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Ca^{2+} channels and Praziquantel: a view from the free world

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Abstract

Targeting the cellular Ca^{2+} channels and pumps that underpin parasite Ca^{2+} homeostasis may realize novel antihelmintic agents. Indeed, the antischistosomal drug praziquantel (PZQ) is a key clinical agent that has been proposed to work in this manner. Heterologous expression data has implicated an action of PZQ on voltage-operated Ca^{2+} channels, although the relevant *in vivo* target of this drug has remained undefined over three decades of clinical use. The purpose of this review is to bring new perspective to this issue by discussing the potential utility of free-living planarian flatworms for providing new insight into the mechanism of PZQ action. First, we discuss *in vivo* functional genetic data from the planarian system that broadly supports the molecular data collected in heterologous systems and the 'Ca^{2+} hypothesis' of PZQ action. On the basis of these similarities we highlight our current knowledge of platyhelminth voltage operated Ca^{2+} channels, their unique molecular pharmacology and the downstream functional PZQ interactome engaged by dysregulation of Ca^{2+} influx that has potential to yield novel antischistosomal targets. Overall the broad dataset underscore a common theme of PZQ-evoked disruptions of Ca^{2+} homeostasis in trematodes, cestodes and turbellarians, and showcase the utility of the planarian model for deriving insight into drug action and targets in parasitic flatworms.

Keywords

Planarians; Schistosomiasis; Platyhelminths; Ca^{2+} signaling; Voltage-operated Ca^{2+} channel

1. Introduction

Over a third of the world's population is estimated to be infected with parasitic worms [1]. As discussed by the authors of this volume, ion channel modifying drugs hold considerable potential for use as antihelmintics with several agents that target ligand-gated ion channels in parasitic worms approved for clinical/veterinary use [2–5]. Voltage-gated ion channels afford similar opportunity for exploitation as druggable targets, given the likely importance of these channels for parasite biology. Our laboratory is particularly interested in the role of platyhelminth voltage-gated Ca^{2+} channels (Ca_V channels), given the fundamental role of

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Ca^{2+} signals in normal cellular and developmental physiology [6, 7]. Indeed, one important drug proposed to disrupt voltage-operated Ca^{2+} entry in platyhelminths is praziquantel (PZQ), and the purpose of this review is to provide broad perspective on evidence linking PZQ to changes in Ca^{2+} homeostasis in both parasitic and free living (planarian) platyhelminths.

PZQ is the key pharmacotherapy used for treating schistosomiasis, as well as cestode infections. It is a crucial treatment: over 200 million people harbor schistosome infections (*Schistosoma mansoni*, *S. japonicum* and *S. haematobium*) and PZQ is the sole therapy available in many areas of endemic disease [8–12]. The associated burden of schistosomiasis manifest through gastrointestinal and liver pathology, anaemia, undernutrition, growth retardation, genitourinary disease (*S. haematobium*) and increased prevalence of co-morbidities, is arguably second only to HIV/AIDS in impact [9, 13]. The low cost (~$0.07/treatment) and high cure rate with a single dose of PZQ has led to initiatives to increase distribution of the drug [10] but there is a continued anxiety that PZQ-resistant strains of schistosomiasis will emerge [12, 14–17]. In the absence of an effective vaccine/vector control for schistosomiasis, the continued efficacy of PZQ in clearing schistosome infections is critical for reducing the devastating burden of this disease in Africa. Therefore, it remains problematic that despite over three decades of clinical use the target of PZQ still remains ambiguous and synthesized structural derivatives prove less efficacious [18–21]. Resolution of the target and effector mechanisms of PZQ is needed to permit rational design of novel drugs to exploit these same pathways and to discover agents that, unlike PZQ, retain efficacy against all stages of the schistosome life cycle [22–24].

We have attempted to bring new perspective to this longstanding roadblock by studying the action of PZQ in planaria [20, 25], free-living turbellarian flatworms (Figure 1A). Planarians have long been used as a model organism in their own right for studying their remarkable powers of regeneration and rejuvenation [26, 27]. The key point of interest is definition of the properties and behavior of neoblasts, the pluripotent stem cell population that empowers regeneration and normal tissue turnover. Neoblasts are the only mitotically active cells in these organisms [28, 29], and by extension neoblast activity drives progression of the parasitic platyhelminths through the various stages of their life cycle [30]. As an experimental model, planarians are easy to maintain in the laboratory as they are free-living and cultures expand rapidly via asexual reproduction (fission). Protocols to culture these free-living organisms are straightforward, contrasting with the logistical complexity of protocols needed to support the schistosome life cycle. The long standing usage of planarians as an experimental model has spurred development of methods and functional genomic techniques (notably RNA interference, RNAi) to interrogate gene expression and function [31, 32], thereby establishing a deep methodological resource for experimentalists working in this system. The basic regenerative assays are robust, simple to execute, and amenable to pharmacological and RNAi screens [33]. Genomic and transcriptomic data are available, and unsurprisingly, recent analyses demonstrate a high degree of gene conservation and protein sequence homology between planarians and schistosomes [34–39].

Here we discuss data concerning the activity of PZQ on free-living planarian flatworms and the potential relevance of these studies for delimiting PZQ targets and effector mechanisms [20, 25]. We believe interrogation of the planarian system can provide new information about the molecular basis of efficacy of existing drugs as well as potentially aid discovery of new agents to mitigate parasitic flatworm infections. That lateral sidesteps between closely related flatworm groupings can provide fresh insight into the workings of this important clinical therapeutic is not in retrospect surprising as we note PZQ was originally introduced as both an anticestodal and antischistocidal agent [40, 41]. Our work on PZQ is but one

evidenced example of a theme of using planarians as a 'parasite model' to provide information pertinent to the study of schistosomes [42–44].

2. The effects of praziquantel on Ca^{2+} signaling in parasitic flatworms

First, let us briefly review evidence that supports a link between PZQ and dysregulated Ca^{2+} homeostasis in parasitic platyhelminths. Early studies on PZQ in schistosomes revealed the drug caused muscle contraction and a sustained paralysis. The PZQ-evoked contraction was dose dependent, rapid (maximum tension < 1 minute) and inhibited by incubation in media with reduced [Ca^{2+}] [45, 46]. Analysis of radioisotopic fluxes revealed the PZQ-evoked muscle contraction was accompanied by a rapid and maintained uptake of $^{45}Ca^{2+}$ from external media, an effect that has been widely reproduced [45, 47–49]. Intracellular Ca^{2+} mobilization was also involved in the sustained contractile response, consistent with a functional relationship between Ca^{2+} entry and Ca^{2+}-induced Ca^{2+} release from intracellular Ca^{2+} stores [46, 50]. Interestingly, certain strains of *S. mansoni* that show a therapeutic resistance to PZQ treatment also exhibit attenuated responses to PZQ in $^{45}Ca^{2+}$ uptake and contractile assays, implying a mechanistic link between these assays and PZQ activity in treating schistosomiasis [49]. PZQ has also been shown to evoke $^{45}Ca^{2+}$ uptake in the trematode *Opisthorchis viverrini* [51], and experiments in cestodes studying the effect of PZQ on Ca^{2+} flux broadly mirror results reported for schistosomes [52, 53].

