

Voltage-gated calcium channel subunits from platyhelminths: potential role in praziquantel
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Abstract

Voltage-gated calcium (Ca^{2+}) channels provide the pathway for Ca^{2+} influxes that underlie Ca^{2+} -dependent responses in muscles, nerves, and other excitable cells. They are also targets of a wide variety of drugs and toxins. Ca^{2+} channels are multisubunit protein complexes consisting of a pore-forming α_1 subunit and other modulatory subunits, including the β subunit. Here, we review the structure and function of schistosome Ca^{2+} channel subunits, with particular emphasis on variant Ca^{2+} channel β subunits ($\text{Ca}_v\beta\text{var}$) found in these parasites. In particular, we examine the role these β subunits may play in the action of praziquantel, the current drug of choice against schistosomiasis. We also present evidence that $\text{Ca}_v\beta\text{var}$ homologs are found in other praziquantel-sensitive platyhelminths such as the pork tapeworm, *Taenia solium*, and that these variant β subunits may thus represent a platyhelminth-specific gene family.

Keywords:

Calcium channels; praziquantel; *Schistosoma mansoni*; *Taenia solium*; Platyhelminthes

1. Introduction

Diseases caused by platyhelminthic parasites represent a worldwide health crisis. Schistosomiasis, caused by trematode flatworms of the genus *Schistosoma*, affects approximately 200 million people, killing as many as 280,000 people per year (van der Werf et al., 2003), with a large percentage of infected individuals residing in Africa (Engels et al., 2002; Chitsulo et al., 2004). Cestodes of the genus *Taenia* and other platyhelminths infect millions more. The drug of choice against platyhelminthic parasites is praziquantel, a compound that is generally ineffective against parasites from other phyla. The platyhelminth-specific efficacy of praziquantel remains unexplained, as its mode of action at the cellular and molecular level is not yet fully understood. Recent evidence suggests that voltage-gated Ca^{2+} channels (VGCCs) in schistosomes possess a subunit with an unusual structure that may underlie their sensitivity to praziquantel. Here we will focus on the structure and function of VGCCs in platyhelminths and present new data demonstrating that the variant VGCC form found in schistosomes is shared by other classes of platyhelminths.

2. Voltage-gated Ca^{2+} channels

VGCCs belong to the superfamily of voltage-gated ion channels that respond to changes in membrane potential by selectively allowing ions to flow down their electrochemical gradient. VGCCs play a unique role within this family by coupling changes in membrane potential to the influx of Ca^{2+} necessary to elicit intracellular signaling (reviewed by Catterall, 2000; Hofmann *et al.*, 1999). In addition to propagating impulses, VGCCs gate the Ca^{2+} influx that underlies excitation-contraction coupling, excitation-secretion coupling, and other Ca^{2+} -dependent processes. The importance of VGCCs to cellular function is underscored by their role as targets

for both therapeutic drugs and components of venoms used by a wide variety of predators.

VGCCs comprise a pore-forming α_1 subunit and smaller auxiliary subunits known as β , $\alpha_2\delta$, and γ (Fig. 1). Although the α_1 subunit largely determines both the pharmacology of the channel and the nature of the current gated by it, the auxiliary subunits play important roles in shaping the response of the channel. The α_1 subunit contains four homologous domains, connected by intracellular loops, that form the sides of the membrane pore (Doyle et al., 1998). Each domain includes six transmembrane segments (labeled S1-S6), with the S4 segment containing positively charged residues that act as a voltage sensor and the extracellular P-loop between S5 and S6 determining the ion selectivity of the channel.

The currents gated by VGCCs are divided into high voltage-activated (HVA) and low voltage-activated (LVA) currents, defined by the membrane potential at which the channels open. HVA currents have been further divided on the basis of pharmacological response. L-type HVA currents are sensitive to classical organic Ca^{2+} channel blockers such as nifedipine, verapamil, and diltiazem. Non-L-type HVA currents include N-type, P/Q-type, and R-type currents, each insensitive to the L-type channel blockers, but distinguished by selective sensitivity to particular toxins.

