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Studijní program: Biologie

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Calcium and calcium-dependent proteins in biology of schistosomes

Vápník a kalcium dependentní proteiny v biologii schistosom

BACHELOR THESIS

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With special thanks to my supervisor Libor Mikeš for guiding me through all difficulties that have occurred throughout my research with patience and professionalism. I would also like to thank all other members of the parasitology lab, especially Jana Bulantová for providing me amazing images of schistosomes, my friends, family and Alžběta, who have been a great support to me throughout my studies.

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze dne

Abstract:

Blood flukes of genus the *Schistosoma* are blood dwelling parasites that affect over 200 million people causing seriously debilitating disease, schistosomiasis. Like in other life forms, calcium represents one of the key elements in schistosomes.

Calcium affects egg hatching, penetration into the host, evasion of hosts immune system and other crucial aspects of *Schistosoma* life. It can regulate those processes in two ways; either directly, or through interaction with calcium-binding proteins. Those proteins are either expressed in every life stage of schistosomes or they can be stage-specific.

It is those properties of calcium and calcium-dependent proteins, that make them a potent vaccine targets. The first pioneer in the calcium dependent protein based vaccines is soon to come to human trials. Until the efficient vaccine is developed, we are dependent purely on chemotherapy against schistosomiasis. At the moment the drug of first choice, praziquantel is used to treat those who suffer from schistosomiasis. Its mode of action is not entirely known, but is evidently directly linked to calcium homoeostasis of schistosomes.

This thesis focuses on calcium and calcium-dependent proteins because of their role – either direct or indirect – in the stated processes. Also, increased understanding of calcium and calcium dependent proteins will surely shed more light on schistosomes and their host-parasite interactions.

Keywords: Schistosoma, Praziquantel, Calcium, Calcium-dependent proteins, Vaccine, Schistosomiasis, PZQ

Abstrakt:

Motolice rodu *Schistosoma* jsou krevní parazité způsobující závažné onemocnění, schistosomózu, které postihuje více než 200 milionů lidí. Vápník je jedním z klíčových prvků pro život schistosom, podobně jako u všech dalších živočichů. U schistosom ovlivňuje například líhnutí vajíčka, penetraci do hostitele, nebo únik před hostitelským imunitním systémem a další důležité procesy. Tyto děje mohou být závislé buď přímo na vápníku, nebo mohou být ovlivňovány pomocí kalcium-dependentních proteinů. Ty mohou být exprimovány buď ve všech životních stádiích, nebo jejich exprese může být specifická pro jednotlivá stádia.

Právě díky těmto vlastnostem jsou kalcium dependentní proteiny zajímavými kandidáty pro vývoj vakcíny. První vakcína založená na kalcium-dependentním proteinu se momentálně připravuje k testování na lidech. Do doby, než bude účinná vakcína vyvinuta, je jedinou možností boje se schistosomózou chemoterapie. V současnosti je lékem první volby praziquantel, jehož mechanismus účinku není ještě zcela objasněn, ale je zřejmé, že ovlivňuje homeostázu vápenatých iontů v parazitovi, čímž způsobí, ať už přímo, či nepřímo, jeho smrt.

Tato práce se věnuje právě vápníku a kalcium-dependentním proteinům, jelikož se výrazně podílejí na výše zmíněných procesech. Obecné pochopení jejich funkcí jistě rozšíří naše znalosti o schistosomách a jejich interakcích s hostitelem.

Klíčová slova: Schistosoma, Praziquantel, Vápník, Kalcum-dependentní proteiny, Vakcína, Schistosomiáza, PZQ

Table of contents:

List of abbreviations:

ATP – adenosine triphosphate

CaM – calmodulin

CDP – calcium dependent protein

ds-RNA – double stranded ribonucleic acid

EDTA – ethylendiaminotetraacetic acid

EGTA – ethylenglycotetraacidic acid

ER – endoplasmic reticulum

FIG – figure

GFP – green fluorescent protein

IgE – Immunoglobulin G

IgG2 – Immunoglobulin G 2

$Ins(1,4,5)P_3$ – inositol-1,4,5-triphosphate

$InsP_3R$ – inositol-1,4,5-triphosphate receptor

kDa – kiloDalton

PZQ – praziquantel

SEM – scanning electron microscope

SR – sarcoplasmic reticulum

S. haematobium – Schistosoma haematobium

S. japonicum – Schistosoma japonicum

S. mansoni – Schistosoma mansoni

TAL – tegumental allergen like

TCTP – translationally controlled tumour protein

T. regenti – Trichobilharzia regenti

T. szidati – Trichobilharzia szidati

TnC – troponin C

VGCC – voltage gated calcium channel

W-7 – N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (CaM antagonist)

1. Introduction

Schistosomiasis, or bilharziasis, is a parasitic infection caused by blood flukes, mainly *S. mansoni, S. japonicum* and *S. haematobium* that inhabit the veins surrounding organs within the abdominal cavity of their final host, the human, and in the case of *S. japonicum,* many other animals (Ross et al., 2002). In their hosts they cause pathologies by laying eggs, resulting in debilitating granulomas in the the liver, kidneys, bladder and chronic morbidities that are associated with inflammation, anemia or impaired growth (Hotez and Fenwick, 2009). The infection is spread from Africa to South America and Asia with more than 200 million people being infected (Gryseels et al., 2006) and close to 800 million being at risk (Steinmann et al., 2006). Under current conditions such as the climate changes and mass migration, a new risk of schistosomiasis spreading to Europe emerges (Houghton and I, 2001;Serre Delcor et al., 2016). Reports are already coming about permanent presence of schistosomiasis in Corsica (Berry et al., 2016).