In addition to causing paralysis, PZQ disrupts the exterior surface of the parasite. Unlike free living turbellarians, whose surface is covered by a layer of epithelial cells, parasitic platyhelminths possess a unique exterior covering, or tegument, which forms a cellular syncytium and interfaces with the host environment. PZQ results in a rapid (as little as 30 seconds) lesion or 'blebbing' of the tegument in many cestodes and trematodes [54–56]. In schistosomes, tegument disruption is also Ca^{2+} dependent, as PZQ has no effect on the surface of worms incubated in Ca^{2+} free media [57], and tegument disruption is also attenuated in parasites which show a therapeutic resistance to PZQ [58]. Studies on schistosomes have shown that the Ca_v1 inhibitor verapamil is also capable of causing tegument lesions, and is lethal *in vitro* [59]. While at first this seems paradoxical, it is consistent with recent drug screening data (see section 4.2.2) and supports the concept that tegument integrity is highly sensitive to Ca^{2+} fluxes.

The role of voltage-operated Ca^{2+} entry in muscle physiology – Ca_v channels were subsequently shown to mediate neuropeptide and depolarization-evoked contractions in schistomes [60, 61] - suggests a focus on the Ca_v complex as a possible site of action of PZQ. Such an idea received critical molecular support following the cloning and heterologous expression of schistosome $Ca_v\beta$ subunits [62–64]. The key observation was that co-expression of a specific β subunit ($Ca_v\beta_{var}$, from either *S. mansoni* or *S. japonicum*) with a mammalian $Ca_v2.3$ channel resulted in PZQ regulable currents [62]. Specifically peak channel current was increased ~1.5–2-fold in the presence of PZQ, consistent with observations of PZQ activation of Ca^{2+} entry observed in schistosomes. This property was not exhibited by the other schistosome $Ca_v\beta$ subunit or mammalian $Ca_v\beta$ subunits, unless two serine residues were mutated to mimic residues in $Ca_v\beta_{var}$ [63, 64]. These minimal changes in $Ca_v\beta$ sequence were sufficient to confer PZQ regulation of $Ca_v2.3$ currents in *Xenopus* oocytes [63, 64]. Furthermore, engineering even a single serine replacement into the schistosome $Ca_v\beta_{var}$ was sufficient to abrogate PZQ regulation of Ca_v currents [63]. Therefore, the importance of this dataset was to reveal that specific, and minimal, changes in $Ca_v\beta$ sequence could impart a PZQ-evoked potentiation of Ca^{2+} entry currents. These data established a molecular basis for PZQ efficacy at the single amino acid level.

On the basis of this data, the hypothesis was proposed that PZQ acts to disrupt the association $Ca_V\beta_{var}$ and $Ca_V\alpha$ subunits [62]. The variant serine residues are found in consensus PKC sites in the region of the $Ca_V\beta$ subunit (the β-interaction domain, BID) important for association with the $Ca_V\alpha$ subunit. Differential phosphorylation would presumably iterate the availability of a PZQ binding site within the broader Ca_V complex. As expression of the $Ca_V\beta_{var}$ subunit unusually decreased Ca_V current amplitude, displacement of $Ca_V\beta_{var}$ from $Ca_V\alpha$ complexes at the cell surface would be expected to relieve this inhibition thereby increasing Ca^{2+} entry into schistosomes if replicated *in situ*. This proposal is not in itself unreasonable: several drugs are known to target accessory subunits/modulators of ion channels (discussed in [65]), and the approach of targeting protein-protein interaction interfaces is receiving increasing attention as a therapeutic strategy. Existing examples provide precedent for targeting Ca_V channels [66–68]. However the key problem for the '$Ca_V\beta$ displacement hypothesis' is simply the lack of supporting structural or biochemical data in the decade since the idea was proposed. Even so, this problem with a single mechanistic hypothesis should not detract from the convincing molecular evidence linking PZQ sensitivity to $Ca_V\beta$ sequence, although further insight is clearly needed.

3. The effects of praziquantel on planarian regeneration

Our research into the biological activity of PZQ in planarians, grew from the serendipitous finding that PZQ miscued the head to tail regenerative polarity of the planarian *Dugesia japonica* [20]. This result derived from a simple, manual screen in which trunk fragments were excised by amputation of head and tail structures, and incubated in drug-containing solution before the solution was exchanged and the excised fragments left to regenerate for a week (Figure 1B). Surprisingly, we found that PZQ exposure invariably caused regeneration of worms with two heads ('bipolar' head), rather than worms with a normal anterior-posterior (AP, head to tail) polarity [20]. In the presence of PZQ, each regenerative blastema yielded head structures (Figure 1B).

Investigation of this unusual property of PZQ revealed that the effect on regenerative polarity was (i) penetrant (at maximal doses of ±PZQ, all regenerating trunk fragments were bipolar); (ii) reflective of a complete duplication of the AP axis, the resulting bipolar worms were viable and able to move and feed; (iii) caused only by an acute exposure to PZQ (a delay of drug exposure after cutting was considerably less effective) and (iv) mimicked by other agents known to impact cellular Ca^{2+} homeostasis and modulated by media Ca^{2+} concentration [20]. The opportunity provided by this result was as an unambiguous phenotype that could be used to screen for genes required for mediating this effect *in vivo*, as the tractability of the planarian system to RNAi at the organismal level allowed interrogation of gene products underpinning the miscued regeneration elicited by PZQ. This is a similar 'chemical genetic' logic to that applied in other models to identify drug targets or genes involved in specific biological processes [69, 70]. Therefore, we used the bipolar regenerative phenotype to derive new insight into both the target of PZQ and effectors of this target (e.g. epistatic pathways) in planarians. That two-headed regeneration was phenocopied by known modulators of Ca^{2+} signaling was especially intriguing, given the previously discussed literature in schistosomes documenting acute effects of PZQ on Ca^{2+} homeostasis.

3.1 Testing the 'Ca^{2+} hypothesis' of PZQ action by RNAi

Our approach toward functional genetic testing of the 'Ca^{2+} hypothesis' of PZQ action depended on a strategy of stepwise cloning of Ca_V channel subunits prior to knockdown of these targets by *in vivo* RNAi. First analyzed were $Ca_V\beta$ subunits: these subunits were more easily cloned than the larger $Ca_V\alpha$ pore-forming subunits and have merit as target(s) because of their role in supporting Ca_V channel expression the cell surface [71, 72]. Knockdown of

$Ca_V\beta$ subunit provided a route for functional impairment of Ca^{2+} influx through Ca_V channels without recourse to a fuller characterization of the pore forming $Ca_V\alpha$ subunits.

As observed with other platyhelminths studied to date (reviewed in [73]), planarians express two $Ca_V\beta$ subunits, $Ca_V\beta1$ and the larger $Ca_V\beta2$ subunit (which lacks the serine residues in the BID domain [20], ~$Ca_V\beta_{var}$ in schistosomes). Both subunits exhibit conservation of $Ca_V\beta$ domains (SH3, HOOK, guanylate kinase-like regions) and residues critical for $Ca_V\alpha$ interaction that have been well studied in the mammalian $Ca_V\beta$ proteins [72]. In the trunk fragment regeneration assay, knockdown of either of these planarian $Ca_V\beta$ subunits attenuated the ability of PZQ to miscue regeneration (Figure 1C, [20]). Furthermore, in intact worms that were continually exposed to a higher dose of PZQ, individual or combinatorial ablation of the $Ca_V\beta$ subunits conferred resistance to PZQ in lethality assays [20]. Both results suggested that *in vivo* PZQ efficacy was dependent upon $Ca_V\beta$ function, and by inference the activity of Ca_V channels.

