Ca^{2+} signaling, voltage-gated Ca^{2+} channels, and praziquantel in flatworm neuromusculature

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SUMMARY

Transient changes in calcium (Ca^{2+}) levels regulate a wide variety of cellular processes, and cells employ both intracellular and extracellular sources of Ca^{2+} for signaling. Praziquantel, the drug of choice against schistosomiasis, disrupts Ca^{2+} homeostasis in adult worms. This review will focus on voltage-gated Ca^{2+} channels, which regulate levels of intracellular Ca^{2+} by coupling membrane depolarization to entry of extracellular Ca^{2+}. Ca^{2+} channels are members of the ion channel superfamily and represent essential components of neurons, muscles, and other excitable cells. Ca^{2+} channels are membrane protein complexes in which the pore-forming α_1 subunit is modulated by auxiliary subunits such as β and $\alpha_2\delta$. Schistosomes express two Ca^{2+} channel β subunit subtypes: a conventional subtype similar to β subunits found in other vertebrates and invertebrates; and a novel variant subtype with unusual structural and functional properties. The variant schistosome β subunit confers praziquantel sensitivity to an otherwise praziquantel-insensitive mammalian Ca^{2+} channel, implicating it as a mediator of praziquantel action.

The causative agents of schistosomiasis are trematode flatworms of the genus *Schistosoma*. Approximately 200 million people worldwide are thought to be infected, most of whom live in Africa (Engels *et al.*, 2002), with as many as 280,000 deaths per year attributed to the disease (van der Werf *et al.*, 2003). The current treatment of choice against schistosomiasis is praziquantel. Praziquantel is known to affect calcium (Ca^{2+}) homeostasis in adult schistosomes, though the precise molecular target of the the drug is not known. This review will discuss Ca^{2+} signaling in schistosomes and other flatworms, focusing on the structure and function of voltage-gated Ca^{2+} channels and recent information regarding the role Ca^{2+} channel subunits appear to play in praziquantel action.

CALCIUM SIGNALING

Ca^{2+} is an essential and versatile intracellular messenger. Normally low (submicromolar) levels of Ca^{2+} within the cytoplasm are interrupted by Ca^{2+} pulses that trigger Ca^{2+}-dependent responses. Indeed, it can be argued that virtually all reactions in excitable cells are regulated either directly or indirectly by Ca^{2+} (Augustine, Santamaria & Tanaka, 2003). The time scale over which Ca^{2+}-dependent regulation operates ranges from microseconds to hours, and Ca^{2+} signals are often highly localized (reviewed by Bootman, Lipp & Berridge, 2001; Augustine, Santamaria & Tanaka, 2003). Cells have developed exquisitely tuned components for temporal and spatial regulation of free Ca^{2+} levels in the cytoplasm (reviewed by Berridge, Bootman & Roderick, 2003). These mechanisms comprise a "Ca^{2+}-signaling toolkit" consisting of a large and diverse collection of signaling units that, alone or in combination with one another, distribute Ca^{2+} signals with varying temporal and spatial properties. These signaling units include receptors, channels, pumps and exchangers, Ca^{2+} buffers, Ca^{2+}-binding proteins, and Ca^{2+}-sensitive enzymes and processes (see Fig.1).

The primary pathways for transient increases in intracellular Ca^{2+} are via entry of

extracellular Ca^{2+} or by release of intracellular stores of Ca^{2+}. Major intracellular stores of Ca^{2+} are found in the endoplasmic reticulum (ER) and, in muscle cells, the sarcoplasmic reticulum (SR). Release of Ca^{2+} from these stores is mediated by inositol-1,4,5-triphosphate (IP3) receptors and by ryanodine receptors, calcium release channels encoded by two distantly-related gene families. Several ligands and secondary messengers, including Ca^{2+} itself, also influence Ca^{2+} release. Entry of Ca^{2+} from the external medium can be mediated by several components, including voltage- and receptor-gated channels, transient receptor potential (TRP) channels, and second messenger-gated channels (*eg*, cyclic nucleotide-gated channels). One of the major gateways for entry of extracellular Ca^{2+} is through voltage-gated Ca^{2+} channels. This review will concentrate primarily on these channels, which, in addition to contributing to impulse propagation, couple membrane depolarization to rapid influxes of Ca^{2+} that can regulate fast Ca^{2+}-dependent cellular responses.

CALCIUM SIGNALING IN FLATWORMS

Several components that are essential for maintenance of Ca^{2+} homeostasis have been described in schistosomes and other platyhelminths (reviewed by Noel *et al*., 2001), representing a subset of the cellular factors likely responsible for modulating intracellular Ca^{2+} levels in worms (see Fig. 1 in Redman *et al*., 1996). For example, sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCAs), intracellular ATP-powered pumps that sequester Ca^{2+} into the SR, have been identified in schistosomes. Two *S. mansoni* SERCAs can be distingushed physiologically (Cunha, Reis & Noël, 1996) and two different *S. mansoni* SERCA-like cDNAs have been reported (deMendonça *et al*., 1995). Expression of one of these SERCA cDNAs, SMA2, rescues SERCA-deficient yeast, and is associated with an internal-membrane-associated, Ca^{2+}-dependent ATPase activity with kinetic properties and a pharmacological profile similar to mammalian SERCA isoforms (Talla *et al*., 1998).

Ryanodine receptors are gated by a ligand (Ca^{2+}) and/or by mechanical coupling with voltage-gated Ca^{2+} channels, and are found in the ER of noncontractile tissues as well as in the SR of muscle cells (reviewed by Shoshan-Barmatz & Ashley, 1997; Rossi & Sorrentino, 2002). [^{3}H]ryanodine binding (Silva *et al*., 1997) and pharmacological experiments on dissociated muscle fibers (Day *et al*., 2000) provide evidence for ryanodine receptors in schistosomes and other flatworms, and ESTs coding for homologs of this channel are found in the transcriptomes of both *S. mansoni* and *S. japonicum*.