Physiological and pharmacological distinctions between VGCCs are mirrored in the molecular variation among α_1 subunits. Phylogenetic analysis divides α_1 subunits into three clusters known as Ca_v1 , Ca_v2 , and Ca_v3 , which correspond to L-type, non-L-type, and LVA channels, respectively (Catterall et al., 2005). Although mammals and other vertebrates possess three or four subtypes of each channel type differing in tissue and subcellular distribution, only single genes for each type are found in the invertebrate species that have been studied. For example, thorough analysis of the fully sequenced genomes of *Caenorhabditis elegans* and

Drosophila melanogaster has revealed one homolog each of Ca_v1, Ca_v2, and Ca_v3 α_1 subunits, each of which presumably fulfills the roles taken by multiple gene products in mammalian tissues.

A further source of variation in VGCC currents is the influence of auxiliary subunits, of which the β subunit has been most extensively studied (reviewed by Walker & DeWaard, 1998; Birnbaumer et al., 1998; Hanlon & Wallace, 2002; Dolphin, 2003; Richards et al., 2004). The β subunit is a cytoplasmic protein that has four subtypes in vertebrates, but only single homologs in most invertebrate species. The β subunit increases current density and ligand binding to the α_1 subunit, in part by masking an endoplasmic reticulum retention signal on the α_1 subunit, enhancing its trafficking to the plasma membrane. β subunits also modulate various kinetic properties of the channel, such as the voltage-dependence of activation and inactivation, the rate of inactivation, and recovery from inactivation.

The primary site on the α_1 subunit for interaction with the β subunit is a region of the intracellular loop between domains I and II labeled the alpha interaction domain (AID). Early studies described a similarly conserved site in the β subunit, the 30-residue β interaction domain (BID; DeWaard *et al.*, 1994). Subsequent work has resolved the crystal structure of the conserved core of the β subunit, both alone and complexed with the AID of the α_1 subunit (Chen et al., 2004; Opatowski et al., 2004; Petegem et al., 2004; commentary by Yue, 2004). These studies have verified the prediction (Hanlon et al., 1999) that Ca_v β s are members of the membrane-associated guanylate kinase (MAGUK) family of proteins. MAGUKs are often concentrated at synapses and are thought to play important roles in clustering ion channels and neurotransmitter receptors (reviewed by Dimitratos et al., 1999). MAGUK proteins typically comprise one or more PDZ domains (Ponting et al., 1997) located N-terminally to a Src-

homology 3 (SH3) domain, a bridging region (the HOOK domain), and a guanylate kinase (GK)-like domain. $\text{Ca}_v\beta$ s differ from other members of the MAGUK family in important respects, lacking the N-terminal PDZ domains and a nucleotide binding site within the GK domain.

Although the newly revealed crystal structure of β subunits indicates that the AID of the α_1 subunit binds not to the BID, but within the modified GK domain, the BID plays an essential structural role in β subunits (Chen et al., 2004). It spans the SH3 and GK domains and the connecting HOOK region, contains two β -strands that are integral parts of the SH3 and GK domains, and may be necessary for proper folding of the β subunit.

Most of the heterologous characterization of α_1 and β subunits has been conducted with genes cloned from mammalian species. Mammalian subunits were the first cloned, and many invertebrate homologs that were subsequently identified were not as amenable to expression in heterologous expression systems. Until molecular biological approaches began to be applied in recent years to studying helminthic Ca^{2+} currents, their direct characterization depended upon electrophysiological recordings, which are technically difficult in flatworms. For example, although Ca^{2+} influx has been implicated in contractility, no native Ca^{2+} currents from schistosome cells have yet been published, though a preliminary report has indicated that these currents can be found in muscle fibers (Cobbett and Day, 2002), along with previously described K^+ currents (Day et al., 1993). Some information on native Ca^{2+} currents has been gained from experiments in other platyhelminths, but pharmacological characterization has proved challenging, which is one reason why the molecular target of praziquantel (see below) remains undefined (for review see Greenberg, 2005).

3. Voltage-gated Ca^{2+} channel subunits in schistosomes

Molecular cloning approaches have now begun to clarify the structure and functional properties of schistosome VGCC subunits (Kohn et al., 2001a, b; reviewed by Greenberg, 2005). Three full-length HVA α_1 subunits have been cloned from adult *S. mansoni*. Although none has yet been heterologously expressed, two can be classified as non-L-type α_1 subunits and one as L-type based on sequence similarity to mammalian proteins (Kohn et al., 2001b). The presence of two non L-type α_1 subunits in schistosomes is unusual. In other invertebrates that have been examined, including several complete invertebrate genomes, only single L-type and non L-type α_1 subunits are found (Littleton and Ganetzky, 2000; Jeziorski et al., 2000).