The next logical step was to characterize the planarian $Ca_V\alpha$ subunits and ascertain whether similar outcomes occurred following $Ca_V\alpha$ RNAi. This was important given increasing evidence for roles of $Ca_V\beta$ subunits independent from the core Ca_V complex [72], and the known promiscuity of ion channel accessory units [65]. Subsequent characterization of these planarian $Ca_V\alpha$ subunits both confirmed and refined this theme [25]. The ability of PZQ to miscue regeneration could be attenuated by knockdown of a specific HVA $Ca_V\alpha$ subunit (Ca_V1A), thereby linking PZQ efficacy to the functionality of a specific Ca_V complex *in vivo*. Surprisingly, knockdown of a second $Ca_V\alpha$ subunit (Ca_V1B) had the opposite effect, increasing the ability of PZQ to miscue trunk fragment regeneration (Figure 1C, [25]). Therefore, two Ca_V1 isoforms differentially modulated the ability of PZQ to miscue regeneration, with the effects of PZQ being selectively blocked by Ca_V1A RNAi. These data suggest that in planarians, PZQ action is mediated via a specific Ca^{2+} channel complex (Ca_V1A), although the sensitivity to PZQ can be modulated by the expression levels of other Ca_V1 isoforms.

The opposing effects of the Ca_V1 channels seemed inconsistent with a simple model where both channels were directly coupled to muscle depolarization. Indeed examination of the expression patterns of both Ca_V1A and Ca_V1B by *in situ* hybridization revealed a bias toward neuronal expression where Ca_V1 channels have been shown to function in other systems [74]. The opposing effects of the Ca_V1 isoforms could then be reconciled to neurons with differing function (excitatory vs inhibitory), or alternatively differential functional coupling within the same cell. An action on the nervous system was also consistent with Ca^{2+} imaging experiments performed in preperations of dissociated planarian cells, where acute PZQ exposure evoked Ca^{2+} signals, and the greatest PZQ-evoked $^{45}Ca^{2+}$ upotake occurred in a neuronally enriched cell fraction [25]. The effectors of the PZQ-evoked Ca^{2+} influx were also neuronally derived. PZQ exposure served to decrease transcriptional effects mediated by neuronally derived Hedgehog (Hh) signals [25], which are recently discovered mediators of planarian regenerative outcomes [75, 76]. Consistent with effects of Ca_V1A RNAi on the bipolar regenerative phenotype, both PZQ-evoked $^{45}Ca^{2+}$ uptake in a neuronally enriched cell fraction and PZQ-evoked transcriptional changes in Hh effectors were also attenuated by Ca_V1A RNAi [25].

So, to summarize, the data studying planarian regeneration are broadly consistent with evidence spanning several decades of research on parasitic platyhelminths, and as such provide *in vivo* genetic support for the broader 'Ca^{2+} hypothesis' of PZQ action. In both systems, PZQ causes an acute Ca^{2+} influx, and PZQ efficacy is dependent on $Ca_V\beta$ subunits (despite measuring disparate molecular and organismal level outcomes). Impairment of $Ca_V\beta$ function by mutation, or RNAi, impairs PZQ activity. The planarian data further narrows

PZQ efficacy to the expression of a specific Ca_V1 complex (Ca_V1A), highlights an action on neuronal signaling and shows that organismal PZQ sensitivity is regulated by Ca_V channel expression. Obviously, the data do not necessarily assign Ca_V1A as the direct target for PZQ action as RNAi effects could simply result from epistatic interactions. Such a conclusion could be drawn only following heterologous expression analyses and demonstration of a gain of function of a novel PZQ-evoked Ca^{2+} influx. The planarian dataset also reminds us that a broader interest in flatworm developmental signaling is not outlandish (PZQ-evoked changes in Hedgehog and Wnt signaling, [25]) given the morphological transitions that are the *sine qua non* for a parasitic lifecycle.

New contrasts also emerge. For example, both $Ca_V\beta$ subunits regulate PZQ activity in planarians, not just $Ca_V\beta_{var}$. Knockdown of either planarian $Ca_V\beta$ subunit ablated PZQ-evoked bipolarity [20], suggesting that rather than differences between individual $Ca_V\beta$ subunits being important, it is the differences relating to the *in vivo* pairings of $Ca_V\beta$ subunits with specific $Ca_V\alpha$ complexes that is paramount for generating PZQ-sensitive Ca^{2+} currents. Such an explanation would be consistent with reports of PZQ modulation of Ca^{2+} entry in organisms lacking $Ca_V\beta_{var}$ [77, 78], and refocus attention on resolving the properties of the pore-forming $Ca_V\alpha$ complexes.

4. What do we know about the properties of platyhelminth $Ca_V\alpha$ subunits?

4.1 Ca_V diversity

Platyhelminths possess a surprisingly diverse repertoire of $Ca_V\alpha$ subunits. Whereas well characterized invertebrate model systems possess only single representatives of each of the three, conventional Ca_V channel families (Ca_V1, Ca_V2 & Ca_V3), bioinformatic mining of sequenced platyhelminth genomes reveals the existence of a broader portfolio of $Ca_V\alpha$ subunits (Table 1). Notably, analysis of the genomes of the three principle species of schistosomes (*S. mansoni* [79], *S. japonicum* [80] and *S. haematobium* [81]) reveals that each species possesses four Ca_V subunits, comprising two representatives from each of Ca_V1 and Ca_V2 classes (Table 1). Best characterized of these are the Ca_Vs from *Schistosoma mansoni* : *SmCa$_V$1A* (originally named *Sm.Ca$_V$1*), *Sm.Ca$_V$1B*, *Sm.Ca$_V$2A* and *Sm.Ca$_V$2B*, which were first identified over a decade ago [82]. While others have suggested the existence of additional, schistosome Ca_V subunits from *in silico* prediction [83], these variants likely represent other classes of four-repeat channels entirely (XP_002575006, a NALNC-like channel [84]) or conform to these known Ca_V subunits (XP_002571932, Ca_V2B [83]).