Several receptors and channels on the plasma membranes of schistosomes likely also modulate levels of intracellular Ca^{2+}. For example, both FMRFamide-related peptides (Day *et al*., 1994) and serotonin (Day, Bennett & Pax, 1994) elicit Ca^{2+}-dependent contraction in dissociated muscle fibers from *S. mansoni*. Recently, Agboh *et al*. (2004) have cloned and expressed an ATP-gated P2X receptor from *S. mansoni* that has high permeability to Ca^{2+} and may play an important role in Ca^{2+}-dependent processes such as neurotransmission and muscle contraction. In addition, several Ca^{2+}-binding proteins from schistosomes that have been described may serve to buffer or sequester Ca^{2+} as well as acting as targets of Ca^{2+} regulation. Information gleaned from the schistosome genome and transcriptome projects should over the next several years provide material for post-genomic studies of the cellular components that participate in regulation of Ca^{2+} homeostasis.

PRAZIQUANTEL

Praziquantel (Fig. 2), discovered in the 1970s, was subsequently introduced for the treatment of schistosomiasis (reviewed by Andrews *et al*., 1983). It is a pyrazinoisoquinoline with an asymmetric center, and standard preparations are composed of equal proportions of the active, levo (–) and the inactive, dextro (+) optical isomers. The activity of the (–) enantiomer has been established in experiments performed both *in vivo* (Andrews *et al.*,

1983; Liu et al., 1986; Tanaka *et al.*, 1989; Wu *et al.*, 1991; Xiao *et al.*, 1999) and *in vitro* (Staudt *et al.*, 1992; Xiao & Catto, 1989).

Praziquantel has activity against all species of schistosomes and shows minimal side effects. As a consequence, it has become the drug of choice against schistosomiasis. Indeed, with the added benefit of dramatic reductions in price, praziquantel has in essence become the sole antischistosomal agent that is available commercially (Fenwick *et al.*, 2003; Hagan *et al.*, 2004). Praziquantel is also active against other trematode and cestode infections, though generally not against nematodes (reviewed by Andrews, 1985), and schistosomes show stage- and sex-dependent differences in praziquantel sensitivity (Xiao, Catto & Webster, 1985; Sabah *et al.*, 1986; Pica-Mattoccia & Cioli, 2004).

Praziquantel effectiveness has been proven repeatedly in large-scale schistosomiasis control efforts in different regions. However, the drug has been severely underutilized in sub-Saharan Africa (reviewed by Fenwick *et al.*, 2003; Hagan *et al.*, 2004). The recently inaugurated Schistosomiasis Control Initiative (www.schisto.org) was launched as a response to this problem, and has as its goal the establishment of sustainable schistosomiasis control programs in this neglected region.

Since praziquantel serves in effect as the only antischistosomal treatment in widespread use, the possibility of emerging drug resistance is troubling. Others (see, eg, Cioli, 2000; Doenhoff *et al.*, 2002) have discussed this problem in detail. It is clear, however, that the potential for emerging resistance to praziquantel by schistosomes is of special concern considering that the molecular target and mode of action of the drug remain uncertain. Thus, despite the widespread use of praziquantel and nearly three decades of research, the exact mechanism of praziquantel action is still unresolved (reviewed by Day, Bennett & Pax, 1992; Redman *et al.*, 1996; Harder, 2002; Cioli & Pica-Mattoccia, 2003). This review will focus on recent advances in identifying the molecular target of praziquantel,

highlighting those experiments that point to a critical role for voltage-gated calcium (Ca^{2+}) channel proteins.

Praziquantel acts selectively against members of the phylum Platyhelminthes. Accordingly, the molecular target (or targets) for praziquantel might be encoded by a novel gene found exclusively in the flatworms. Schistosome genomes and transcriptomes contain several sequences that show no clearcut homology with genes found in other phyla (reviewed by Hu *et al*., 2004; LoVerde *et al.*, 2004; McManus *et al.*, 2004; Verjovski-Almeida *et al.*, 2004). On the other hand, the target for praziquantel might be a member of a gene family found in other phyla as well as in the platyhelminths, but with platyhelminth-specific structural signatures required for interaction with the drug. Even minor differences in critical domains of a protein, including single amino acid alterations, can have major consequences for the functional and pharmacological properties of typical receptors and channels (see, eg, Heinemann, Terlau & Imoto, 1992; Satin *et al.*, 1992).

Though elucidating the mode of action of praziquantel has proved a daunting task, the effects of the drug on adult schistosomes do provide clues to potential targets for the drug. Praziquantel produces a well-documented effect on intracellular Ca^{2+} levels in adult schistosomes (reviewed by Andrews, 1985; Day, Bennett & Pax, 1992; Redman *et al*., 1996). Within seconds of exposure to the drug, adult schistosomes exhibit a rapid, sustained contraction of the worm's musculature (Fetterer, Bennett & Pax, 1980) and vacuolization and disruption of the parasite tegument (Becker *et al.*, 1980; Mehlhorn *et al.*, 1981), an effect associated with the subsequent exposure of parasite antigens on the surface of the worm (Harnett & Kusel, 1986). Both of these responses are thought to be linked to a praziquantel-dependent disruption of Ca^{2+} homeostasis (reviewed by Day, Bennett & Pax, 1992; Redman *et al.*, 1996).

Praziquantel elicits a rapid uptake of $^{45}Ca^{2+}$ in adult male schistosomes (as well as a

much slower influx of Na^+; Pax, Bennett & Fetterer, 1978). The effects of praziquantel on both contraction of the worm's musculature and disruption of the parasite tegument are Ca^{2+}-dependent processes. Removal of Ca^{2+} from the medium blocks both responses (Pax, Bennett & Fetterer, 1978; Wolde Mussie *et al.*, 1982; Xiao *et al.*, 1984). However, neither of these inhibitory effects appear immediately. For example, inhibition of the praziquantel-dependent contraction of the musculature requires at least 10 minutes to occur, a delay thought to correspond to the time required for depletion of sequestered intracellular Ca^{2+} stores. These results indicate that though extracellular Ca^{2+} is not required for the initiation of praziquantel-dependent action, it is required for maintenance of the response.

Based on comparisons between praziquantel response in intact and detegumented parasites, it appears that both the tegument and the sarcolemma contain praziquantel-sensitive sites (Blair, Bennett & Pax, 1992). Thus, intact worms that are bathed in a medium with a high magnesium (Mg^{2+}):Ca^{2+} ratio exhibit a praziquantel-dependent biphasic muscle contraction instead of the tonic contraction that occurs in standard media. Detegumented worms continue to respond to praziquantel, but they show only a single, pronounced phasic contraction in high Mg^{2+}, indicating that a tegumental site is necessary for the full response. Furthermore, unlike intact worms, which show a transient response to praziquantel in Ca^{2+}-free medium, application of praziquantel to detegumented worms in Ca^{2+}-free medium produces no muscular contraction. Interestingly, praziquantel (1-2 μM) has been reported to interact with both sarcolemmal and intracellular sites to produce a sustained Ca^{2+}-dependent contraction in the penile retractor muscle from the mollusc *Lymnaea stagnalis* (Gardner & Brezden, 1984).

