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Calmodulins from *Schistosoma mansoni*: Biochemical analysis and interaction with IQ-motifs from voltage-gated calcium channels

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ABSTRACT

The trematode *Schistosoma mansoni* is a causative agent of schistosomiasis, the second most common parasitic disease of humans after malaria. Calcium homeostasis and calcium-mediated signalling pathways are of particular interest in this species. The drug of choice for treating schistosomiasis, praziquantel, disrupts the regulation of calcium uptake and there is interest in exploiting calcium-mediated processes for future drug discovery. Calmodulin is a calcium sensing protein, present in most eukaryotes. It is a critical regulator of processes as diverse as muscle contraction, cell division and, partly through interaction with voltage-gated calcium channels, intra-cellular calcium concentrations. *S. mansoni* expresses two highly similar calmodulins – SmCaM1 and SmCaM2. Both proteins interact with calcium, manganese, cadmium (II), iron (II) and lead ions in native gel electrophoresis. These ions also cause conformational changes in the proteins resulting in the exposure of a more hydrophobic surface (as demonstrated by anilinonaphthalene-8-sulfonate fluorescence assays). The proteins are primarily dimeric in the absence of calcium ions, but monomeric in the presence of this ion. Both SmCaM1 and SmCaM2 interact with a peptide corresponding to an IQ-motif derived from the α-subunit of the voltage-gated calcium channel $SmCa_v1B$ (residues 1923–1945). Both proteins bound with slightly higher affinity in the presence of calcium ions. However, there was no difference between the affinities of the two proteins for the peptide. This interaction could be antagonised by chlorpromazine and trifluoperazine, but not praziquantel or thiamylal. Interestingly no interaction could be detected with the other three IQ-motifs identified in *S. mansoni* voltage-gated ion calcium channels.

1. Introduction

Calmodulin is a small (~17 kDa) calcium sensor protein which is found in almost all eukaryotes. It is known to function in calcium signalling in a wide range of distinct cellular processes including muscle contraction, calcium homeostasis and inflammation [1]. Structurally, it consists of two globular heads, linked by an extended α-helix [2,3]. Within each globular head are two EF-hand motifs which are capable of binding calcium and other divalent cations. The EF-hand is a compact structure in which the polypeptide backbone turns tightly, creating a space for divalent cation binding in which carbonyl and carboxylate ligands point inwards to coordinate the ion [4,5]. On binding calcium ions, calmodulin undergoes a conformational change in which the protein's surface becomes more hydrophobic [6–8]. This facilitates interaction with a range of target molecules [9,10]. Typically, the linker helix partially "melts" enabling the two heads to move closer together and for the protein to wholly or partially wrap around extended target sequences.

One key target motif is the IQ-motif. This has the consensus sequence IQxxxRGxxxR; however considerable variation on this sequence is possible. The motif is found in proteins as diverse as myosin superfamily members, the cytoskeleton regulating IQGAP family proteins and voltage-gated ion channels. The effect of calcium ions on calmodulin-IQ-motif interactions varies. In some cases, the interaction requires calcium ions whereas others are promoted in the absence of calcium and some are independent of calcium concentrations [11–13]. Structurally, the IQ-motif typically forms an extended α-helix which calmodulin (and structurally similar proteins such as myosin light

Abbreviations: ANS, anilinonaphthalene-8-sulfonate; BS^3, *bis*(sulfosuccinimidyl)suberate; CPZ, chlorpromazine; PZQ, praziquantel; rmsd, root mean square deviation; SmCaM1, one of the two calmodulins from *S. mansoni*; SmCaM2, one of the two calmodulins from *S. mansoni*; TFP, trifluoperazine; ThA, thiamylal; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride

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chains) can wrap around [14–17].