In addition to the α_1 subunits, two subtypes of $\text{Ca}_v\beta$ s have been cloned from schistosomes (Kohn et al., 2001a; 2003a). Again, this finding in itself is noteworthy, in that only one $\text{Ca}_v\beta$ subtype has been identified in the genomes of other invertebrates. One of the schistosome $\text{Ca}_v\beta$ s resembles other invertebrate $\text{Ca}_v\beta$ s in its size and pattern of conserved regions. The other, a variant subtype which we have named $\text{Ca}_v\beta\text{var}$, displays clear similarity to the family of $\text{Ca}_v\beta$ subunits, but differs in several fundamental ways. The predicted protein is significantly larger than conventional $\text{Ca}_v\beta$ s, and similarity at the amino acid level in conserved regions is markedly reduced, suggesting a separate gene family. Although $\text{Ca}_v\beta\text{var}$ contains a domain similar to the BID of conventional β subunits, it lacks two highly conserved serine residues (see Table 1). These conserved serines each constitute consensus protein kinase C (PKC) phosphorylation sites. The $\text{Ca}_v\beta\text{var}$ subunit was simultaneously cloned from two species of schistosomes, *S. mansoni* ($\text{SmCa}_v\beta\text{var}$, formerly called $\text{SmCa}_v\beta\text{A}$) and *S. japonicum* ($\text{SjCa}_v\beta\text{var}$, formerly $\text{SjCa}_v\beta$), but no homologs of the variant subtype could be identified by similarity searches in any vertebrate or other invertebrate genome.

Studies using heterologous expression revealed that schistosome $\text{Ca}_v\beta_{\text{var}}$ possesses functional properties that are not shared with other $\text{Ca}_v\beta$ s (Kohn et al., 2001a). The $\text{Ca}_v\beta_{\text{var}}$ subunit associates with α_1 subunits when expressed in *Xenopus* oocytes, as it causes the current/voltage relationship of α_1 subunits to shift in a hyperpolarizing direction much as conventional β subunits do. However, all conventional β subunits studied to date also increase the amplitude of the current gated by α_1 subunits. In contrast, coexpression of schistosome $\text{Ca}_v\beta_{\text{var}}$ subunits with either a jellyfish (*CyCa_v1*) or human (*Ca_v2.3*) α_1 subunit markedly reduces current through the channel.

Another intriguing aspect of the schistosome $\text{Ca}_v\beta_{\text{var}}$ subunit is its ability to confer praziquantel sensitivity to α_1 subunits. Praziquantel is the drug of choice to treat schistosomiasis, due to its efficacy against all schistosome species and relatively benign side effect profile (Andrews et al., 1983; Fenwick et al., 2003; Hagan et al., 2004). Although praziquantel acts against a wide variety of platyhelminths, it is generally ineffective against other organisms such as nematodes (Andrews et al., 1985). The molecular target of praziquantel has not been defined, but it is known to induce rapid influx of Ca^{2+} , leading to a rapid, sustained Ca^{2+} -dependent muscular contraction (for reviews see Andrews, 1985; Day et al., 1992; Redman et al., 1996; Cioli and Pica-Mattoccia, 2003; Greenberg, 2005).

Co-expression of the schistosome $\text{Ca}_v\beta_{\text{var}}$ subunit with a praziquantel-insensitive mammalian α_1 subunit (*Ca_v2.3*) yields a current that is responsive to praziquantel. Peak Ca^{2+} currents are increased in the presence of 100 nM praziquantel in *Xenopus* oocytes coexpressing the two subunits, but not with either subunit alone (Kohn et al., 2001a). The praziquantel-dependent increase in Ca^{2+} current amplitude is consistent with the reported ability of the drug to induce Ca^{2+} influx. Other $\text{Ca}_v\beta$ s, including the conventional schistosome β subunit, do not confer

praziquantel sensitivity to the mammalian α_1 subunit (Kohn et al., 2003b).