Schistosomes, much like other organisms, have processes that are regulated by calcium and calcium-dependent processes. Apart from those general processes the calcium and calcium-dependent proteins facilitate a massive amount of specific processes that are vital for schistosomes survival and reproduction, such as egg formation and their release from the tissues, penetration to the host, host-parasite interactions, immune system evasion and many others (Wu et al., 2014; Liu et al.,2015; Katsumata et al., 1989).

These are a potent target for vaccines and drugs and thus build increasing pressure on a deeper understanding of calcium related processes in schistosomes. With the boom in the genome and proteomic studies, the identification and understanding of molecules and their mechanisms is becoming more simple and shows some promising results. Until the introduction of efficient vaccine, the only way of fighting schistosomiasis is through chemotherapy. For more than four decades praziquantel (PZQ) has been the first choice cure for schistosomiasis (Chai, 2013). Yet the exact pharmacological mechanism of its effect remains still poorly understood except for the fact that it is calcium homoeostasis related process (Chan et al., 2013). Furthermore, the unsettling reports of resistance to PZQ are emerging (Wang et al., 2012) and no vaccine is in use at the moment.

Main aims of this work are to provide overall insight on, and discuss the most important calcium-dependent proteins and calcium related processes, that could be potential vaccine or drug targets or have effect on host-parasite interactions schistosomes. In this review I will primarily focus on *S. mansoni, S. japonicum,* and *S. haematobium,* that are the main cause of human pathologies. I would also like to briefly introduce the reader to the importance of calcium in schistosomes and their vertebrate hosts and discuss the calcium related mechanism of PZQ, which shows effect on calcium homoeostasis, and to sum up some of calcium related observations in schistosome biology.

2. General biology of schistosomes

Schistosomes are parasitic flatworms, members of the family Schistosomatidae, order Strigeidida, class Trematoda within Platyhelminthes. The family recently consists of fourteen genera with *Schistosoma* and *Trichobilharzia* being the most important genera affecting human host, and *Schistosoma* causing seriously debilitating schistosomiasis and the latter causing cercarial dermatitis, which is also known as swimmers itch.

While the schistosomes are quite well known and researched organisms, mainly for the pathologies caused in humans, the primarily avian members of the genus *Trichobilharzia* are much less researched relatives. Schistosomes are endoparasites of vertebrates (mammals and birds) and have water snail intermediate hosts.

In this work I would like to focus on the genus *Schistosoma*, especially the species *Schistosoma mansoni, S. haematobium* and *S. japonicum*, which are also known as the blood flukes, causing the human disease schistosomiasis or bilharziasis in a large part of the world – see FIG. 1.

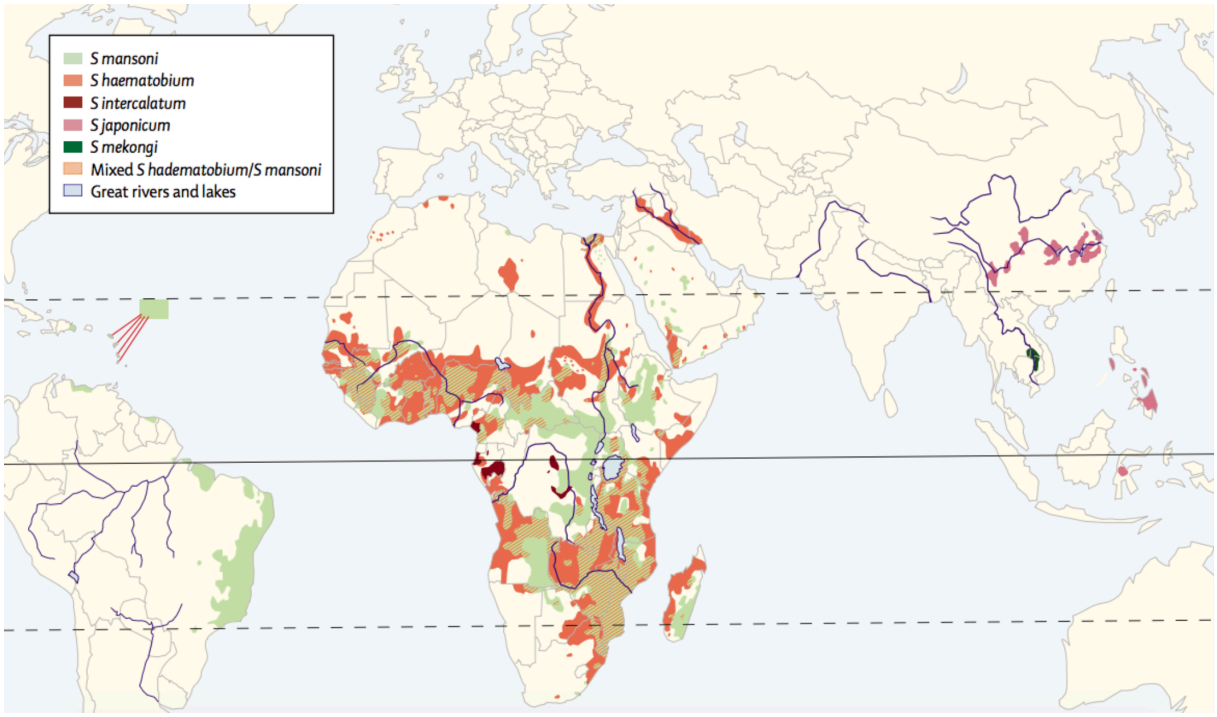

Figure 1 | **The distribution of schistosomes causing human schistosomiasis.**

Image adapted from (Gryseels et al., 2006)