Voltage-gated calcium channels (Ca_v channels) regulate the influx of calcium into cells. These channels are multimeric protein complexes consisting of a large, pore-forming Ca_v α_1 subunit, attached to two smaller auxiliary subunits, termed Ca_v β and Ca_v $\alpha_2\delta$ [18]. The cytoplasmic β subunit acts to modulate the activity of the channel whilst directing expression to the plasma membrane [19], whereas the heterodimeric $\alpha_2\delta$ subunit acts as a transmembrane receptor for various anti-epileptic drugs [20]. Although both auxiliary subunits play important regulatory roles in the protein complex, it is the α_1 subunit that primarily defines the pharmacological and functional profile of the channel. Ca_v α_1 subunits are defined by the type of current they gate, and can be distinguished by various kinetic parameters or pharmacological characteristics [18]. For example, long-lasting, L-type Ca_v channels have a high voltage of activation (HVA), and a slow inactivation rate [18]. These channels are formed by Ca_v1-type α_1 subunits (including the $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$ and $Ca_v1.4$ sub-families) and are further characterised by their sensitivity to various antagonistic drugs, most notably those of the dihydropyridine (DHP) class [21–23]. Mammals typically express a complement of ten Ca_v α_1 subunits, whereas invertebrate species tend to contain a single representative from each Ca_v1, Ca_v2 and Ca_v3 subtypes [18,24]. However, these trends do not apply to members of the Schistosoma genus [25]. Initial work revealed the existence of three *Schistosoma mansoni* voltage-gated calcium channels ($SmCa_v$), designated $SmCa_v1A$, $SmCa_v2A$ and $SmCa_v2B$ [26]. Subsequent annotation of the *S. mansoni* genome indicated the existence of a fourth, designated $SmCa_v1B$ [27]. Furthermore, as $SmCa_v1$ α_1 subunits ($SmCa_v1A$ and $SmCa_v1B$) share highest sequence identity with vertebrate Ca_v1 (L-type) channels, whereas $SmCa_v2$ isotypes ($SmCa_v2A$ and $SmCa_v2B$) share highest sequence identity with Ca_v2 (P/Q, N and R-type) channels, this suggests that schistosomes encode four HVA channels, yet possess no equivalent to the LVA (T-type) channel [25].

Depending on local or global calcium ion concentrations, Ca^{2+}-CaM binding at the C-terminal region of the Ca_v α_1 subunit can either attenuate the influx of calcium ions through the channel pore, in a process called calcium-dependent inactivation (CDI), or it can promote calcium influx, in a process called calcium-dependent facilitation (CDF) [28–32]. Furthermore, the C-terminal region of the α_1 subunit is believed to function as a binding site for apo-CaM, allowing constitutive tethering of the calcium sensor [28,33,34].

Schistosomiasis (bilharzia) is a parasitic disease which results from infections by worms from the genus Schistosoma. It is second only to malaria in terms of the number of humans affected by parasitic infections and the majority of cases are in the developing world [35]. In schistosomes (and some other helminth parasites) disruption of calcium homeostasis is one consequence of the administration of the anthelminthic drug praziquantel (PZQ) [36–42]. A number of authors have hypothesised that PZQ acts by disruption of voltage-gated calcium channels; however, the molecular details of this have yet to be discovered [38,39]. Nevertheless, this suggests that calcium signalling molecules are worthy of further study as potential targets for novel anti-schistosomal drugs. It has been shown previously that *Schistosoma mansoni* has two calmodulin isotypes. These two isotypes are highly similar, differing only by two amino acid residues towards the C-termini [43]. Despite this high level of similarity the available evidence suggests that the proteins are derived from separate genes [43]. The calmodulin antagonist trifluoperazine (TFP) reduced miracidium-to-sporocyst transformation in a dose-dependent manner suggesting a critical involvement for one or both calmodulin isotypes in this process [43]. This compound has also been shown to collapse the tegumental membrane potential and to disrupt the ultrastructure of the tegument [44]. Earlier work demonstrated that calmodulin is also required for egg hatching in *S. mansoni* [45]. Here, we present the biochemical characterisation of the two known calmodulin isotypes from *S. mansoni* including the identification of a likely binding site in one of the parasite's voltage-gated calcium channels.