Site-directed mutagenesis has revealed that the absence of the two consensus PKC sites in the BID region of $\text{Ca}_v\beta_{\text{var}}$ subunits is responsible in part for both the current reduction and the ability to confer praziquantel sensitivity (Kohn et al., 2003a, b). Restoring the consensus PKC sites in $\text{Ca}_v\beta_{\text{var}}$ by substituting serine residues at either or both of these sites restores the ability of the subunit to enhance currents through α_1 subunits like other $\text{Ca}_v\beta$ s, while sensitivity to praziquantel is eliminated. When one such mutant containing a single serine is further altered to eliminate a basic residue and disrupt the consensus PKC site, the phenotype reverts to that of the wildtype schistosome $\text{Ca}_v\beta_{\text{var}}$. Thus, the novel effects of the variant subunit result from a lack of PKC sites in the BID rather than the absence of serines. The importance of PKC sites within the BID of $\text{Ca}_v\beta_{\text{var}}$ is consistent with the finding that phosphorylation of VGCC subunits is a critical regulator of channel properties (reviewed by Rossie, 1999; Kamp and Hell, 2000; Keef et al., 2001). Although recombinant mammalian β subunits can be phosphorylated by PKC *in vitro* (Puri et al., 1997), the specific sites of phosphorylation have not yet been determined.

In recent years, several reports of schistosome isolates with reduced susceptibility to praziquantel have appeared (reviewed by Cioli et al., 1993; Day and Botros, 2006; Brindley, 1994; Geerts and Gryseels, 2001). The finding that a single-base mutation at a critical site can eliminate the ability of $\text{Ca}_v\beta_{\text{var}}$ to confer praziquantel sensitivity might suggest such a mechanism for acquiring praziquantel resistance. *S. mansoni* strains reported to have reduced praziquantel sensitivity show no changes in primary structure or expression levels of schistosome β subunits (Valle et al. 2003). However, Egyptian isolates with reduced praziquantel susceptibility show differential stability and reproductive fitness, which may indicate that there are several pathways for acquiring reduced sensitivity to praziquantel (William et al., 2001b),

and schistosomes may make use of more than one mechanism.

If the variant β subunit found in schistosomes is in fact an essential target of praziquantel, the $\text{Ca}_v\beta\text{var}$ gene should be found in other organisms that exhibit sensitivity to the drug. An important target of praziquantel is *Taenia solium*, the pork tapeworm, which represents a significant health problem in developing countries (Carpio et al., 2002; Flisser et al., 2003; Garcia et al., 2003). Praziquantel is effective in treating both the intermediate porcine host and humans infected with either cysticerci or adult *T. solium* (Andrews et al., 1983; Cioli and Pica-Mattoccia, 2003; Garcia et al., 2003). As in schistosomes, the precise manner in which praziquantel acts upon *Taenia* has not been extensively studied.

4. Variant $\text{Ca}_v\beta$ s in *Taenia solium* and other platyhelminths.

We used reverse transcription of RNA plus PCR (RT-PCR) to search for β subunit homologs in *T. solium* cysticerci obtained from muscle tissue of an infected pig (Veterinary School, Universidad Nacional Autónoma de México). Using degenerate primers designed against conserved regions of β subunit genes, we amplified fragments of two distinct β subunit cDNAs from *T. solium*, and the full cDNAs were then isolated via standard cloning techniques and sequenced.

One cDNA, named $\text{TsCa}_v\beta$, is 2249 bp long and encodes a protein of 640 amino acids with a predicted molecular weight of 72.5 kDa that resembles the conventional β subunits found in *S. mansoni* and in many other invertebrate and vertebrate organisms (Fig. 2A). The second cDNA, $\text{TsCa}_v\beta\text{var}$, more closely resembles $\text{SmCa}_v\beta\text{var}$ and $\text{SjCa}_v\beta\text{var}$, the variant β subunits identified only in schistosomes to date. The cloned $\text{TsCa}_v\beta\text{var}$ cDNA is 2772 bp in length and contains an open reading frame encoding a protein of 809 amino acids with a predicted

molecular weight of 86.6 kDa (Fig. 2B). Two spliced forms of the TsCa_vβvar cDNA were isolated during the process of cloning. One lacks a cassette of 18 amino acids between residues 684-701 within an unconserved region near the carboxyl terminus. A second cDNA lacks the 133 amino acids between residues 391-523, including a segment that is moderately conserved with the schistosome subunits. Although neither of the two alternate cDNAs is a product of mispriming during PCR, it is still unclear whether they represent splicing artifacts or functionally relevant mRNAs.