Similarly in planarians, RT.PCR based cDNA cloning identified four high-voltage activated (HVA) subunits in *D. japonica*, (*Dj-Ca$_V$1A* and *Ca$_V$1B*; *Dj-Ca$_V$2A* and *Dj-Ca$_V$2B*, [25]) that were named as an extension of the original *S. mansoni* nomenclature (Table 1). These planarian Ca_V1 channels show ~60% amino acid identity to their schistosome counterparts [25]. Both platyhelminth families conform to the four domain Ca_V structure with long cytoplasmic loops connecting the first three domains (I and II, II and III) and a shorter loop connecting domains III and IV (Figure 2A). Ca_V1B is the larger family member in both planarians (longest splice variant, 2689 amino acids) and schistosomes (predicted up to 2570 amino acids), a size approaching the upper range of $Ca_V\alpha$ subunits characterized to date [85]. Overall identity with mammalian Ca_V1 proteins is lower (<50%), notwithstanding a clear conversation of this architecture as well as key channel regulatory motifs (Figure 2A). These include: (i) residues important ion permeation and selectivity - notably, the selectivity filter glutamate residues ('EEEE' motif), as well as the outer pore tryptophan ring and conserved aspartate residue downstream from the domain II selectivity filter glutamate suggested to be involved in delivering a Ca^{2+} ion to the selectivity filter [86]; (ii) COOH terminal motifs (EF, $PreIQ_3$, IQ) and transmembrane residues involved in Ca_V channel

regulation/inactivation [87, 88]; and (iii) the region in the domain I–II linker (the alpha-interaction domain, AID), that mediates association with $Ca_V\beta$ subunits.

The planarian complement of Ca_V channels holds further diversity compared with schistosomes owing to the presence of an additional low volateg activated (LVA, Ca_V3) isoform (Table 1). All five isoforms are also present in *Schmidtea mediterranea* genome [36] and evidenced at the transcript level by *de novo* transcriptome sequencing [89]. Although Ca_V3 was verified only as a partial clone in *D. japonica* [25], the protein exhibits highest homology to Ca_V3 channel sequences from other invertebrates, and notably contains aspartate residues in the domain III and IV P loops conforming to the EEDD-type selectivity filter motif diagnostic of LVA channels (compared with the EEEE motif harbored in all HVA Ca_V channels). These aspartate residues are key features of LVA channels that impart divergent permeation and activation properties compared with HVA channels [90]. Bioinformatic prediction of further sequence of planarian Ca_V3 (from the *S. mediterranea* genome) reveal conservation of the selectivity filter glutamate residue together with the highly conserved adjacent aspartate residue in domain II and the glutamate residue in the P-loop of domain I. Therefore, free living planarians possess a fifth $Ca_V\alpha$ subunit (Ca_V3) not found in the genomes of parasitic platyhelminths (Table 1).

Profiling available sequencing projects confirms platyhelminths are endowed with unexpected diversity in $Ca_V\alpha$ subunits compared to ‘classic’ invertebrate model systems (Table 1). This diversity holds clear ramifications for understanding organismal physiology. The lineage specific duplication of HVA channels, absent in other lophotrochozoans is clearly independent from the vertebrate Ca_V radiation and presents opportunity for unique neofunctionalization of these genes in platyhelminth physiology. This may be particularly important given the lack of Ca_V3 channels in parasitic platyhelminths and the additional absence of Na_V channels in schistosomes [79]. Schistosomes therefore possess a smaller complement of voltage-gated cation influx channels for supporting excitable tissue physiology that exists in the free-living turbellarians. This narrow repertoire of voltage-gated channels, perhaps reflecting the massive gene loss associated with the evolution of parasitism [35, 91], provides opportunity for chemotherapeutic attack and highlights the need for understanding the molecular basis of neuronal and tissue excitability in these pathogens that is clearly divergent from their hosts.

Finally, it is well known that mammalian Ca_V subunits increase their diversity through alternative splicing and this molecular diversity can impart different functionalities [92–94]. For example, 40 splice variants at a dozen loci have been identified within the human $Ca_V1.2$ subunit [95] and specific variants show altered pharmacological, regulatory or electrophysiological signatures [93, 95]. We have identified, but not characterized, splice variants of both Ca_V1A and Ca_V1B in planarians (Figure 2A), and suggest the functional repertoire of Ca_V activity in platyhelminths is likely further expanded by heterogeneity introduced by alternative splicing. In summary, the presence of multiple $Ca_V\alpha$ genes and associated variants in platyhelminths provides molecular substrate for customization and fine-tuning of responses in the excitable tissues of these organisms.

4.2 Ca_V properties

What do we know about the molecular pharmacology of platyhelminth Ca_V channels? The answer is currently little. Practical difficulties in heterologously expressing Ca_V clones have precluded direct functional insight into the pharmacological profile of these channels. Interpretation of whole animal responses to known Ca_V modulators is also risky – as the lack of responsiveness to agents established to target mammalian Ca_Vs may simply result from pharmacokinetic considerations (e.g. failure of drug accumulation, xenobiotic defenses) rather than a divergent molecular pharmacology of the channel itself. For example, in *C.*

elegans, nifedipine lacks efficacy against intact worms but is able to antagonize the nematode Ca_V1 channel (EGL-19) in dissociated specimens [96–98]. Most of our knowledge about flatworm Ca_V pharmacology therefore depends on three approaches – (i) recordings of endogenous currents and contractile responses in dissociated preparations, (ii) hits to known Ca_V modulators in pharmacological screens for anithelminthics, and (iii) bioinformatic prediction of pharmacological properties from cloned flatworm Ca_V sequences. We will briefly discuss these approaches in turn.

4.2.1 Endogenous Ca_V responses—Ca_V channel activity can be resolved by studying excitable cells in flatworm musculature and nervous systems. However, electrophysiological recording has not proved trivial. Endogenous currents are generally small (e.g. I_{max} <100pA in *S. mansoni* muscle fibres [60, 61] and ~200pA in *Dugesia* muscle fibres [99]) and prone to rapid rundown within several minutes. Therefore most data derives from studying the contractile response of intact organisms [45, 48, 50] or dissociated muscle fibres [60, 61, 99]. Contractions of isolated *S. mansoni*, *D. japonica* and *Bdelloura candida* muscle fibres require extracellular Ca^{2+} and can be inhibited by blocking Ca_V function with known Ca_V modulators [60, 61]. The major classes of Ca_V1 ligands are the dihydropyridines (DHPs; antagonists and agonists, e.g. nifedipine and S-(−)-Bay K8644), benzothiazepines (BZs; antagonists, e.g. diltiazem) and phenylalkylamines (PAs; antagonists, e.g. verapamil). Both depolarization and neuropeptide-evoked contractions are inhibited by nicarpidine, as well as verapamil and methoxyverapamil at higher concentrations [60, 61]. Other DHPs, diltiazem, or peptide neurotoxins, fail to inhibit contractile responses [60, 61]. Consistent with this profile, recent data has shown the voltage-operated Ca^{2+} currents in muscle are reversibly inhibited by verapamil (DHP derivatives were not tested [29]). Intriguingly, this general pharmacological signature parallels the drug profile from the planarian regeneration assay (nicardipine > verapamil, other Ca_V1 modulators without effect [20]). Less is known about Ca_V currents in the nervous system: although dissociated neurons from an ectoparasitic turbellarian (*B. candida*) displayed depolarization-evoked Ca^{2+} currents that were attenuated by verapamil (~30% decrease, [100]). Therefore, our knowledge about endogenous Ca_V currents remains quite limited. Key data that is lacking in schistosomes is the resolution of an endogenous PZQ-evoked Ca^{2+} signal in muscle fibres.