The effects of praziquantel on Ca^{2+} homeostasis could point to a direct action of the drug on membrane permeability to Ca^{2+}. However, early experiments indicated that praziquantel is not acting as a Ca^{2+} ionophore (Pax, Bennett & Fetterer, 1978). On the other

hand, it has been reported that praziquantel alters the structure of membrane bilayer phospholipids or membrane fluidity (Harder, Goossens. & Andrews, 1988; Lima *et al.*, 1994), which could result in changes in membrane permeability to Ca^{2+} or to indirect effects on membrane receptors and channels.

The target of praziquantel might also be one of the several cellular factors involved in regulating intracellular levels of Ca^{2+} discussed above and reviewed by Redman *et al*. (1996.) To date, there is little direct evidence implicating or eliminating these factors as possible targets in praziquantel action. However, Cunha & Noël (1997) have reported that high concentrations of praziquantel (100 μM) have no effect on schistosome (Na^{+}+K^{+})-ATPase or (Ca^{2+}-Mg^{2+})ATPase activities.

Recently, voltage-gated Ca^{2+} channels have been identified as candidate targets of praziquantel action (Kohn *et al*., 2001a, 2003a, b). As important entry sites for extracellular Ca^{2+}, voltage-gated Ca^{2+} channels play a critical role in regulating levels of intracellular Ca^{2+}. However, until recently, the role of voltage-gated Ca^{2+} channels in praziquantel action had not been tested directly, as Ca^{2+} currents had never been recorded from schistosome cells (see below). Nevertheless, pharmacological studies by Blair, Bennett & Pax (1992) on praziquantel-induced contraction in both intact and detegumented worms led them to suggest that Ca^{2+} channels might be involved in the action of the drug. Interestingly, high concentrations (50 μM) of praziquantel prolong the Ca^{2+}-dependent plateau phase of the cardiac action potential in rats, which is carried by voltage-gated Ca^{2+} channels (Chubb *et al.*, 1978). On the other hand, methoxyverapamil (D-600), an inhibitor of one class of mammalian Ca^{2+} channels (L-type), does not block the praziquantel-dependent Ca^{2+} influx in schistosomes, though it does block the tonic contraction of these cells resulting from increased K^{+} concentrations (Fetterer, Pax & Bennett, 1980). However, recent results from expression of cloned Ca^{2+} channel proteins indicates a significant role for voltage-gated Ca^{2+}

channels in praziquantel action.

VOLTAGE-GATED CA^{2+} CHANNELS

Voltage-gated ion channels, which are part of the ion channel superfamily, underlie electrical excitability in cells. They form an ion-selective pore through the membrane which, when activated by a change in membrane potential, allows ions to flow down the electrochemical gradient across the cell membrane. Voltage-gated channels include those selective for potassium, sodium, and Ca^{2+}. Voltage-gated Ca^{2+} channels are membrane protein complexes that form Ca^{2+}-selective pores gated by depolarization. Like other voltage-gated channels, Ca^{2+} channels contribute to impulse propagation, but they are also essential regulators of intracellular Ca^{2+} levels. By providing a pathway for rapid Ca^{2+} influxes, Ca^{2+} channels couple depolarization of the cell to a wide array of Ca^{2+}-dependent responses including muscle contraction and neuroscretion in muscles, nerves, and other excitable cells (reviewed by Catterall, 2000; Hofmann, Lacinova & Klugbauer, 1999). Thus, voltage-gated Ca^{2+} channels are essential to the behavior and survival of the animal. This point is further underscored by the fact that organisms as diverse as fish-hunting cone snails and spiders produce toxins targeted against specific Ca^{2+} channel subtypes to immobilize prey.

The pore-forming subunit of voltage-gated Ca^{2+} channels is the α_1 subunit. In addition to the α_1 subunit, voltage-gated Ca^{2+} channels typically contain associated auxiliary subunits that modulate the properties of the channel (see Fig. 3). The α_1 subunit is made up of four linked homologous domains, each of which contains six transmembrane regions (S1-S6). The predicted structure of the α_1 subunit fits within the ion channel superfamily (Doyle *et al*., 1998). The most basic ion channels are tetrameric structures comprised of the fifth and sixth transmembrane regions (S5 and S6, respectively) and the P loop, a region between S5 and S6 that forms the selectivity filter of the pore. The residues that define a channel's ionic

selectivity and many of its pharmacological properties reside in this region. In the voltage-gated channels, the fourth transmembrane segment within each domain (S4) contains a positively charged amino acid (lysine or arginine) at every third residue and is thought to serve as the voltage sensor of the channel.

Two major classes of Ca^{2+} currents that have been characterized in both vertebrate and invertebrate cells are Low Voltage Activated (LVA; t-type) and High Voltage-Activated (HVA). HVA currents can be further subdivided into L-type, which, in vertebrates, are sensitive to the dihydropyridine class of Ca^{2+} channel antagonists (nifedipine, nimodipine, etc.); and a variety of dihydropine-insensitive currents which are collectively known as non L-type. Heterologous expression of cloned Ca^{2+} channel subunits has been used to demonstrate a correspondence between these different Ca^{2+} currents and particular subtypes of Ca^{2+} channel α_1 subunits. Thus, LVA currents are gated by the Ca_v3 class of α_1 subunits (reviewed by Perez-Reyes, 2003), while HVA L-type currents and non L-type currents are gated by Ca_v1 α_1 subunits and Ca_v2 α_1 subunits respectively. Similar studies have indicated that the pharmacological differences found between vertebrate L-type and non L-type channnels (*eg*, in dihydropyridine sensitivity) are not as clearcut in invertebrates (reviewed by Jeziorski, Greenberg & Anderson, 2000a). Thus, expression of invertebrate α_1 subunits that are clearly members of the L-type family based on structure produces currents that are relatively insensitive to dihydropyridines and other potent modulators of vertebrate L-type channels.