2.1. Schistosoma life cycle

To illustrate life cycles of schistosomes, *Schistosoma mansoni, S. haematobium and S. japonicum* cycles are explained in FIG. 2

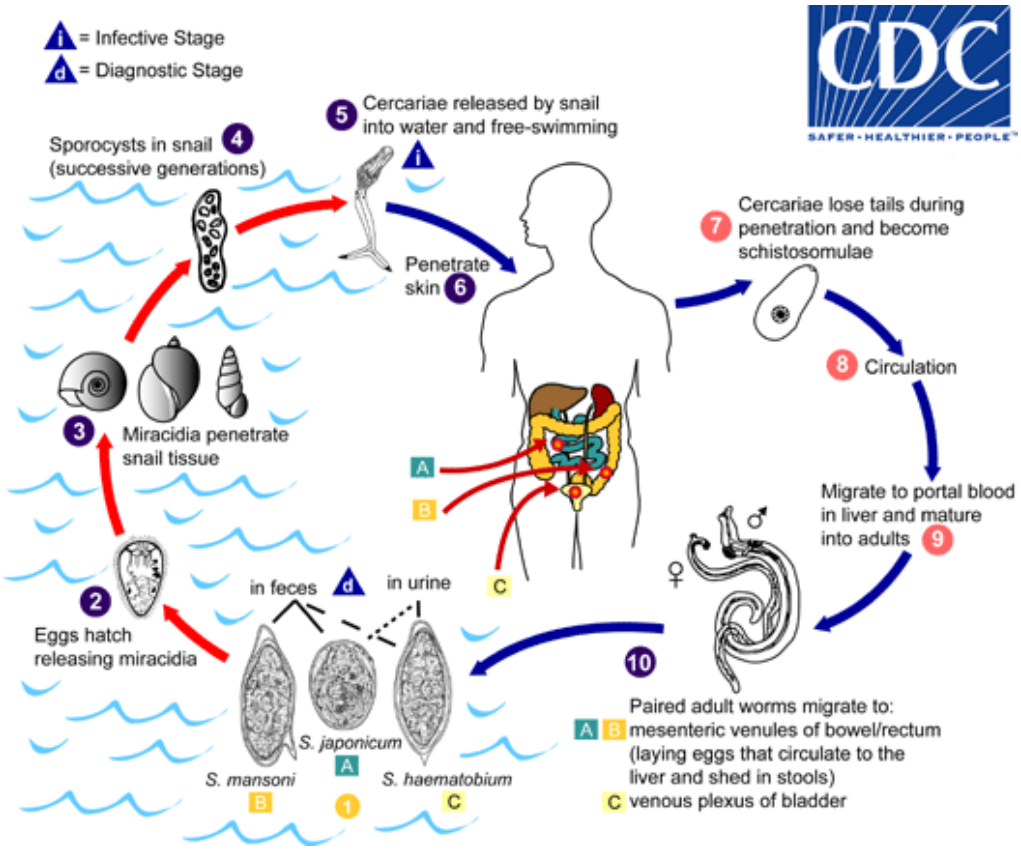

Figure 2 | **The life cycle of schistosomes**

1) The eggs are produced by adult flukes and expelled in urine (*S. haematobium [C]*) or faeces (*S. mansoni [B], S. japonicum [A]*)
2) Under optimal conditions – water environment – miracidia hatch from eggs.
3) Miracidia actively search and penetrate the intermediate hosts – freshwater snails (*Bulinus* for [C], *Biomphalaria* for [B] and *Oncomelania* for [A].) (Jordan, 1988)
4) In the snail, two generations of sporocysts and cercariae are produced.
5) The infective cercariae find their way out of the snails and seek the definitive host, human.
6) While penetrating the hosts skin, cercariae shed their tails and are becoming schistosomules.
7) Schistosomula
8) The schistosomules migrate through the host's tissues and enter the circulatory system.
9) The schistosomules migrate in the veins and mature into adults.
10) Once they pair with their sexual partners in hepatoportal veins, schistosomes migrate to specific locations within hosts. ([A] – mesenteric veins in the proximity of small intestine, [B]- veins around large intestine. However, locations of [A] and [B] can sometimes interchange. [C] is mostly located in a venous network of the urinary bladder, but can sometimes occur around the rectum.
In these locations, schistosomes mate and produce eggs that progress towards the lumen of the intestines, and/or urinary bladder in case of *S. haematobium*.

Image and information adapted from DPDx (http://www.cdc.gov/parasites/schistosomiasis/biology.html)

2.2. Biology of *Schistosoma* life stages

2.2.1. Egg

Schistosome eggs can vary greatly in sizes (dozens to hundreds of μm) but are generally oval and consist of the vacuole, vitelline membrane, and ciliated larva – miracidium. The eggs require time to mature in the human tissue (6 days in those of *S. mansoni*), and only mature eggs succesfully leave the host as they are able to produce the proteins that facilitate the movement through tissue and eventually into the lumen of either bladder (*S. haematobium*) or intestine (*S. mansoni*) (Maule and Marks, 2006; Rollinson and Simpson, 1988).

