit was causing a non-specific inhibition of transcription or translation. However, we have shown that the influence of γ does not cause a generalized down-regulation of all heterologously expressed ion channels, because the I–V relationship for the voltage-dependent potassium channel Kv3.1b expressed in Xenopus oocytes was unaffected by co-expression of the γ subunit. Furthermore, immunocytochemistry experiments confirmed that the expression of Kv3.1b in COS-7 cells was unaltered by the co-expression of the γ subunit and there was little effect on expression of the VDCC β1b subunit.

Our results bear a striking resemblance to the dominant-negative synthesis suppression of the Cav2.2 subunit induced by the co-expression of truncated Cav2.2 constructs (Raghib et al., 2001). In that investigation, it was proposed that the translation of full-length Cav2.2 was halted when domain I or domain I–II of Cav2.2 interacted with the initially synthesized transmembrane α-helices of the nascent full-length Cav2.2. We do not yet know if a similar mechanism might be invoked for the down-regulation of Cav2.2 by γ.

Further evidence indicates that the influence of γ expression may be upon newly synthesized Cav2.2 protein. Transient expression of the γ subunit in sympathetic neurones did not significantly alter endogenous (largely N-type) calcium current densities. The γ subunit therefore does not affect the function of pre-existing functional N-type channels.

It appears that the influence of γ on VDCC expression and function is via a mechanism unlike that reported previously for the other stargazin-like proteins. Nevertheless, in the present study, inhibition of expression by the γ subunit is apparently specific for VDCC subunits, particularly Cav2.2, because its co-expression with Kv3.1b did not inhibit the expression of the potassium channel.

That γ is actually a subunit of a functional plasma membrane calcium channel complex appears unlikely, at least for Cav2.2. This is reinforced by the absence of significant endogenous γ in sympathetic neurones, where the current is predominantly N-type. Future work will examine in which cell types γ is expressed endogenously in the brain, and whether its endogenous function is to down-regulate Cav2.2 expression.

Materials and methods

Text-based in silico cloning

The GenBank and EMBL databases were searched for sequences possessing homology to the full-length sequence of the mouse stargazin gene (Letts et al., 1998). ESTs and genomic sequences from both databases were clustered to identify overlapping identical sequence belonging to the same transcript using the GCG package (Wisconsin Package Version 9.0, Genetics Computer Group) and a program developed by GlaxoSmithKline Research and Development Bioinformatics group, termed ESTBlaster (Gill et al., 1997). Multiple sequence alignments of conceptually translated proteins were aligned using the Clustal_W algorithm (Omiga 1.1, Oxford Molecular Group, Oxford, UK).

Cloning of stargazin-like genes

An RT–PCR approach was used to amplify the complete ORFs or partial sequences of predicted γ-subunits either from cDNA derived from RNA or from overlapping partial clones. Human brain total RNA (Invitrogen, Paisley, UK) or mouse cerebellar total RNA purified from the cerebella of 31-day-old mice using the RNeasy kit (Qiagen, Crawley, UK) were used to generate cDNA using the Superscript Pre-amplification System (Invitrogen) primed with random hexamers.
Cloning mouse γ7. The γ7, 487 bp fragment predicted by in silico cloning was amplified from 25 ng of Superscript-synthesized human brain cDNA using 25 pmol each of primer pair 5′-CCGGAGAAAGT-GTCGCTG-3′ and 5′-TCATTTGTAGGACACCTGG-3′. This was then extended by 5′ and 3′ RACE protocols using Marathon Ready total human brain cDNA (BD Biosciences Clontech, Basingstoke, UK). The complete nucleotide sequence of γ7 was assembled from two of the generated RACE clones and the original 487 bp clone by a splice overlap PCR protocol using a primer pair specific for the predicted ORF. The cDNA was sequenced then subcloned into a modified pMT2 expression vector (Swick et al., 1992). Human γ7-mycHis was constructed by amplifying the complete ORF minus the stop codon from the pMT2-γ7 clone and inserting the fragment in-frame into the multiple cloning site of pC DNA3.1 myc/his A (+) (Invitrogen) using the Nof and Apal restriction sites.

Cloning mouse γ7. The nucleotide sequence of the human γ7 was compared with the HTGS subset of GenBank using BLASTn with default parameters. The complete coding sequence was found within chromosome 19 BAC ACO79557 and used to design gene-specific primers 5′-ATGAGTCACTGCAGCAGCCG-3′ and 5′-TCA-GCACGGGAAATGGGAGA-3′. The complete ORF was amplified in a single PCR from 20 ng of mouse cerebellar cDNA and cloned into pC DNA3.1 myc/his A(+). The PCR product was sequenced and subcloned into pMT2M as described (Swick et al., 1992). For expression in Xenopus oocytes/COS-7 cells or SCG neurones, respectively.