2. Materials and methods

2.1. Molecular modelling

Initial molecular models of SmCaM1 and SmCaM2 in the "closed" conformation were generated using Phyre2 in the intensive mode [46,47]. These were energy minimised using YASARA (http://www.yasara.org) [48]. To generate calcium-bound forms of the two proteins, the minimised models were aligned (using PyMol, www.pymol.org) with the most similar structure identified by Phyre2 (potato calmodulin, 1RFJ [49]). New models were generated by saving either SmCaM1 or SmCaM2 with the calcium ions from 1RFJ. To generate the final, calcium-bound structures, these models were then minimised with YASARA. The templates for the remaining *S. mansoni* calmodulin (SmCaM1 and SmCaM2) homology models were: Ca^{2+}-CaM in the "open" conformation, *Rattus rattus* calmodulin (PDB: 3CLN) [50]; Ca^{2+}-CaM in the "collapsed" conformation, Bos taurus calmodulin (PDB: 1PRW) [51]; Apo-CaM in the homodimeric state, *Rattus norvegicus* calmodulin (PDB: 1QX5) [52]; Ca^{2+}-CaM-IQ peptide complex, *Homo sapiens* calmodulin in complex with an IQ peptide derived from the human $Ca_v1.2$ channel α_1 subunit, $HsCa_v1.2$ (PDB: 2F3Y) [16]. These structures were viewed and edited in PyMol, version 4.40 (http://www.pymol.org/) using the mutation function to alter the sequences to match the *S. mansoni* ones. The resulting SmCaM1 and SmCaM2 models were then saved as new molecules, and then computationally solvated and energy minimised using the YASARA [48]. To validate the models, Ramachandran plots were calculated using RAMPAGE (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) [53].

2.2. Expression and purification of SmCaM1 and SmCaM2

Expression vectors for *S. mansoni* calmodulins (SmCaM1 and SmCaM2) were generated by ligation independent cloning into pET-46 Ek/LIC (Merck, Nottingham, UK). This vector introduces nucleotides encoding a hexahistidine tag (amino acid sequence MAHHHHHHVD-DDDK) into the coding sequence. Amplicons were generated by PCR from *S. mansoni* adult-stage cDNA (Schistosomiasis Resource Centre, distributed by BEI Resources, Manassas, VA, USA; Strain PR-1; NR-48633; Lot 62,506,671) using primers designed according to the manufacturer's instructions. These amplicons were purified and inserted into the vector using the manufacturer's protocol. DNA sequences were verified (GATC Biotech, London).

Recombinant *S. mansoni* calmodulin proteins were expressed in *Escherichia coli* Rosetta(DE3) (Merck). Competent cells were transformed according to the manufacturer's instructions, and plated overnight on LB-agar plates supplemented with 100 μg ml^{-1} ampicillin, and 34 μg ml^{-1} chloramphenicol. For the recombinant expression of both SmCaM1 and SmCaM2, single colonies were picked and cultured overnight, shaking at 30 °C, in 5 ml of LB (Miller) broth (Foremedium, UK), supplemented with 100 μg ml^{-1} ampicillin and 34 μg ml^{-1} chloramphenicol. These cultures were used to inoculate larger cultures (1 l LB broth, supplemented with 100 μg ml^{-1} ampicillin and 34 μg ml^{-1} chloramphenicol), which were grown at 22 °C for a further 10 h before induction with 0.4 *g* IPTG (1.7 mM final concentration). IPTG-induced cultures were grown overnight at 16 °C, and cells harvested by centrifugation at 4200 *g* for 30 min. Pellets were resuspended in approximately 20 ml of buffer R (50 mM Hepes − OH, pH 7.5; 150 mM sodium chloride; 10% (v/v) glycerol), and frozen at - 80 °C until required.

Recombinant proteins were purified by cobalt affinity chromatography, as previously described [54]. Briefly, cells were thawed, disrupted by sonication (five 30 s pulses at 100 W, separated by 30 s intervals to prevent overheating), and the suspension clarified by centrifugation (22,000 *g* for 20 min). The sonicate was passed through a