The eggs secrete proteins that induce inflammation of the surrounding tissues and allow their escape from tissue to the lumen (Wu et al., 2014). They are also the main source of *Schistosoma* – related pathologies such as granuloma formation, liver fibrosis, hepatosplenic inflammation or intestinal disease (*S. mansoni, S. japonicum*) or obstructive inflammatory disease in urinary system, higher susceptibility to HIV and urethritis that can progress to bladder cancer (*S. haematobium*) (Gryseels et al., 2006; Steinmann et al., 2006; Kjetland et al., 2012). In total, the calcium binding proteins add up to 2% of *S. mansoni* eggs soluble antigen content according to a proteomic study (Mathieson and Alan, 2009).

2.2.2. Miracidium

Miracidium is the first free, freshwater swimming larval stage of schistosomes which is actively seeking its snail hosts in water (Jordan, 1988). It is covered by anucleated ciliated cells and carries a terebratorium carrying sensory organelles, which are ciliated. The anterior part of miracidium contains apical gland and two lateral gland cells (Rollinson and Simpson, 1988).

Positive phototaxis and negative geotaxis were observed in miracidia of *S. mansoni* and *S. japonicum*. However, *S. haematobium* shows the exact opposite behaviour. This phenomenon is believed to be related to the ecology of host snails (Rollinson and Simpson, 1988). Miracidia have also shown a strong attraction to snail excretory and secretory products, i.e. mucopolysaccharides, sialic and butyric acid (MacInnis, 1965; Haberl et al., 1995). The fixation to the host snail occurs in the mucus layer of the foot, where, after series of exploratory movements penetration occurs as well. The terebratorium membrane folds act as a sucker while apical glands facilitate the penetration itself. *S. japonicum* miracidia use different strategy and majority of their miracidia prefer natural openings of the host for penetration (Xia and Jourdane, 1991).

Unlike most digenea, the schistosome miracidium does not lose its ciliature during the penetration but after it (Jordan, 1988).

2.2.3. Sporocyst

After the host snail is infected, the miracidia form new tegument, also known as neodermis, from subepidermal cells, which is then covered with numerous microvilli and allows evasion of the host immune system. When the metamorphosis is complete, miracidium becomes a mother sporocyst and starts gaining nutrients from the snail plasma (Walker, 2011). The localization of sporocysts is different in the individual species of schistosomes. In *S. haematobium* and *S. mansoni* the sporocysts develop in the vicinity of the penetration point while *S. japonicum* is invading various host locations with a particular interest in snails cavital organs (Jourdane and Mingyi, 1987). The mother sporocysts then produce daughter sporocysts asexually. These are covered in tegument and are ready to be released from mother sporocyst, measuring from 100μm to 250μm in length. The secondary sporocysts travel to the digestive gland of the snail through the circulatory system of the host. During the migration no cercariogenesis takes place. Cercariogenesis happens once the sporocysts are in final location. At this time, a major increase in size is also observed (Rollinson and Simpson, 1988).

The secondary/daughter sporocysts are capable of producing not only cercariae but also another generation of identical sporocysts (Rollinson and Simpson, 1988). This is often used when cloned schistosomes are needed – the sporocysts are simply transplanted into another snail where they produce clones (Jourdane and Theron, 1980).

2.2.4. Cercaria

High production of cercariae from the host snail occurs from 3-6 weeks after penetration by the miracidium (Rollinson and Simpson, 1988). When the cercariae escape from the sporocyst birth pores (Walker, 2011) using their escape glands that disappear during the emergence from the snail (Stirewalt, 1974), they seek their way out of the host snail into the free water where they search for their definitive host. Strategies of searching for the host are different in various *Schistosoma* species yet all thrive to penetrate into the host, as their time is limited due to their inability to obtain food and thus being solely dependent on their glycogen reserves from the sporocyst. The time is believed to be limited to 24 hours after leaving the sporocyst. The successful infection after 24 hours is highly unlikely due to glycogen exhaustion (McKerrow and Salter, 2002).

The main mechanisms of attraction are temperature gradient and the concentration gradient of amino acids and other vertebrate skin products. For example, arginine is a strong attractant, and once the cercariae comes in contact with this amino acid, they start producing it themselves and thus attracting other cercariae from close vicinity (Haas et al., 1994). This is probably a behaviour increasing the odds of successful penetration and infection of the definitive host. The strategies of

infection are specific for different species; *S. mansoni* cercariae identify host using combination of amino acids, water turbulence and temperature gradient, *S. japonicum* invade the host seemingly purely by chance without utilizing chemical gradients, as they prove not so efficient in the mud, where cercariae of *S. japonicum* await their host. Also, their tegument transformation can be induced by warmth alone, which results in high cercariae loss, on the other hand it allows *S. japonicum* to invade a very broad spectre of hosts. The cercariae of *S. haematobium* are the most sensitive to thermal gradient (5x more sensitive than *S. mansoni*) migrating along gradient as low as 0.03°C/mm (Haas, 2003).