Phylogenetic analysis reveals that the conventional and variant β subunits form two subfamilies that are related but separate (Fig. 3). As described above, an important structural distinction between the two types of subunits resides within the BID, in which variant β subunits lack two conserved PKC sites. TsCa_vβ differs from other conventional β subunits by containing threonine in place of the first serine, but threonine can also be phosphorylated by PKC (Woodgett et al., 1986). In both SmCa_vβvar and SjCa_vβvar, the two serines are replaced by cysteine and alanine. Within the TsCa_vβvar sequence the serines are replaced by asparagine and alanine (Table 1), which is consistent with the BIDs of the variant schistosome subunits. As in SmCa_vβvar and SjCa_vβvar, the remainder of the BID is conserved with respect to conventional β subunits.

We investigated whether variant β subunits are found in other classes of platyhelminths by screening two turbellarians for β subunit BID regions. Adapter-ligated, amplified cDNA pools (Matz, 2002) were generated from *Dugesia dorotocephala* (Carolina Biological Supply) and *Bdelloura candida* (collected from the legs and gills of *Limulus polyphemus* maintained at the Whitney Laboratory, University of Florida). cDNA fragments amplified as described in Fig. 2 from each species encoded a BID lacking the two serine residues. As in the variant schistosome

subunits, the turbellarian sequences incorporate cystine and alanine residues in the corresponding positions (Table 1). A second fragment resembling the BIDs of conventional β subunits was also amplified from *B. candida*, indicating that both forms of the β subunit are expressed in three different classes of platyhelminths.

These findings demonstrate that a homolog of the variant β subunits identified in the trematodes *S. mansoni* and *S. japonicum* is also expressed in the cestode *T. solium*, as well as in two species of turbellarians. Although it remains to be determined whether a homologous protein is found in all platyhelminths, our results establish a stronger correlation between the presence of the β var subtype and sensitivity to praziquantel. As noted above, the β var isoform may be unique to platyhelminths, as no sequence resembling that of variant $\text{Ca}_v\beta_s$ has yet been found outside of the phylum. For example, only a single, conventional β subtype has been found in the mollusc *Lymnaea stagnalis* (Spafford et al., 2003). Furthermore, a thorough BLAST search for β subunit homologues within the fully sequenced genomes of *C. elegans*, *D. melanogaster*, and *Ciona intestinalis* detects only a single conventional β subunit in each. A distant homolog of known β subunits is present in some genomes (e.g. the w10c8.1 gene in *C. elegans*), but the translated sequence deviates significantly from both β subunits described here, and the *C. elegans* protein appears not to modulate α_1 subunits when expressed in *Xenopus* oocytes (M.C. Jeziorski, unpublished observations).

5. Conclusions

The results presented here provide further evidence for the hypothesis that a variant β subunit of VGCCs is the clinically relevant target of praziquantel in schistosomes and other platyhelminths. Heterologous expression has demonstrated that the $\text{Ca}_v\beta$ var subunit can

associate with an α_1 subunit, induce alterations in channel kinetics and current amplitude, and confer sensitivity to praziquantel in a normally insensitive subunit. The increase in Ca^{2+} current in such channels in the presence of praziquantel is consistent with previously described physiological effects of the drug. Conserved PKC sites that exist in all known conventional $\text{Ca}_v\beta$ subunits, but in no $\text{Ca}_v\beta\text{var}$ subunits, account for the differential responses of the two families of subunits to praziquantel. We now show that the $\text{Ca}_v\beta\text{var}$ subunit is not limited to schistosomes, but is also found in *Taenia solium*, an important clinical target of praziquantel, as well as two species from a third class of platyhelminths. To date, the presence or absence of the $\text{Ca}_v\beta\text{var}$ subunit in a given organism is consistently correlated with the presence or absence of sensitivity to praziquantel.

Nevertheless, several key questions remain before the $\text{Ca}_v\beta\text{var}$ subunit can be definitively established as the essential target of praziquantel. Due to technical obstacles in expressing cloned schistosome α_1 subunits, the effects of the $\text{Ca}_v\beta\text{var}$ subunit have been studied only upon non-helminthic channels. We do not yet know whether $\text{Ca}_v\beta\text{var}$ associates with one or all of the α_1 subunits present in schistosomes, or whether the association is limited to L-type, non-L-type, or as yet undescribed T-type (LVA) channels. Furthermore, it is not clear whether $\text{Ca}_v\beta\text{var}$ competes with the conventional $\text{Ca}_v\beta$ subunit, or whether the two have complementary or exclusive roles in modulating trafficking, kinetic response, or pharmacology of the α_1 subunit. $\text{Ca}_v\beta\text{var}$ may associate with multiple α_1 subunits, but induce praziquantel sensitivity in only a subset. In addition, the precise mechanism of praziquantel interaction with Ca^{2+} channel subunits remains unresolved. Does it interact directly with the β subunit, interfere with α_1/β interaction, or act indirectly on Ca^{2+} channels containing these variant $\text{Ca}_v\beta$ s?