In the HVA Ca^{2+} channels, the α_1 subunit is associated with and modulated by auxiliary subunits (reviewed by Hofmann, Lacinova & Klugbauer, 1999; Catterall, 2000; Arikkath & Campbell, 2003) that include α_2/δ and β subunits (Fig. 3), as well as γ subunits (not shown). Ca^{2+} channel β subunits ($Ca_v\beta s$) are cytoplasmic proteins that have been studied extensively and are critical components of Ca^{2+} channel complexes. When coexpressed with

β subunits, α_1 subunits show increases in current density and ligand binding. $Ca_v\beta$s appear to play a role in membrane trafficking of the α_1 subunit, likely in part by masking an ER retention site on the α_1 subunit (Bichet *et al.*, 2000). β subunits also affect a variety of the biophysical properties of Ca^{2+} channels, including the voltage-dependence of channel activation and steady state inactivation, rates of inactivation (reviewed by Walker & DeWaard, 1998; Birnbaumer et al., 1998; Hanlon & Wallace, 2002; Dolphin, 2003), and the rate of recovery from inactivation (Jeziorski, Greenberg & Anderson, 2000b).

The primary site on the α_1 subunit for binding of $Ca_v\beta$s is the Alpha Interaction Domain (AID; Pragnell *et al.*, 1994), an 18 amino acid region in the intracellular loop between Domains I and II of the α_1 subunit. Recent X-ray crystallographic studies (Chen, *et al.*, 2004; Opatowski *et al.*, 2004; Petegem *et al.*, 2004) indicate that the AID indeed does complex with $Ca_v\beta$s, forming an amphipathic helix with the most highly conserved residues on one side of the helix in contact with a complementary groove on the β subunit.

Using homology modeling, Hanlon *et al.* (1999) proposed that $Ca_v\beta$s are members of the membrane-associated guanylate kinase (MAGUK) family of proteins. MAGUKs are scaffolding proteins, often concentrated at synapses where they play important roles in clustering of ion channels and neurotransmitter receptors (reviewed by Dimitratos *et al.*, 1999). Typically, MAGUKs contain one or more PDZ domains located N-terminal to a Src-homology 3 (SH3) domain, a bridging region (the HOOK domain), and a guanylate kinase (GK)-like domain. Resolution of the crystal structure of the conserved core of β subunits, both alone and in complex with the AID of the α_1 subunit, are consistent with $Ca_v\beta$s indeed being members of the MAGUK family, though with distinct characteristics (Chen, *et al.*, 2004; Opatowski *et al.*, 2004; Petegem *et al.*, 2004; commentary by Yue, 2004). For example, the PDZ domains typically found in other MAGUKs appear to be absent in $Ca_v\beta$s, and the orientation of the SH3 and GK domains has been modified. In addition, the GK

domain of $Ca_v\beta$s does not contain a nucleotide binding site. Instead, the modified GK domain forms a deep hydrophobic groove (the AID-binding pocket, or ABP) with which the AID of the α_1 subunit interacts.

Several lines of evidence indicated that a highly conserved ~35 amino acid region in $Ca_v\beta$s dubbed the Beta Interaction Domain (BID) serves as the primary site for β subunit interaction with the α_1 subunit (DeWaard, Pragnell & Campbell, 1994). However, the recent crystal structures have cast doubt on this hypothesis. Instead, the BID region is found buried within the β subunit protein, and is therefore unlikely to be involved directly in protein-protein interactions such as binding to the AID. However, as Chen *et al*. (2004) have noted, the BID nonetheless appears to play an essential structural role in β subunits, spanning the SH3 and GK domains and their connecting HOOK region, and containing two β-strands that are integral parts of the SH3 and GK domains. Indeed, the BID and surrounding areas are particularly highly conserved regions of β subunit proteins.

SCHISTOSOME AND FLATWORM CA^{2+} CURRENTS

Do voltage-gated Ca^{2+} channels in schistosomes play a role in praziquantel action? Are they the molecular targets of praziquantel? These questions may be answered by characterizing the structure, function, and pharmacological sensitivities of Ca^{2+} channels from schistosomes and other flatworms. These studies may additionally provide insights into the physiology of excitable cells in platyhelminths, about which relatively little is currently known, and should also provide clues about the evolution of ion channels. Furthermore, structural and functional characterization of flatworm Ca^{2+} channels might provide highly specific targets for new antiparasitic agents. For example, a recent patent (Walter & Kuris, 2003; see also Bonn, 2004) claimed that high concentrations of verapamil and nifedipine, two compounds that block L-type voltage-gated Ca^{2+} channels in vertebrates, suppress egg

production in *S. mansoni* and another trematode, *Echinostoma caproni*. Clearly, a more thorough understanding of the pharmacological properties of platyhelminth Ca^{2+} channels will be required to determine whether these compounds are acting on worm voltage-gated Ca^{2+} channels or other targets.

Isolated muscle cells from *S. mansoni* exhibit Ca^{2+}-dependent contractility (Day, Bennett & Pax, 1994). To date, however, there have been no published reports of native Ca^{2+} currents from schistosome cells. Indeed, Day *et al*. (1993) found no inward currents of any type in their voltage-clamp analysis of muscle cells isolated from adult *S. mansoni*, most likely because of technical limitations with these cells.

In contrast, Ca^{2+} currents have been recorded from other flatworms. For example, neurons from the polyclad flatworm *Notoplana acticola* contain a normal complement of ionic currents that are implicated in the generation of action potentials. These include cadmium-sensitive Ca^{2+} currents (Keenan & Koopowitz, 1984). Similarly, using voltage-clamp analysis, voltage-gated Ca^{2+} currents have been recorded in nerve and muscle cells of *Bdelloura candida*, a triclad ectoparasitic flatworm that resides on the legs and gills of hoseshoe crabs (*Limulus polyphemus*; Blair & Anderson, 1993, 1994). Both cell types possess Ca^{2+} currents that activate at -30 mV, reach peak amplitude in approximately 5 ms, and inactivate slowly. The neuronal Ca^{2+} current shows relatively little sensitivity to organic Ca^{2+} channel blockers such as nifedipine and verapamil, and is not blocked by the cone snail toxin ω-conotoxin GVIA. This neuronal Ca^{2+} current also exhibits no sensitivity to 10 μM praziquantel (Blair & Anderson, 1996). Unfortunately, the muscle Ca^{2+} current was too unstable for determination of pharmacological sensitivities. More recently, Cobbett & Day (2003) recorded Ca^{2+} currents from muscle cells of the triclad turbellarian *Dugesia tigrina*. However, these currents were also too unstable for systematic pharmacological analysis.