To penetrate the host, cercariae use secretory glands in the head region, which serve as storage for attachment and penetration molecules. Cercariae have two pairs of circumacetabular (also called preacetabular) glands, three pair of postacetabular glands and a head gland (Ligasová et al., 2011). The glands are unicellular, containing secretory vesicles that consume majority of the cell (Stirewalt, 1974). Postacetabular glands contain adhesive molecules that facilitate attachment to the host, and the preacetabular glands contain elastases used for succesful penetration of the host skin (Lewert et al., 1966, as cited in Stirewalt, 1974; Salter et al., 2002). Surprisingly high concentrations of calcium have been found in the cercariae preacetabular glands by (Gordon and Griffiths, 1951), and it has been the subject of discussions ever since (Dresden and Asch, 1977).

The acetabular glands of cercariae disappear within the first day after penetration (Curwen and Wilson, 2003), which together with the shedding of the characteristic bifurcated tail is one of the first signs of morphological and functional transformations into schistosomula.

2.2.5. Schistosomula and adult worms

When the cercariae penetrate the host, they must shed their glycocalyx, a protective membrane on surface of cercariae, in order to successfully evade the host immune system as glycocalyx is highly immunogenous (Stirewalt, 1974; Da'dara and Krautz-Peterson, 2014).

Schistosomula migrates through the skin towards the venous system of the host. The major mechanism of movement is likely – thanks to the compression of cells surrounding the schistosomula – the insinuation of hosts cells (Curwen and Wilson, 2003). This topic, however caused some debates and it is probable that this mechanism is not solely based on insinuation an that proteolytic enzymes from cercarial secretory glands, that disappear within one day after penetration, play an important role in host invasion (McKerrow and Salter, 2002; Curwen and Wilson, 2003). An important feature of schistosomules and adult worms is their tegument, which helps them evading hosts immune reactions and nutrient uptake from hosts bloodstream (Rollinson and

Simpson, 1988). The tegument evolves from cercarial trilaminate outer membrane into heptalaminate membrane within three hours after penetration (Hockley and McLaren, 1973). It consists of invaginated plasma membrane overlaid by the membranocalyx, and a lipid bilayer (Braschi et al., 2006; Hockley and McLaren, 1973; Sotillo et al., 2015).

One of the large challenges for the schistosomula is changing its metabolism from aerobic to anaerobic and getting to the hepatoportal system, where the maturation occurs, through heart and lungs. On its way up to 70% of schistosomules are eliminated in lung capillaries (Wilson, 1990). Once the schistosomules get to the liver venous system they mature within 4-6 weeks, pair up with a worm of opposite sex and start feeding on blood (Gryseels et al., 2006).
For SEM image of worm pair see FIG. 3.

Figure 3 | **Paired adults of *S. mansoni***
The grey bar represents 200 μm. Magnified 70x
Image courtesy of Jana Bulantová, Ph.D.

The adults digest the erythrocytes in blind gut which is partially bifurcated and partially single (Wilson, 2012). They express sexual dimorphism, with the male being shorter and thicker and keeping female that is longer and thinner in his *canalis gynaephorus*. Worms then migrate to either hepatoportal veins (*S. mansoni, S. japonicum*) or veins surrounding the urinary bladder (*S. haematobium*), where they start producing eggs (Gryseels et al., 2006). The time required for egg production ranges from five to eight weeks.

Adult schistosomes usually live from 3 to 5 years, but can live for up to 30 years causing serious pathologies, which can result in death to their mammalian host. The reproduction potential of one pair can reach up to 600 billion schistosome eggs (Gryseels et al., 2006).

3. Calcium and its role in animals

3.1. General properties of calcium

Calcium is one of the important elements for most of organisms. It is a soft **alkaline earth metal** that is solid under normal conditions. It was first recognised in 1808 by Humphry Davy and was named after Latin for lime; *calx*. It has multiple isotopes of which the most stable and thus abundant is **^{40}Ca**. Another isotope of significance is the radioactive ^{45}Ca that is used in calcium localization and transport studies in biology. Calcium is the **fifth most abundant element** in the Earth crust by mass, constituting 3% of its mass (Bertini et al., 1994). The ionic radius of Ca^{2+} is always larger than the radius of other common divalent ions in organisms, such as Mg^{2+}. For example, the EF-hand (helix-loop-helix structural motif) domain binds calcium 10 000 times more than it does bind magnesium (Bertini et al., 1994). The recent realisations of Ca^{2+} ions importance in cell and organism functioning have led to a formation of a new term, "calciomics", in order to describe the various and complicated roles of Ca^{2+} in biological systems (Brini et al., 2013).

In organisms it is mostly found as variety of salts. In bones and teeth of higher organisms it is present as Ca^{2+} phosphate salts. In lower organisms the main compounds of skeletal structures consist mainly of Ca^{2+} carbonates and sulphates. Calcium exists in least **three** basic **forms**; the already mentioned **salts, ions** and complexed **in organic compounds**. Structurally calcium is involved in processes such as: **second messengers, muscle proteins, neurotransmitters, mechanical stress, and biomembranes**. Chemical involvement consists mainly of its **hormonal** activities (Brini et al., 2013). Almost every process in the animal physiology is dependent or at least influenced by calcium (Wiercinski, 1989).