4.2.2 Drug Screens—While interest in flatworm Ca_V channels has predominantly related to their status as a candidate target for PZQ (Section 3), this relationship underscores the potential of Ca_Vs as antihelminthic targets in their own right. Ca_V1 modulators have emerged as preliminary 'hits' in recent unbiased screens (<2,000 compounds) aimed at discovering new antischistosomals. Verapamil passed an initial phenotypic typing (<3% compounds) against schistosomula but not subsequent screening [101]. Methoxyverapamil and felodipine were also identified as compounds (2 from 30) that delayed miracidial transformation [102]. Nicardipine is also known to be lethal to miracidia [103]. Given the inherent snapshot nature of such unbiased screens (often building from a single compound dosage at a single life cycle stage), it is best to draw only general conclusions. Notably, the pharmacological theme from the tissue assays (section 4.2.1) is extended - BZs and common DHP blockers seem poorly represented, while PAs possess some effectiveness.

4.2.3 Predicted pharmacology of Platyhelminth Ca_Vs—In the absence of a large experimental dataset, what can sequence analysis alone tell us about the pharmacological signature of the platyhelminths Ca_Vs? Sequence comparison between Ca_V channels with different pharmacological profiles has guided mutagenesis to reveal mutants with impaired or *de novo* drug sensitivities and thereby identify key residues for ligand binding. Worth bearing in mind is the planarian data showing opposing activities of Ca_V1A and Ca_V1B on PZQ efficacy *in vivo* [25]: a*gonists* at Ca_V1A would be desired to phenocopy PZQ activity,

whereas *antagonists* of Ca_v1B should sensitize organisms to PZQ. Therefore, a structural basis for loss of agonist efficacy at Ca_v1A, and impairment of antagonist potency at Ca_v1B may help explain why agents widely used to modulate vertebrate Ca_vs fail to act as schistocidal agents. Given existing Ca_v1 blockers are widely used cardiovascular therapeutics, this discussion is not meant to infer clinical utility, simply to illuminate why conventional Ca_v ligands fail to support the 'Ca^{2+} hypothesis' of PZQ action. For example, this analysis may illuminate why PZQ activity, mediated by Ca_v1A activation, cannot be blocked by conventional Ca_v1 antagonists or why antagonists at Ca_v1B are in themselves toxic (note results from Ca_v blockers in drug screens, section 4.2.2). Auguring from *in silico* analysis alone is obviously hazardous: often the effects of single amino acid variation can be counterintuitive when analyzed in isolation, and the lack of biophysical characterization of the platyhelminth channels is a problem when drug affinity is closely linked to channel state (e.g. dihydropyridine block is dependent on the voltage sensitivity of mammalian L-type Ca_vs). Nevertheless, the large body of experimental and modeling studies [104–110] addressing the structural basis of ligand sensing at Ca_v channels (including studies in invertebrates [97, 111–113]), permits worthwhile speculation on the pharmacological profile of the flatworm Ca_vs.

All three major classes of Ca_v ligands (DHPs BZS & PAs) share interactions with critical ligand-sensing residues [97, 105–110], as well as proposed direct interactions with a Ca^{2+} ion coordinated within the selectivity filer [106–108]. Many of the residues known to interact with these drugs are conserved in the platyhelminth Ca_vs (for example, 24/28, 12/14 and 13/14 residues experimentally evidenced to regulate DHP, BZ and PA binding in mammalian $Ca_v1.2$ channels are identical in the planarian Ca_v1A channel). However, such analysis is too crude to predict drug effectiveness as single amino acid differences are sufficient to abrogate DHP agonism and antagonism [104]. More detailed scrutinization is required.

First, consider DHP binding. Two groups of residues are important for DHP binding to Ca_v1 channels – residues that comprise the actual DHP-binding pocket and residues that allosterically impact DHP-binding [96, 114]. Comparison of these residues between rat ($Ca_v1.2$), planarian (*Dj*-Ca_v1A & *Dj*-Ca_v1B) and *Schistosoma mansoni* (*Sm*.Ca_v1A & *Sm*.Ca_v1B) Ca_v clones (Figure 2B) reveals the extent of sequence variation. Some of these changes are conservative and/or exist in the *C. elegans* channel sequence, which retains sensitivity to many DHPs *in vitro* [96], so they are likely not key determinants of DHP action. However, other common and unique variants merit discussion. First, are two amino acids in domain III that are variant in both the planarian and schistosome Ca_v1 channels (Q1043 & M1161 in rat $Ca_v1.2$). The M1161 substitution (M to I in the platyhelminth Ca_v1 channels) was identified as a polymorphism in a *C. elegans egl-19* mutant (~Ca_v1) that conferred resistance to nemadipine-evoked growth defects [97]. This feature may in itself confer low sensitivity to DHP blockade to the platyhelminth Ca_v1 channels [25]. The M1161 residue in domain IIIS6 is thought to contribute to a hydrophobic pocket which interacts with the 'portside' methyl group of DHP ligands [107], and mutagenesis of this residue (to alanine) has been shown to decrease DHP binding affinity (~10-fold [109]) and blockade (~100-fold [97]) in $Ca_v1.2$ channels. Second, the highly conserved glutamine in IIIS5 (Q1043 in rat $Ca_v1.2$) shows variation (Q/E, or Q/V in *Dj*-Ca_v1A). This residue is also unique to DHP modulation (not required for PA or BK binding), and is thought to hydrogen bond with one of the carbonyl oxygen of DHP ligands [107]. Restoration of this residue to glutamine can have variable effects, with evidence for increased, decreased or no effect on DHP blockade depending on the context of the mutation (in isolation, or in concert with other changes [111]) and the channel backbone [115]. Variation at this residue may also impact the potency of DHP agonists (see [111]). The Q/E mutant when introduced into a rat Ca_v backbone significantly reduced potentiation by (S)-(−)-Bay K8644 suggesting the

complete amide side chain of the glutamine residue was needed for full agonist efficacy [115]. Both schistosome Ca_v1 variants have an asparagine at this location, and this variation may contribute to the lack of potency of (S)-(−)-Bay K8644 in phenocopying PZQ as a schistocidal agent.

Beyond examples of variation common to all platyhelminth Ca_v1 proteins, *Sm*.Ca_v1A is also notable in showing poor conservation of other residues known to be important for DHP binding (only 20/28 identical residues). The additional variation encompasses dual tyrosine residues in the IIIS6 (Y1152 in $Ca_v1.2$ vs F1062 in *Sm*.Ca_v1A) and IVS6 transmembrane domains (Y1463 vs H1363) which are also considered hydrogen bonding partners with DHP ligands. Indeed, coupled with variation at Q1043 discussed above, it appears *Sm*.Ca_v1A shows non-identity across the triad of residues suggested critical for DHP coordination [107]. The variation of S1115 in planarian Ca_v1A (represented by alanine) also merits comment. S1115 is located three residues proximal to the selectivity filter glutamate residue in the domain III P loop. The S1115A mutational change in rat $Ca_v1.2$ reduced the affinity for DHP antagonists (~60-fold reduction in nitrendipine blockade), and also abrogated responsiveness to the DHP agonist S-(−)-Bay K8644 [113]. This alanine is also present in other invertebrate Ca_v channels that exhibit a blunted pharmacological responsiveness to DHPs [112, 116]. Reverse engineering of the same mutation (A to S) restored sensitivity to nitrendipine and S-(−)-Bay K8644 [112].