Clearly, determining the properties of native Ca^{2+} currents in schistosomes and other

flatworms presents major challenges. These difficulties may be bypassed by using a molecular approach. Expression of cloned Ca^{2+} channel subunits from schistosomes and other flatworms in heterologous systems may present an alternative means of elucidating the physiological properties and pharmacological sensitivities of these channels. However, analysis of native currents within schistosome cells will eventually be necessary for a thorough understanding of the properties of these channels and their physiological roles within the parasite.

SCHISTOSOME CA^{2+} CHANNEL SUBUNITS

Adult schistosome adults express at least three subtypes of HVA Ca^{2+} channel α_1 subunits (Kohn *et al.*, 2001b). Phylogenetic analysis shows that two of these subtypes cluster with the non L-type class of HVA α_1 subunits, while the third is most closely related to the L-type class of α_1 subunits. Although mammals contain multiple subtypes of L-type and non L-type α1 subunits, other invertebrates examined to date contain only a single representative of each of these classes (Littleton & Ganetzky, 2000; Jeziorski, Greenberg & Anderson, 2000a). Schistosomes (and presumably other platyhelminths) therefore appear to be unique among the invertebrates in that they have two subtypes of non L-type α_1 subunits rather than one.

Schistosomes also express at least two subtypes of $Ca_v\beta$s (Kohn *et al.*, 2001a; 2003b). This finding is also unprecedented among the invertebrates; to date, only a single β subunit gene has been identified in the genomes of other invertebrate species. Even more noteworthy, one of these schistosome β subunit subtypes has particularly unusual structural features and functional properties. To date, no representatives of this variant subtype have been identified in any vertebrates or invertebrates other than the platyhelminths (see Fig. 4).

The variant $Ca_v\beta$ subtype proteins are clearly part of the broader β subunit family, although they have very distinct structural features. For example, they are from 25% - 50%

larger than other β subunits, including the conventional *S. mansoni* $Ca_v\beta$. However, the most striking feature of these variant $Ca_v\beta$s can be found in the BID, where two serine residues that are conserved in other $Ca_v\beta$s and that correspond to consensus protein kinase C (PKC) phosphorylation sites are replaced by cysteine and alanine (Fig. 5).

The variant schistosome β subunits also exhibit distinctive functional properties (Kohn *et al.*, 2001a). Conventional β subunits enhance currents when coexpressed with α_1 subunits. In contrast, coexpression of a variant β subunit in *Xenopus* oocytes with a jellyfish (*Cy*Ca_v1) or human ($Ca_v2.3$) α_1 subunit results in a dramatic reduction in current compared to the levels found when the α_1 subunit is expressed alone. Yet, other than this novel effect on current levels, the modulatory effects of the variant schistosome $Ca_v\beta$s are similar to those of conventional $Ca_v\beta$s. For instance, like other $Ca_v\beta$s, the variant β subunits shift the current/voltage relationship of α_1 subunits in a hyperpolarizing direction. Thus, although the variant $Ca_v\beta$s have some highly unusual properties, they appear to behave in most respects as genuine, functional β subunits.

A particularly exciting property of the variant schistosome $Ca_v\beta$s is that they can confer praziquantel sensitivity to an otherwise praziquantel-insensitive mammalian α_1 subunit (Kohn *et al.*, 2001a). When the mammalian $Ca_v2.3$ α_1 subunit is expressed alone in *Xenopus* oocytes, it does not respond to 100 nM praziquantel. On the other hand, when $Ca_v2.3$ is coexpressed in *Xenopus* oocytes with one of the variant schistosome $Ca_v\beta$s, peak currents are increased 1.5- to 2-fold in the presence of 100 nM praziquantel. Other $Ca_v\beta$s, including the conventional schistosome β subunit, do not confer praziquantel sensitivity (Kohn *et al.*, 2003b). This praziquantel-dependent increase in Ca^{2+} influx found with Ca^{2+} channels containing the variant β subunit is consistent with the effects of the drug on Ca^{2+} homeostasis in schistosomes and implicates the variant schistosome $Ca_v\beta$s in the action of

praziquantel.

Using site-directed mutagenesis, the specific amino acid residues involved in determining the unusual functional properties and pharmacological sensitivities of the variant β subunits have been localized. Both the reduction in current levels and the responsiveness to praziquantel map to the two conserved consensus PKC sites in the BID that are not present in these variant β subunits (Kohn *et al.*, 2003a, b). If serine residues are substituted at either or both of these sites in the variant $Ca_v\beta$ from *S. mansoni* the consensus PKC sites are restored (see Fig. 5). This mutated β subunit, like conventional $Ca_v\beta$s, now enhances currents through α_1 subunits and does not confer sensitivity to praziquantel. A double mutation containing a serine at one of these sites, but also containing a second mutation that eliminates the consensus PKC sequence, behaves like the wildtype schistosome subunit. Thus, the absence of PKC sites in the BID region, rather than simply the presence of residues other than serines, appears to be responsible for the novel effects of the variant subunit. Similarly, elimination of both PKC sites in the BID of a conventional β subunit (mammalian β2a) results in a β subunit that can now confer praziquantel sensitivity to the mammalian α_1 subunit (Kohn *et al*., 2003b).

Thus, the absence of consensus PKC sites in the β subunit BID is associated with the capability of a β subunit to confer susceptibility to praziquantel, while the presence of a single consensus PKC site in the BID is sufficient to transform a variant $Ca_v\beta$ into a β subunit with characteristics typically found for conventional β subunits. Based on these results, we have hypothesized that the unusual modulatory properties and pharmacological sensitivities of the variant $Ca_v\beta$s from schistosomes are dependent on the absence of the consensus PKC phosphorylation sites found in the BIDs of other β subunits.

Phosphorylation of voltage-gated Ca^{2+} channel subunits by PKC and other protein

kinases plays a central role in regulating channel properties (reviewed by Rossie, 1999; Kamp & Hell, 2000; Keef, Hume & Zhong, 2001). A recombinant mammalian β2a subunit has been shown to be phosphorylated by PKC, with a stoichiometry estimated to be 1-2 moles of phosphate per mole of β2a protein (Puri *et al*., 1997). There are several consensus PKC sites in β subunits, but the exact sites that are phosphorylated by PKC have yet to be defined either *in vitro* or *in vivo*.