All these facts predetermine calcium to be the key player in fighting the schistosomes through understanding how they use it, identifying their weaknesses and targeting them. In this chapter, I would like to discuss the importance of calcium in animals with emphasis on vertebrate hosts of schistosomes.

It is not the aim of this work to describe the entire spectre of cellular and organismal calcium use. However, I consider it to show certain significant and most known calcium-dependent processes to illustrate its major role in both cell biology and the biology of the organism as a unit. Those mechanisms are explained later in the chapter "calcium signalling toolkit".

To keep this thesis as related to the topic as possible, I decided to exclude description of any calcium-related mechanism in plants.

3.2. Calcium regulation in potential vertebrate hosts of schistosomes

The most abundant metal ions dispersed in organisms are those of Na, Ca, K, Mg. Sometimes, Zn is also added to the count. While Na^+ and Ca^{2+} are the most common in extracellular spaces, the other three are main intracellular metals. While most of the Na (up to 75%) and almost all the K^+ is free in the cells, the other three metals, especially the Ca^{2+} are present in bound forms. The total cell Ca^{2+} concentration ranges from 1 to 10mM. This accounts for the calcium, which is both ionised or bound to ligands, small organic molecules, and specific binding proteins or Ca^{2+} sequestered within endoplasmic, or sarcoplasmic reticulum or Golgi apparatus. The concentration of Ca^{2+} in the cytoplasm, the location of most of its signalling targets, is significantly lower (in order of hundreds of nM), than extracellular concentrations. This low concentration is achieved thanks to membrane transport systems such as channels, pumps, and exchangers that pump Ca^{2+} from the cytosol (Brini et al., 2013).

3.3. Calcium as a cell signalling agent

In order to understand the variety of calcium related processes, we must understand how is this versatility even possible. It is important to realise that the regulation consists of multiple combinations of Ca^{2+} signalling components, that create a Ca^{2+} "signalling toolkit" (Berridge et al., 2000). This toolkit can then be assembled in a variation of combinations that create very different signals. Even more combinations can be achieved through so-called crosstalk, which are Ca^{2+} interactions with other signalling pathways.

3.3.1. Signalling toolkit

- The Ca^{2+} signalling toolkit is divided into four functional units, consisting of the signalling, which is triggered by stimuli, generating Ca^{2+}-mobilizing signals.
- The Ca^{2+}-mobilizing signals then activate the ON mechanisms that cause Ca^{2+} influx into the cytoplasm.
- The Ca^{2+} functions as a messenger to stimulate various Ca^{2+}-sensitive processes.
- The last member of the toolkit are the OFF mechanisms that are composed of pumps, channels, and exchangers, and whose task is to re-establish the cytosolic concentration to the resting state. The relationship between those is illustrated in FIG. 4 below (Berridge et al., 2000).

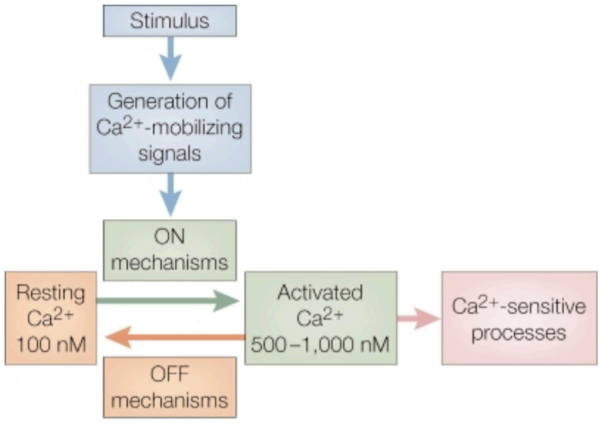

Figure 4 | **The four units of Ca^{2+} signalling network.**

Image adapted from Berridge et al., 2000

3.3.1.1. Production of Ca^{2+}-mobilizing signals

Those signals are produced by both intracellular and extracellular sources of Ca^{2+}. The intracellular sources, such as ER (endoplasmic reticulum), or SR (sarcoplasmic reticulum), have already been mentioned above. The release from those is controlled by different channels with the inositol-1,4,5-triphosphate receptor ($InsP_3R$) and ryanodine receptor (RYR) being the most known and researched. The activator of these channels is Ca^{2+} itself. The process is very important and is also known as Ca^{2+} induced Ca^{2+} release.

An example of the important stimuli that can activate these mechanisms is $Ins(1,4,5)P_3$ that diffuses into the cell and activates $InsP_3R$ and release of the Ca^{2+} from ER (Berridge et al., 2000).

3.3.1.2. ON mechanisms

The ON mechanisms depend on Ca^{2+} channels which control the release of Ca^{2+} from internal storages or control the entry of external Ca^{2+}. For the latter, we differentiate the entry channels by the mechanism of their activation. There are multiple known mechanisms such as voltage-operated channels, receptor-operated channels or store-operated channels. The receptor-operated channels open upon binding stimuli such as ATP, acetylcholine, or glutamate. The store-operated channels are of particular interest as they are believed to provide the Ca^{2+} that controls a large number of cellular processes (Berridge et al., 2000).