Northern blots
Human 12-lane multiple tissue blots and brain II and IV blots (BD Biosciences Clontech) were hybridized at 65°C in ExpressHyb solution (BD Biosciences Clontech) according to the manufacturer’s instructions. The 5′- and 3′-end probes corresponding to nucleotides 576–583 or 80–482 were assembled according to the Strip-EZ DNA Probe Synthesis Removal Kit [Ambion (Europe) Ltd, Huntingdon, UK]. The following cDNA clones were hybridized: rabbit CaV2.2 (D14157), N-terminal CaV2.2 (Raghib et al., 2001), rabbit CaV2.2 Δ3UTR, rabbit CaV2.2 Δ3UTR (form II, M67515), rat β1b (X61394, except R471S, V435, V449A, W492R, V511A, a gift from Dr T.P.Snutch), mouse β2Ib (AF247139, common brain splice variant), mut-3b GMP (M62653, except S72A and S65G, a gift from Dr T.E.Hughes) and rat K7.3.1b (M68880). The 3′-UTR of rabbit CaV2.2 was removed to generate CaV2.2 Δ3UTR, by introducing an Spel site immediately after the stop codon by blunt-anchored and subcloning the CaV2.2 back into the vector. All cDNAs were subcloned into expression vector pMT2 (Swick et al., 1992), with the exception of K7.3.1b that was in pRC-CMV.

Cell culture and transfections
Monkey COS-7 cells were cultured as previously described (Brice et al., 1997). Transfection was performed with Geneporter transfection reagent (Gene Therapy Systems, San Diego, CA). Cells were plated onto coverslips 2±3 h prior to transfection. The DNA and Geneporter reagent coverslips 2±3 h prior to transfection. The DNA and Geneporter reagent were mixed and applied to the cells. The α, β, γ2α and γ7 subunits were mixed and transacted in a 1:1:1:1 ratio by DNA weight. In the absence of γ2α and/or γ7 subunit, blank pMT2 vector was substituted. After 3.5 h, 1 ml of medium containing 20% serum was added to the cells, which were then incubated at 37°C for 3 days. Prior to electro-physiological recording, cells were re-plated using a non-enzymatic cell detachment solution (Sigma, Dorset, UK) and maintained at 27°C for between 2 and 6 h. Cells for immunocytochemistry were not re-plated.

Electrophysiology
Xenopus oocytes. Xenopus oocytes were prepared, injected and utilized for electrophysiology as previously described (Canti et al., 1999), with the following exception. Plasmid cDNA microinjection for CaV2.2-injected oocytes contained: 5 mM Ba(OH)2, 80 mM TEA-OH, 2 mM CsOH and 5 mM HEPES (pH 7.4 with methanesulfonic acid). The membranes were blocked with 3% bovine serum albumin (BSA) for 5 h at 55°C and then incubated overnight at 20°C with either 1.2 μg/ml of an anti-peptide Ab raised in rabbits against residues 484±861 within the II–III loop of rabbit brain CaV2.2 (Raghib et al., 2001), or a 1:1000 dilution of anti-rat β1b antisera. Secondary Ab was used at 10 and 5 μg/ml, respectively. Texas red or fluorescein isothiocyanate (FITC)-conjugated streptavidin were used at 3.33 μg/ml. In co-labelling experiments, K7.3.1b was labelled with goat anti-rabbit Texas red-conjugated secondary Ab at 10 μg/ml (Molecular Probes). In some experiments, cells were incubated for 20 min with Oregon Green–phalloidin (6.6 μM, Molecular Probes). The nuclear dye 4′,6-diamidino-2-phenylindole (DAPI; 300 nM, Molecular Probes) was also used to visualize the nucleus. Cells were examined on a confocal scanning laser microscope (Leica TCS SP, Milton Keynes, UK). All images were scanned sequentially to eliminate cross-talk, and photomultiplier settings were kept constant in each experiment.

Western blotting
COS-7 cells were processed as previously described (Raghib et al., 2001). Samples (50 μg of total protein/lane in Figure 7B and 10 μg of total protein/lane in Figure 7C) were separated by SDS–PAGE using 4–20% gradient gels and then transferred electrophoretically to polyvinylidene difluoride membranes. The membranes were blocked with 3% bovine serum albumin (BSA) for 5 h at 55°C and then incubated overnight at 20°C with either 1.2 μg/ml of an anti-peptide Ab raised in rabbits against residues 484±861 within the II–III loop of rabbit brain CaV2.2 (Raghib et al., 2001), or a 1:1000 dilution of anti-rat β1b antisera. Secondary Ab was used at 1:1000 dilution of goat anti-rabbit IgG–horseadish peroxidase conjugate) was added and the membranes incubated for 1 h. Following extensive washing, bound Abs were detected using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Little Chalfont, UK).

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