CONCLUSIONS AND FUTURE QUESTIONS

The variant $Ca_v\beta$s found in schistosomes appear to be involved in praziquantel action, while the addition of a PKC site in the BID of these subunits can abolish susceptibility to the drug. It therefore follows that one mechanism for schistosomes to acquire praziquantel resistance might be by acquiring one or both of these PKC sites by mutating the cysteine or alanine residues to serine. Schistosome isolates with reported reduction in praziquantel sensitivity have been tested for the presence of these (and other) mutations (Valle *et al.*, 2003). These isolates did not exhibit changes in primary structure of schistosome β subunits, nor did they show changes in expression levels of those subunits. Thus, a reduction in praziquantel susceptiblity in these strains apparently does not depend on altered $Ca_v\beta$ structures or expression levels. However, since various Egyptian isolates with reduced praziquantel susceptibility show differential stability (William *et al.*, 2001), there may be alternative pathways for acquiring praziquantel susceptibility, with different strains of parasites acquiring resistance to praziquantel via various mechanisms.

There are several unresolved issues regarding schistosome Ca^{2+} channels and their role in praziquantel action. Of primary importance is the need to characterize schistosome Ca^{2+} channel α_1 subunits, either alone or in combination with the two schistosome $Ca_v\beta$ subtypes. These experiments will answer several questions, including whether all possible

schistosome α_1/β combinations are capable of forming functional channels. More specifically, are only certain α_1/β combinations capable of forming praziquantel-sensitive channels, or can any schistosome channel that contains a variant β subunit respond to the drug? Furthermore, which schistosome cells express these combinations? Once specific praziquantel-sensitive channels are identified, the question of precisely how praziquantel acts on these channels will still remain. For example, praziquantel might be interacting directly with the variant β subunit, or it might instead interfere with α_1/β subunit interactions, or it could be acting indirectly, via effects on interacting proteins or on the membrane itself to affect Ca^{2+} channels that contain these variant $Ca_v\beta$s. Finally, what are the downstream players in the cascade of events that ultimately leads to paralysis and tegumental disruption? As described above, the components that regulate Ca^{2+} homeostasis in cells are connected with one another through a network of interaction and regulation. The components comprising these interdependent networks might also be affected by praziquantel. In schistosomes, these interactions are further complicated by the complexity of the adult tegument and sub-tegumental compartments (see Fig. 1 of Redman *et al*., 1996). The tegument of the adult is formed by a membrane that has a double-bilayer structure (McClaren & Hockley, 1977) and is electrically coupled to underlying muscle cells (Thompson, Pax & Bennett, 1982).

Independent of their role in praziquantel action, the biological function of these variant schistosome $Ca_v\beta$s is particularly intriguing. All other known β subunits enhance Ca^{2+} currents, while these variant β subunits inhibit currents. Are there special characteristics of schistosome α_1 subunits or other components of the Ca^{2+} signaling system that might necessitate the deveolpment of this unusual type of Ca^{2+} channel modulation by β subunits? Are there any other organisms that have developed a similar strategy? Answers to some of these questions may result from expression of channel subunits in heterologous systems,

while other insights may come from the schistosome genome and transcriptome. Ultimately, however, a full understanding of the role played by Ca^{2+} channels in schistosome physiology, Ca^{2+} signaling, and prazquantel action will require a thorough understanding of the properties of native schistosome Ca^{2+} (and other) currents.

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FIGURE LEGENDS

FIGURE 1. A subset of the components that regulate intracellular Ca^{2+} levels. Various receptors (R) act through second messengers to effect or modulate release of Ca^{2+} from the endoplasmic/sarcoplasmic reticulum (ER/SR) via IP3 receptors (IP3R) and ryanodine receptors (RyR). Voltage-gated Ca^{2+} channels (VGCCs) and other channels which are permeable to Ca^{2+} (CCs; *eg*, ligand-gated channels, second-messenger-operated channels, TRP channels) also increase Ca^{2+} levels, both directly from entry of Ca^{2+} from the external medium and indirectly, since release of Ca^{2+} from internal stores is controlled by Ca^{2+} itself. Ca^{2+} is removed from the cytoplasm by various exchangers and pumps, including the plasma-membrane Ca^{2+}-ATPase (PMCA) and the Na^{+}/Ca^{2+} exchanger (NCX), which extrude Ca^{2+} to the outside. The sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps Ca^{2+} back into intracellular stores. Ca^{2+} buffers (Buffers) bind a large proportion of cytoplasmic Ca^{2+}, effectively removing it from the activating pool. Mitochondria also play an important role in regulating levels of Ca^{2+} in the cytoplasm. They rapidly sequester Ca^{2+} through a uniporter, as well as releasing Ca^{2+} through the NCX.

FIGURE 2. Chemical structure of praziquantel.

FIGURE 3. Structure of voltage-gated Ca^{2+} channels. This simplified representation shows the pore-forming α_1 subunit, which consists of four homologous domains surrounding a central pore. Domains II - IV are shown, and Domain I is cut away to show the structure. Each of the four domains is composed of six transmembrane regions (S1-S6). The P loop dips into the membrane between S5 and S6, forming 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 associated β and $\alpha_2\delta$ subunits, which modulate α_1 function. The γ subunit has been omitted. 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. The size of subunits and domains are not to scale in this cartoon.

FIGURE 4. Phylogenetic tree of Ca^{2+} channel β subunits. Amino acid sequences were aligned using Clustal X (Thompson *et al.*, 1997), and a tree constructed using the neighbor-joining method, as implemented in MEGA 2.1 (Kumar *et al.*, 2001). The schistosome conventional and variant β subunits are shaded, and the variant β subunits are boxed with a dashed line. Numbers represent bootstrap values (1000 replications). Sequences and NCBI accession numbers are: Human β1, NP_954856; Rabbit β1, AAA31180; Human β2, NP_000715; Rabbit β2, CAA45576; Human β3, NP_000716; Rabbit β3, CAA45578; *Xenopus laevis* (toad) β3, AAA75519; Human β4, NP_000717; Rat β4, A45982; *Caenorhabditis elegans* (nematode) β, AAB53056; *Schistosoma mansoni* conventional β, AY033599; *Lymnaea stagnalis* (snail) β, AAO83844; *Loligo bleekeri* (squid) β, BAB88219; *Musca domestica* (housefly) β, A54844; *Drosophila melanogaster* (fruitfly) β, AAF21096; *Cyanea capillata* (jellyfish) β, AAB87751; *S. japonicum* variant β, AAK51116; *S. mansoni* variant β, AAK51117; *C. elegans* β-like sequence (w10c8.1), AAK21500.