3.3.1.3. Ca^{2+}sensitive processes

Once the ON mechanism generates the Ca^{2+} signal, the Ca^{2+}-sensitive processes translate this signal into an adequate cellular response. The Ca^{2+} signalling toolkit has many different Ca^{2+}-binding proteins. Those can be divided into Ca^{2+} buffers and Ca^{2+} sensors. As an example of a

Ca^{2+} buffer, we can mention parvalbumin or calretinin. They are involved in regulating the strength and duration of Ca^{2+} signals. The Ca^{2+}sensors such as troponin C (TnC) or calmodulin (CaM) respond to increased concentration of $Ca^{2+}$by activating various processes. They activate down-stream effectors thanks to a conformational change that occurs once $Ca^{2+}$is bound to their EF hands. Both CaM and TnC have four EF hands that bind Ca^{2+}. CaM regulates many processes, such as contraction of smooth musculature, metabolism, gene transcription and many others. TnC regulates the interaction of actin and myosin during muscle contractions (Berridge et al., 2000). There are many other Ca^{2+}binding proteins that will be either presented below or are less important to the topic.

3.3.4. OFF mechanisms

It is important to re-establish the usual concentrations of Ca^{2+}in the cytoplasm once it has carried out its signalling function. For this reason, a series of mechanisms have developed in the cell. Most significant are pumps and exchangers such as Ca^{2+}-ATPase pumps, Na^{+}/ Ca^{2+} exchangers or sarcoplasmic reticulum ATPase. When mentioning the OFF mechanisms, the mitochondrion should be mentioned for its ability to rapidly sequester Ca^{2+} when the Ca^{2+} signal is being developed and then slowly release it during the recovery phase. This is important in shaping the patterns of Ca^{2+} signals (Duchen, 1999, as cited in Berridge et al., 2000).

One of the best ways to illustrate the Ca^{2+} signalling toolkit of the cell is a diagram of one. FIG. 5 below shows one of those as presented by (Berridge et al., 2000) in their 2000's article which has been a valuable source of information for this work.

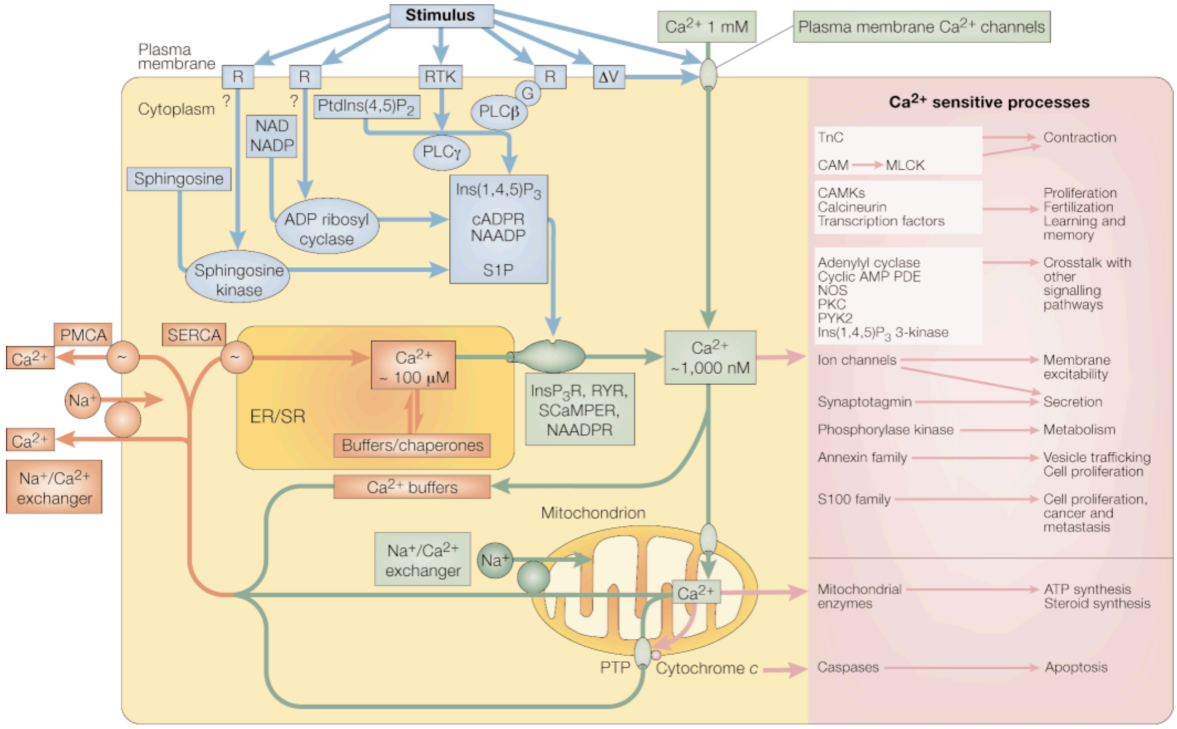

Figure 5 | **Elements of the Ca^{2+} signalling toolkit**

The cellular signalling toolkit can produce various Ca^{2+} signals as the picture shows. Note that differently coloured parts of the picture represent different units of the signalling toolkit that are stated in FIG. 4 i.e. the blue colour represents Ca^{2+}-mobilizing signals, red represents OFF mechanisms, etc.

The Ca^{2+}-mobilizing signals are triggered through stimuli affecting a variety of cell surface receptors (R) such as receptor tyrosine kinases (RTK) and G-protein (G)-linked receptors. Generated signals are inositol-1,4,5-triphosphate ($Ins(1,4,5)P_3$), which is generated by hydrolysed phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$). The enzymes responsible for this are from phospholipase C family (PLCβ, PLCγ). ADP-ribosyl cyclase is responsible for generating cyclic ADP-ribose (cADPR) and nicotinic acid dinucleotide phosphate (NAADP), generating them from nicotinamide-adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). Sphingosine kinase is responsible for generating S1P from sphingosine.