Therefore, sequence prospecting provides ample reason to explain the low sensitivity of platyhelminth Ca_vs to conventional DHP blockade. The DHP that is most effective at retaining antagonistic effects against parasitic [60, 61] and free-living [20] flatworms Ca_v channels is nicardipine. Is there a structural basis for this observation? Nicardipine possess an ionizable alkylamino group on the 5-position of the pyridine ring and recent studies have suggested that this bulky substituent may interact with unique residues beyond the conventional DHP binding site [117], providing a possible explanation as to why nicardipine can target platyhelminth Ca_v channels. One recent study brought focus on the role of a residue in IIS6 (A752), which is conserved in the all schistosome and planarian Ca_v1 channels [114]. Nicardipine therefore may present a structural framework for iteration of DHP-based ligands to identify novel antagonists of platyhelminth Ca_vs.

What about blockade by the other classes of Ca_v1 antagonists? Again the majority of residues experimentally implicated in PA and BZ binding appear conserved in the platyhelminth channels (up to 13/14 key residues examined for each class). Again the devil is in the detail, and one possible explanation for the lower sensitivity of these agents is the occurrence of a serine substitution of an important alanine residue in the platyhelminth Ca_v1B variants (and *Dj*-Ca_v1A). Studies of mammalian Ca_v1 channels, have implicated a triad of residues within the IVS6 transmembrane region (‘YAI triad’) as necessary for high affinity phenylalkylamine blockade through interactions with the amino group proximal methoxylated aromatic ring of many PAs [106, 118]. The alanine to serine substitution in the platyhelminth Ca_v1 channels correlates with a substitution (A1467S in rat $Ca_v1.2$) that has been shown to decrease the affinity of desmethoxyverapamil block by ~11-fold [118]. The importance of the YAI triad also holds for BZ binding, as these residues comprise part of the shared binding site for the two different classes of ligand [119]. Although this alanine residue is conserved in *Sm*.Ca_v1A, this particular Ca_v variant is predicted to harbour substitutions of the two other residues in the critical ‘YAI’ triad (‘HAV’ in *Sm*.Ca_v1A, resulting from a Y1463H and a conservative I1470V substitution) as well as another critical tyrosine residue for PA binding in IIIS6. This tyrosine residue in IVS6 (Y1463 in rat Cav1.2 numbering), and the second tyrosine residue in IIIS6 (Y1152 in rat Cav1.2 numbering) are both thought to form hydrogen bonds with the methoxy groups of the two PA aromatic rings [106] are both represented by different residues (Y/F and Y/H) in Sm.Ca_v1A. These changes

would be predicted to decrease PA sensitivity of *Sm*.Ca_V1A on the basis of prior mutagenesis studies: the Y1152F substitution in rat Cav1.2 caused an increase (~18-fold) in the concentration of desmethoxyverapamil needed for current blockade [110], and a double mutant (Y1152F, Y1463F) was reported to decrease the potency of desmethoxyverapamil blockade by 100-fold [110]. The Y1463 residue in IVS6 is also important for regulating the potency of diltiazem blockade [120]. Two further residues that regulate BZ (but not PA) block also show variation between the platyhelminth Ca_Vs. First, *Sm*.Ca_V1A contains an alanine in domain IVS6 (A1364 in *Sm*.Ca_V1A) which is represented by methionine in the other platyhelminth Ca_Vs (and vertebrate $Ca_V1.2$). The corresponding mutation M1464A has been shown to decrease sensitivity to diltiazem by ~3-fold [120]. Restoration of this methionine in an invertebrate Ca_V1 also increased the potency of isradipine (a DHP) blockade [111]. Second, *Sm*.Ca_V1B (and *Dj*-Ca_V1A) contain a conservative substitution in IIIS6 at position 1150 (I1150V in $Ca_V1.2$ numbering): the I1150A mutation has also been shown to decreases BZ sensitivity by ~3-fold [120].

Collectively, these observations suggest a molecular basis for the poor susceptibility of the platyhelminth Ca_V channels to blockade by the Ca_V1 blockers widely used in mammalian systems. This divergence explains why conventional Ca_V1 blockers have provided only equivocal support for the hypothesis that PZQ stimulates L-type Ca_V channels, and caution against the lack of such evidence being used to argue against an activity of PZQ against schistosome Ca_Vs. Overall, while the divergent pharmacological profiles of the platyhelminth and mammalian Ca_V1 channels is frustrating for impeding molecular dissection of the action of PZQ and understanding Ca_V1 physiology in flatworms, there is a silver lining; the realization that divergent pharmacology may ultimately augur selectivity in the activity of novel ligands targeting flatworm Ca_Vs as novel drug leads for treating schistosomiasis.

5. Refractoriness to PZQ action

Molecular insight into the targets/effectors of PZQ action and adaptatory mechanisms will likely come from analysis of situations where PZQ efficacy is decreased. These include *de novo* mechanisms that emerge in individual strains (‘drug resistance’), natural variation in PZQ potency during the schistosome life cycle [22–24], as well as comparative phylogenetic analysis probing PZQ effectiveness. Some brief comments on these scenarios in the context of the ‘Ca^{2+} hypothesis’ of PZQ action are worthwhile. While PZQ activity is Ca_V1 dependant when measuring acute responses (Ca^{2+} influx, contraction) in cultured flatworms, it is obvious that the anhelminthic effect of PZQ applied to an *in vivo* infection involves a broader array of influences. These encompass the host immune system [121, 122], as well as the mechanisms controlling PZQ pharmacokinetics [123] and the downstream effectors of Ca^{2+} influx in the parasite itself. Therefore, it is not difficult to envisage how changes in this broader interactome could impact PZQ efficacy independently from any alteration in target receptor(s) for PZQ. Therefore, in the context of PZQ resistance, a failure to detect changes in specific Ca_V channel components [124, 125], or a coupling of the Ca_V complex to effector mechanisms [47] should not detract from the ‘Ca^{2+} hypothesis’ of PZQ action. PZQ sensitivity could be decreased by changes in pathways both upstream (drug handling) and downstream components (effectors) independent from alterations in the primary drug target, and unraveling such changes is important for identifying novel druggable targets. Identification of such targets will come from unbiased examinations of gene expression in scenarios of natural and acquired refractoriness to PZQ. Indeed, recent microarray profiling studies provide the first chapter of research exploiting such methods [125–127]. Such data are generally supportive of an adaptive organismal Ca^{2+} toolkit as part of the broader transcriptional adaption to PZQ exposure. Equally, for the free-living planarians it will be important to understand species specific differences in PZQ efficacy. The PZQ-evoked

bipolar regeneration of *D. japonica*, which is a frequently used strain to study drug responsiveness, is not replicated in other planarian species and the molecular basis for this difference is unexplored.

5. Conclusions

The purpose of this review was to discuss the action of PZQ on planarian flatworms and bring new perspective onto *in vivo* targets relevant to the efficacy of this important therapeutic. The planarian data suggest a focus on specific Ca_V1 complexes coupling to neuronally derived signaling pathways. Therefore, our data studying the effects of PZQ on planarian regeneration are broadly supportive of the 'Ca^{2+} hypothesis' of PZQ action despite studying a unique organismal output (tissue regeneration) in an amenable model system not widely exploited for antischistosomal drug research. This review has highlighted the divergent molecular pharmacology of platyhelminth Ca_V channels from their human counterparts, a principle likely shared by downstream Ca_V effectors and a property that could be exploited by directed drug design in future. Obviously, a variety of different targets for PZQ have been proposed, and our intent was not to discriminate amongst these viable ideas beyond illustrating common principles of PZQ action on Ca^{2+} signaling in the different flatworm systems. PZQ may have more than one target *in vivo*, a useful promiscuity that could delay the emergence of drug resistance. Argueably more important than target definition *per se* is the realization that Ca_V channels and their downstream effectors represent novel targets for novel antihelminthic drugs.