FIGURE 5. Comparison of the amino acid sequence of the BID from the variant schistosome β subunits with a consensus β subunit BID sequence. The cysteine and alanine residues which substitute in the variant BIDs for the conserved serines are shaded. The two consensus PKC sites conserved in the consensus BID sequence are underlined, with the serines in bold.

Figure 1.

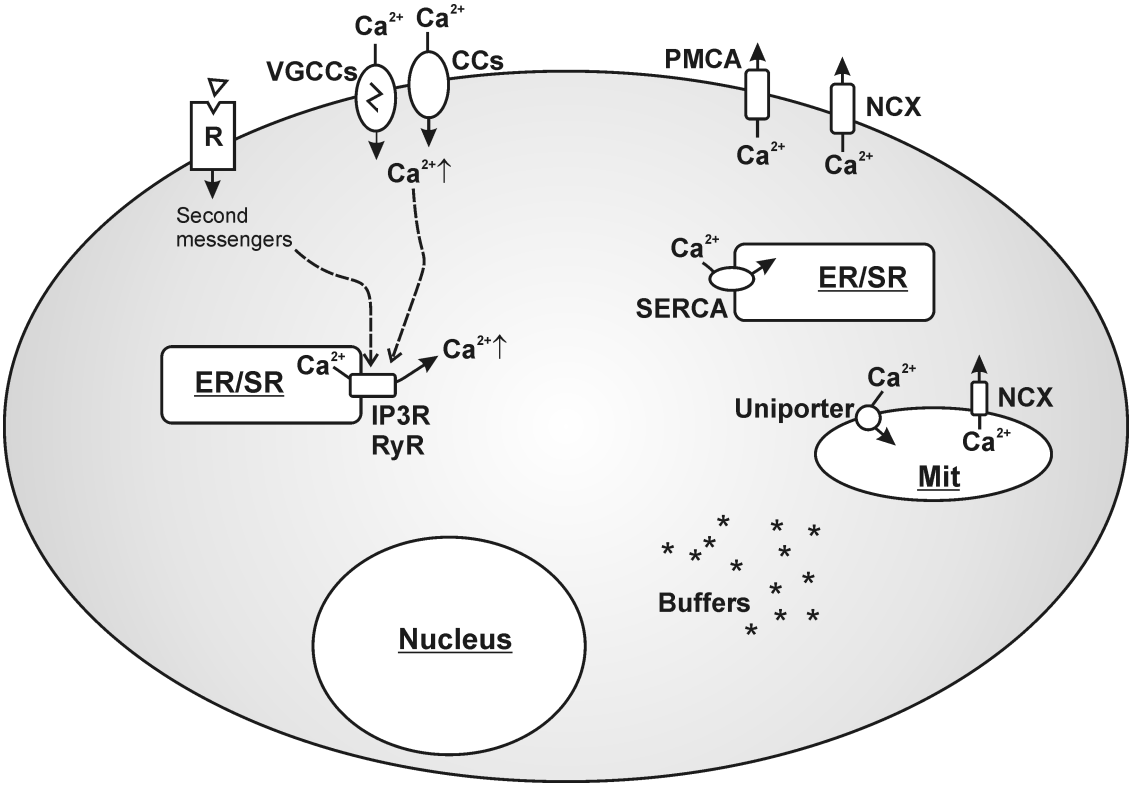

Ca2+
Ca2+
VGCCs
CCs
PMCA
NCX
R
Ca2+
Ca2+
Ca2+↑
Second
messengers
Ca2+
ER/SR
SERCA
ER/SR
Ca2+
Ca2+↑
IP3R
RyR
Ca2+
Uniporter
NCX
Ca2+
Mit
Buffers
Nucleus

Figure 2.

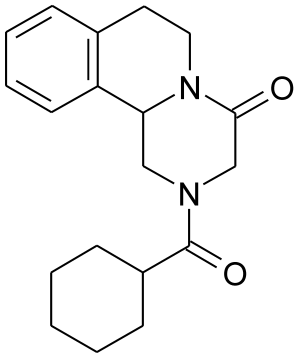


Figure 3.