Beyond the questions related to praziquantel sensitivity are the biological roles these variant $\text{Ca}_v\beta$ s play in platyhelminths. The growing evidence that the $\text{Ca}_v\beta_{\text{var}}$ subunit is a platyhelminth-specific family of β subunits raises intriguing issues about their importance to helminth physiology. Unlike other β subunits, the variant β subunits reduce Ca^{2+} currents in oocytes instead of increasing them. If this response is true of native schistosome currents, what is the importance of this unusual type of modulation? Are these β subunits interacting with other receptors or channels? Genomic, post-genomic, and physiological studies may allow some of these questions to be addressed directly.

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FIGURE LEGENDS

Figure 1. Simplified structure of a vertebrate voltage-gated Ca^{2+} channel. Subunits include the pore-forming α_1 subunit, which consists of four homologous domains surrounding a central pore. Each domain comprises six transmembrane regions (S1-S6). The P loop which dips into the membrane between S5 and S6 forms the selectivity filter of the channel. The S4 transmembrane region contains a series of regularly spaced, positively charged residues (+) and is thought to form the voltage sensor of the channel. Also shown are auxiliary β , $\alpha_2\delta$, and γ subunits, which modulate α_1 function. β and $\alpha_2\delta$ subunit homologs have been identified in schistosomes. Domains of the β subunit defined by recent homology modeling and high resolution structural analysis (see text) are shown. The β subunit interacts via its guanylate kinase (GK) domain with the Alpha Interaction Domain (AID) on the I-II loop of the α_1 subunit. Size of subunits and domains are not to scale. Adapted from elements of Randall and Benham (1999) and Yue (2004).

Figure 2. A. Alignment of the conventional *T. solium* β subunit TsCa ν β with the *S. mansoni* subunit SmCa ν β . **B.** Alignment of the variant *T. solium* β subunit TsCa ν β var with the *S. mansoni* subunit SmCa ν β var. Residues that are identical between sequences are shaded. The position of the β interaction domain is indicated in each figure, and the locations of two deletions identified in certain TsCa ν β var clones are indicated by dashed lines. The alignments were completed using Clustal W 1.82. To clone the two *T. solium* cDNAs, RNA was extracted from *T. solium* cysticerci with TRIzol and reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen) and an oligo-dT primer. Touchdown PCR with the degenerate oligonucleotides AAYAAYGAYGGTGGAT and GCYTTYTGATCATRTC [94° for 30 sec, 49° for 40 sec (minus 0.5°/cycle), 72° for 30 sec for 22 cycles, followed by an additional 30 cycles using a 42° annealing temperature] generated two distinct cDNA fragments. The amplified products were

cloned into the pTOPO II vector (Invitrogen) and sequenced on an automated sequencer. The cDNA ends were amplified using 3'RACE and 5'RACE (Frohman et al., 1988), and the full coding region of each cDNA was determined. For each of the two subunits, multiple cDNAs were cloned from separate sources and sequenced to establish a consensus sequence. The GenBank accession numbers are [AY624029](#) for TsCa_vβ, [AY624030](#) for TsCa_vβvar, [AAK51118](#) for SmCa_vβB, and [AAK51117](#) for SmCa_vβvar.

Figure 3. Phylogenetic relationships among conventional and variant Ca²⁺ channel β subunits, including the distant *C. elegans* β subunit homolog w10c8.1. The tree was constructed using the neighbor-joining method as implemented in MEGA 3.1 (Kumar et al., 2004). Bootstrap values from 100 replicates are shown. The GenBank accession numbers for the sequences are as follows: *S. japonicum* βvar, [AY033597](#); human β1, [NP_954856](#); rabbit β1, [AAA31180](#); human β2, [AAD33730](#); rabbit β2, [P54288](#); human β3, [AAA19799](#); rabbit β3, [CAA45578](#); human β4, [AAB53333](#); rat β4, [A45982](#); *Xenopus* β3, [AAA75519](#); *Fugu* β3, [AAB96361](#); *Caenorhabditis elegans*, [AAB53056](#); *Musca*, [A54844](#); *Drosophila*, [AAF21096](#); *Loligo*, [BAB88219](#); *Lymnaea*, [AAO83844](#); *Cyanea*, [AAB87751](#); and *C. elegans* w10c8.1, [AAK21500](#).