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Highlights

- The antischistosomal action of PZQ may derive from dysregulated Ca^{2+} homeostasis
- New molecular insight supporting this model comes from the planarian model system
- PZQ causes Ca^{2+} influx in a neuronally derived population via a specific Ca_V complex
- The utility of the planarian model for antischistosomal drug research is discussed

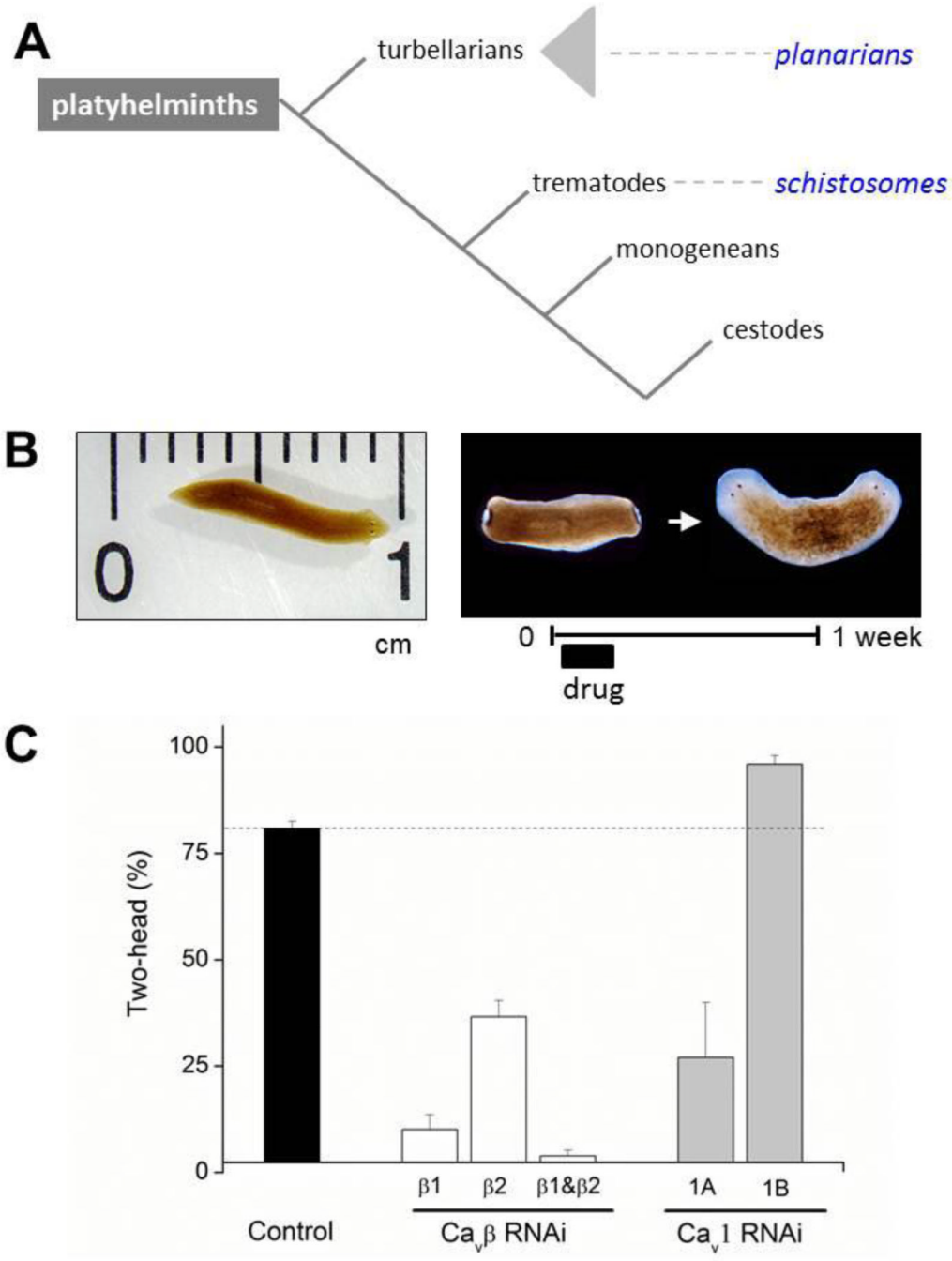

Figure 1. PZQ effects on regeneration of the planarian *Dugesia japonica*

(**A**) Overview of the relationship between free living planarian species to the parasitic platyhelminth groupings. (**B**) Left, image of intact *D. japonica.* Right, bipolar trunk fragment regenation after exposure to ±PZQ (70μM, 48hrs). Anterior structures are visually apparent by the presence of the eyespots. This regeneration assay is robust and no polarity defects are observed in the absence of drug (i.e. by surgery alone). (**C**) Penetrance of PZQ-evoked two-headed regeneration in *D. japonica* following RNAi knockdown of different Ca_v complex subunits. Data are summarized from [20, 25].