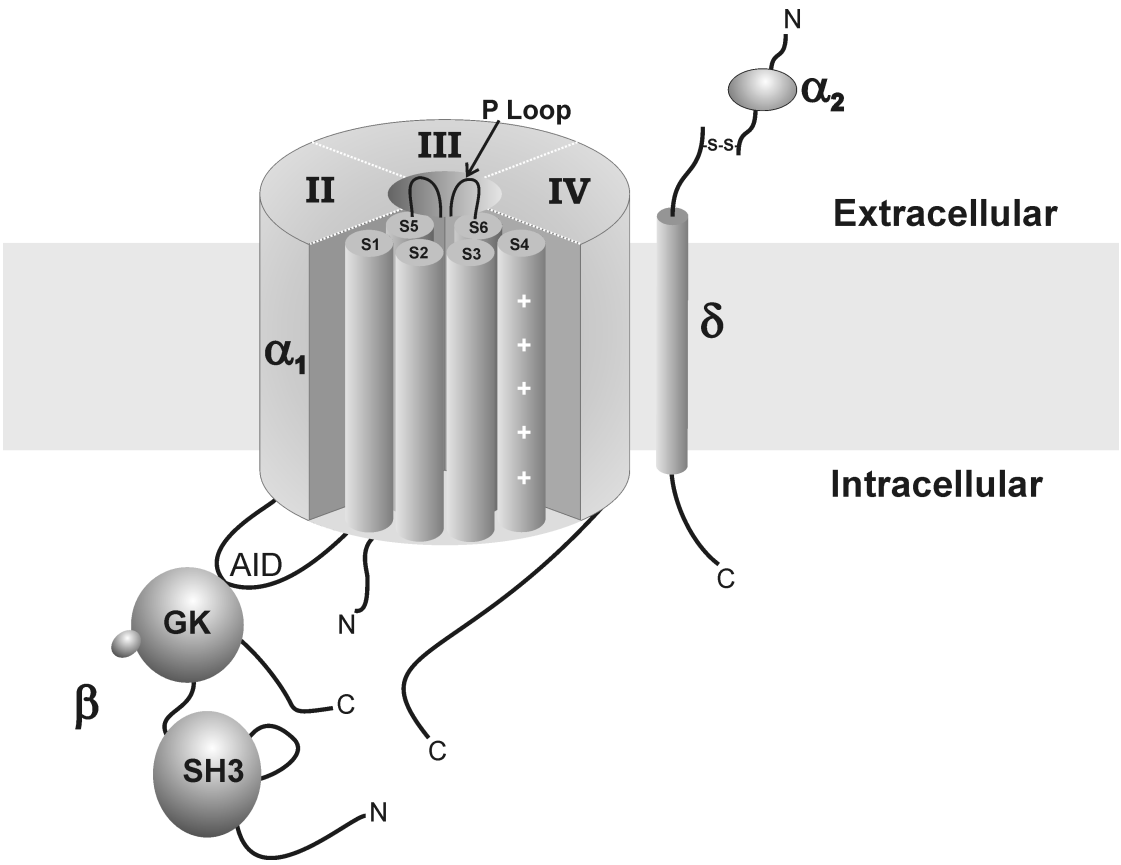

N
α2
P Loop
III
S-S
II
IV
Extracellular
S5
S6
S1
S2
S3
S4
α1
δ
Intracellular
AID
GK
N
β
C
C
SH3
N
C

Figure 4.

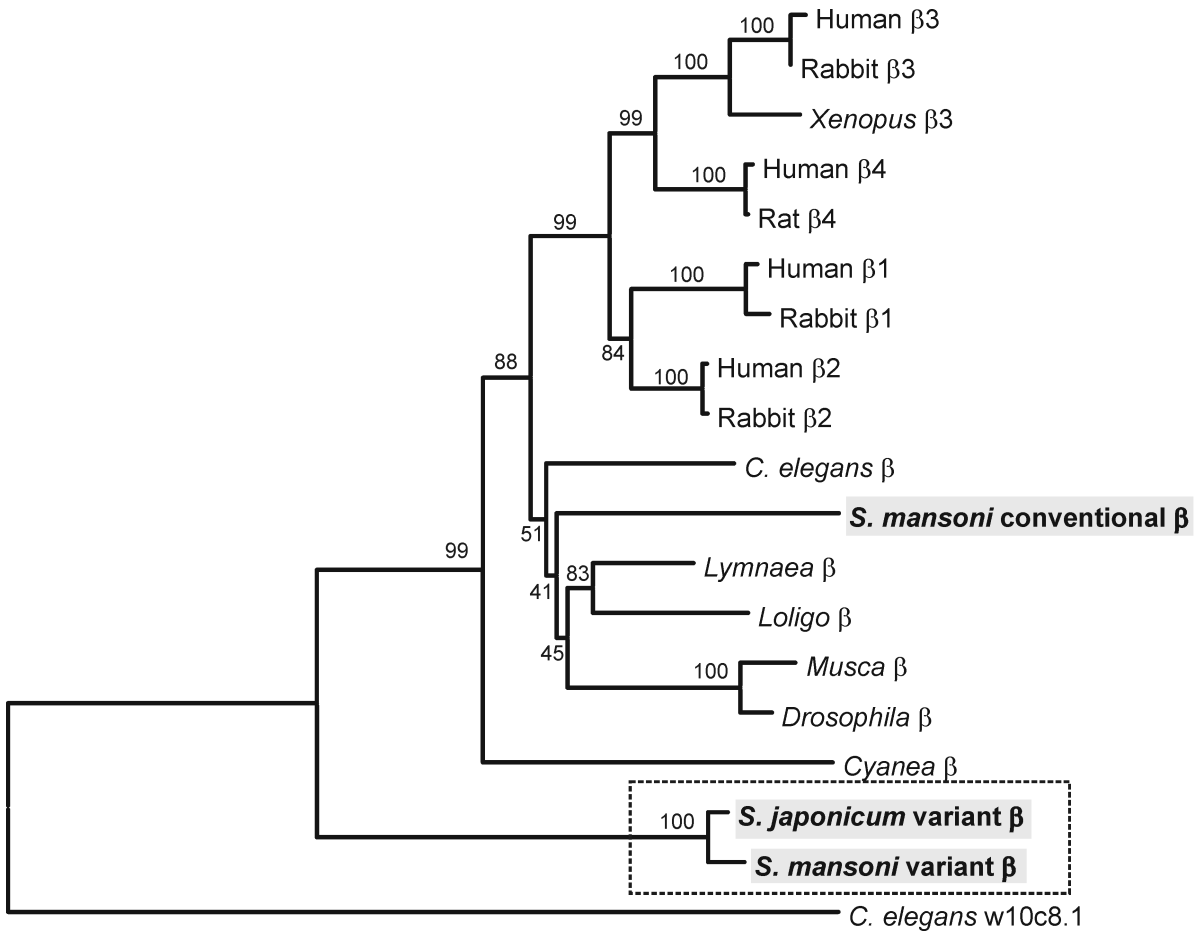
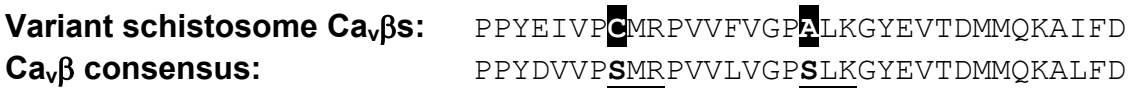

Human β3
Rabbit β3
Xenopus β3
Human β4
Rat β4
Human β1
Rabbit β1
Human β2
Rabbit β2
C. elegans β
S. mansoni conventional β
Lymnaea β
Loligo β
Musca β
Drosophila β
Cyanea β
S. japonicum variant β
S. mansoni variant β
C. elegans w10c8.1
100
100
99
100
99
100
84
100
88
51
99
83
41
45
100
100

Figure 5.


Variant schistosome Caνβs: PPYEIVPCMRPVVFVGPALKGYEVTDMMQKAIFD
Caνβ consensus: PPYDVVPSMRPVVLVGPSLKGYEVTDMMQKALFD