Figure1
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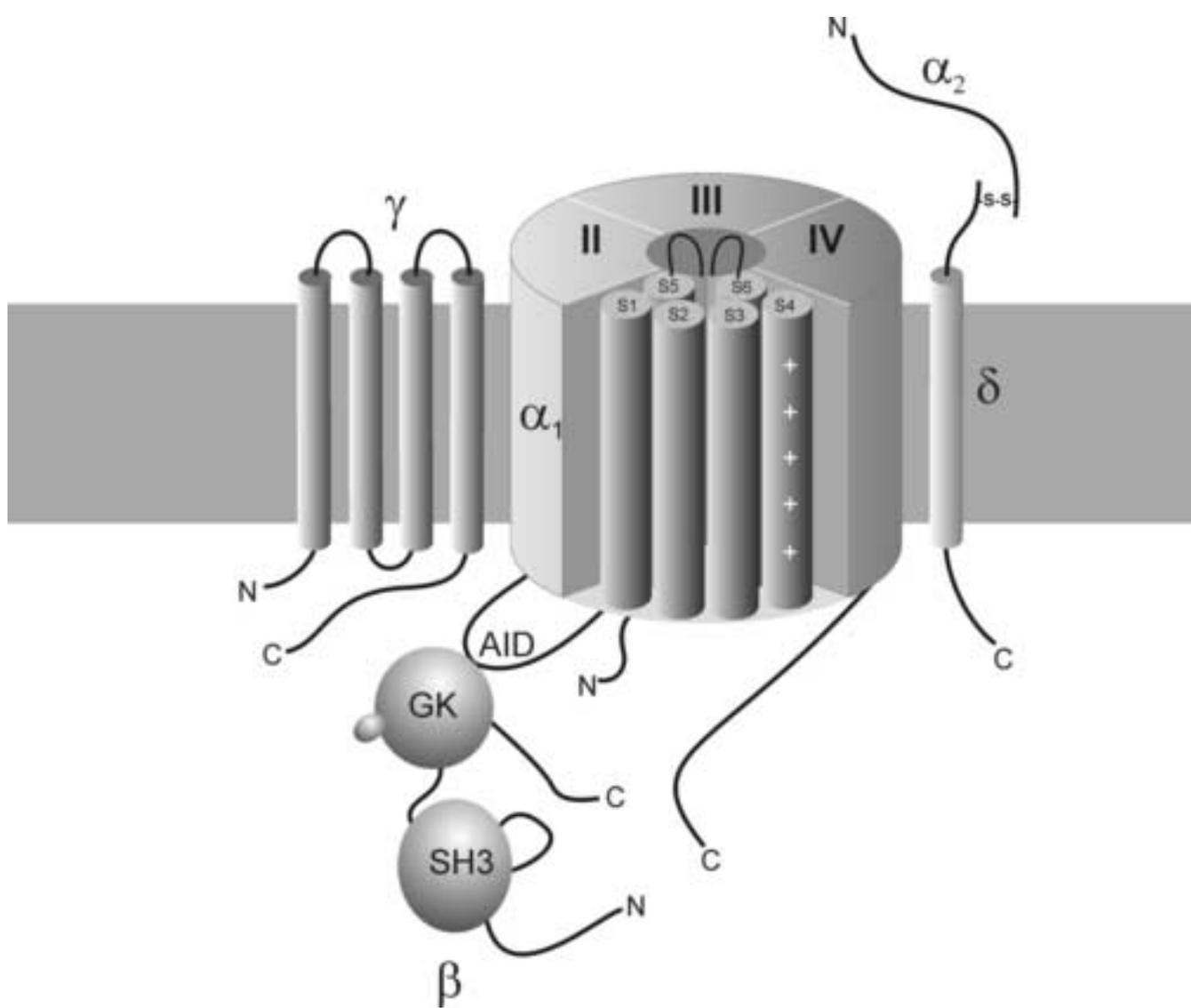


Figure 3

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