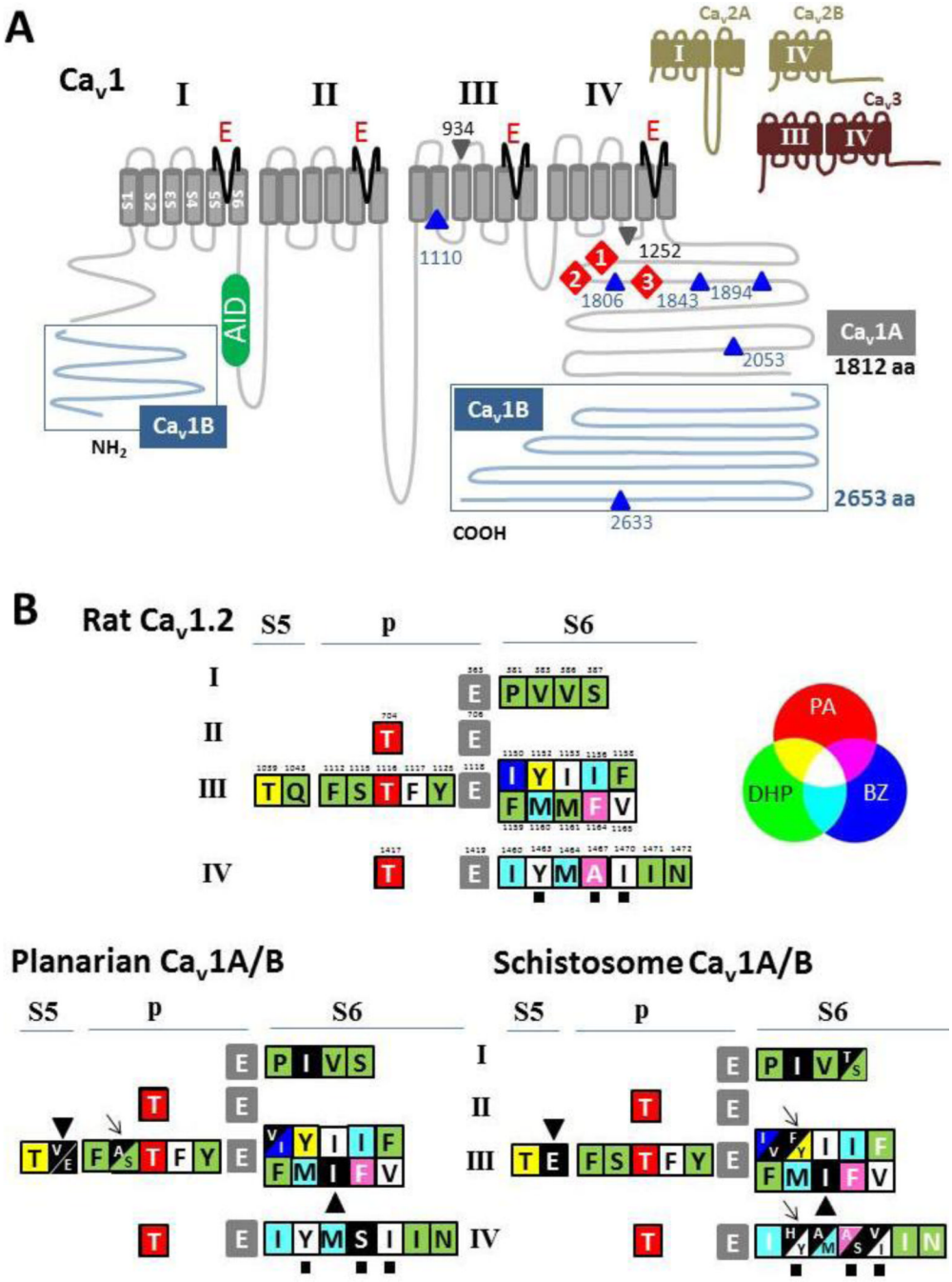

Figure 2. Molecular architecture of planarian Ca$_v$ channels

(**A**) Schematic representation of planarian Ca$_v$1 isoforms (Ca$_v$1A & Ca$_v$1B). While Ca$_v$1B has longer NH$_2$ and COOH terminal regions (blue) than Ca$_v$1A, both isoforms share a similar topology (domains I–IV with six transmembrane helices, S1–S6) and contain similar motifs. These include the 'EEEE' selectivity filter motif (red), the alpha-interacting domain (AID) in the I–II linker (green) and the cytoplasmic COOH-terminal regions (1, EF; 2, preIQ3; 3, IQ, red diamonds) known to be important for Ca^{2+} regulation of mammalian Ca$_v$1 isoforms. Splice isoforms of both Ca$_v$1A (2 variants, grey triangles) and Ca$_v$1B (6 variants, blue triangles) have been identified at the indicated residues in Ca$_v$1A and Ca$_v$1B respectively. *Inset*, schematic depicting characterized regions of the partially cloned

planarian Ca_Vs (Ca_V2A/2B & Ca_V3). (**B**) Overview of residues important for binding Ca_V ligands and their variation in platyhelminth Ca_Vs. Residues identified in experiments as being important for dihydropyridine (DHP), phenylalkylamine (PA) and benzothiazepine (BZ) binding (summarized in [97, 106–108]) are depicted schematically in terms of their localization (domains I to IV, transmembrane regions S5 & S6, and intervening 'p' loop) in rat (numbering of rat Cav1.2, Genbank M67515.1), planarian and schistosome Ca_V channels. Colouring indicates importance of residue for binding a particular class of ligand, as per additive colour map. For example, if a residue is important for binding all three classes of ligand it is shown in white. Selectivity filter glutamates are shown in grey. Residues that show variation in the platyhelminth Ca_Vs are shown in black, and variation that may be critical for ligand binding is highlighted by different symbols (discussed in text). The 'YAI' triad in domain IVS6 is identified by solid squares.

Table 1

Diversity of Lophotrochozoan Ca_V channels.

		Ca_V1			Ca_V2		Ca_V3
		↓			↓		↓
	Caenorhabditis elegans	•			•		•
	Drosophila melanogaster	•			•		•
LOPHOTROCHOZOANS							
	Capitella capitata	•			•		•
	Lottia gigantea	•			•		•
	Lymnaea stagnalis	•			•		•
Platyhelminths							
Turbellarians	*Dugesia japonica*	•		•	•		•
	Schmidtea mediterranea	•		•	•		•
Trematodes	*Schistosoma mansoni*	•		•	•		
	Schistosoma japonicum	•		•	•		
	Schistosoma haematobium	•		•	•		
	Clonorchis sinensis	•		•	•		
Cestodes	*Taenia solium*	•		•	•		
	Echinococcus multilocularis	•		•	•		
	Hymenolepis microstoma	•		•	•		
		↑		↑	↑		↑
		Ca_V1A		**Ca_V1B**	**Ca_V2A**		**Ca_V2B**	**Ca_V3**

Ca_V channel diversity was examined in classic invertebrate model systems, as well as available lophotrochozoan sequence data. Dots indicate the presence of Ca_V channels assigned by homology to indicated Ca_V subunits. Notable outcomes are Ca_V1 and Ca_V2 duplication in platyhelminths compared with other lophotrochozoans and the absence of Ca_V3 channels in parasitic platyhelminths. Sequence identifiers are from NCBI unless otherwise noted: *C. elegans* (NP_741442.1, NP_741734.1, NP_741848.1), *D. melanogaster* (Q24270.2, P91645.3, NP_001245544.1), *C. capitata* (Capca1_51954, Capca1_51958, Capca1_89566)[JGI], *L. gigantea* (Lotgi1_51270, Lotgi1_216445 & Lotgi1_119993, Lotgi1_91235)[JGI], *L. stagnalis* (AAO83838.2, AAO83841.1, AAO83843.2), *D. japonica* (AEJ87267, AEJ87268, AEJ87269, AEJ87270, AEJ87271), *S. mansoni* (Smp_020270, Smp_159990,Smp_020170, Smp_004730)[GeneDB], *S. japonicum* (Sjp_0099010, Sjp_0010120 and Sjp_0010110, Sjp_0005280 and Sjp_0073860, Sjp_0016770, Sjp_0096680), *S. haematobium* (Sha_105781, Sha_200459, Sha_105898, Sha_101457 and Sha_107907)[schistodb.net], *C. sinensis* (GAA55733.1, GAA30063.2, GAA52227.1, GAA56330.1), *T. solium* (TsM_000783400 and TsM_000783500, TsM_000442900, TsM_000598200, TsM_000175700), *Echinococcus multilocularis* (EmuJ_000143800.1, EmuJ_000961000.1, EmuJ_000146300.1, EmuJ_000890600.1)[GeneDB], *Hymenolepis microstoma* (HmN_000242200, HmN_000186400, HmN_000427400 and HmN_000427500, HmN_000387900)[GeneDB]. No identifiers tags are listed for *S. mediterranea* given the current nature of the genome assembly.