The ON mechanisms (green) are consisting of Ca^{2+} channels, located both in the plasma membrane and in intracellular membranes. The plasma membrane channels respond to transmitters, or to depolarisation (ΔV). the intracellular Ca^{2+} channels; the $Ins(1,4,5)P_3$ receptor($InsP_3R$), ryanodine receptor (RYR), NAADP receptor and sphingolipid Ca^{2+} release-mediating protein of the ER (SCaMPER). The Ca^{2+} released by those channels then activate different Ca^{2+} sensors (pink) which then affect various Ca^{2+}-sensitive processes (pink).

The OFF mechanisms (red) purpose is to pump Ca^{2+} out of the cytoplasm.The plasma membrane Ca^{2+} ATPase (PMCA) and NA^{+}/ Ca^{2+} exchanger pump Ca^{2+}out of the cell and the sarcoendoplasmic reticulum Ca^{2+} ATPase is responsible for pumping it back to the SR/ER.

Image and information adapted from Berridge et al., 2000.

4. Calcium in schistosomes

Like in other animal species, calcium plays a key role in most of the processes in schistosome life. Whether it is the already mentioned "signalling toolkit", calcium homoeostasis as the target of current antischistosomal agent praziquantel, high calcium carbonate content in preacetabular glands, calmodulin inhibitor's deadly effect on schistosome miracidia and eggs or other processes either directly or indirectly affected by calcium. In this chapter, I intend to describe some of the most important known molecules that are related to calcium control in schistosomatids. There is a risk that this work might quickly become outdated as new molecules and facts are being frequently published and the knowledge on schistosomal calciomics evolves fairly quickly.

Schistosomes share certain calcium regulating properties with other animals, like mammalians, and are obviously quite different from those in other aspects. For example, schistosomes do not need to worry about the release and adsorbtion of calcium to their skeletal compounds, as they simply do not have any. On the other hand, the parasite's muscularity is quite similar to the smooth muscles of higher animals (Silk and Spence, 1969, as cited in Noël et al., 2001).

4.1. Calcium binding proteins

Calcium binding proteins (CBP) are a wide, heterogeneous protein group. They participate in a wide variety of cellular processes and regulate the cell functions. Most calcium binding proteins bind reversibly and selectively calcium in specific domains and the reaction is usually very fast (Wiercinski, 1989). The EF-hand motif is the most common calcium binding motif amongst proteins (Lewit-Bentley and Réty, 2000). It consists of a helix-loop-helix motif characterized by sequence of, usually, 12 amino acid residues flanked with two alpha helices in arrangement that looks like hand with thumb and index finger as illustrated in FIG. 6 (Wiercinski, 1989).

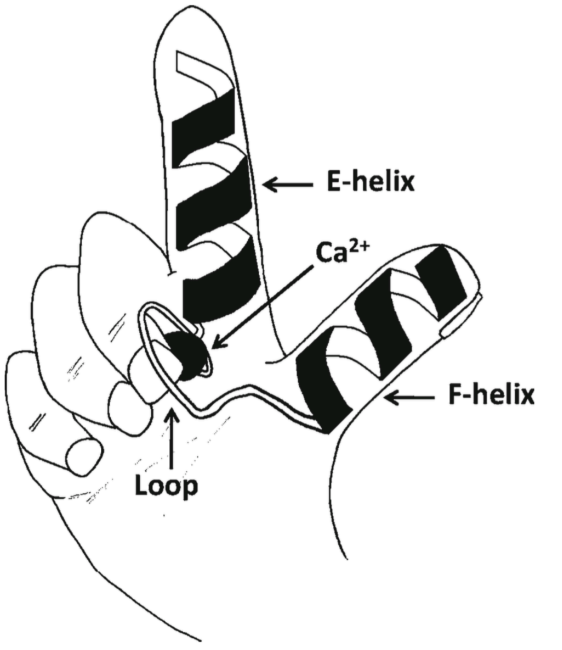

Figure 6 | **EF-hand motif**
Image adapted from (Wiercinski, 1989)

Below some of the most significant calcium binding proteins and their main properties will be presented.

4.1.1. Tegumental calcium binding proteins

Tegumental Ca^{2+}-binding proteins form a protein family specific for schistosomes and other parasitic flatworms. They consist of one or two N-terminal EF-hand domains and dynein light chain domain on the C-terminus (Zhang et al., 2012).

4.1.1.1. *S. mansoni* tegumental allergen like (TAL) proteins

Three *S. mansoni* proteins were analysed; **SmTAL1**, also known as **Sm22.6**, **SmTAL2**, known as **Sm21.7** and **SmTAL3**, which can also be found under the assignation **Sm20.8** (Thomas et al., 2015).

The analysis showed that SmTAL1 and SmTAL2 can bind calcium and other ions with their two, and one calcium binding structures(-EF-hands), respectively. SmTAL1 was capable of binding Mn, Sr and Ni^{2+} and possibly Fe^{2+} ions. SmTAL2 showed binding Mn, Cd^{2+}, Mg and possibly Sr and Ba ions (Thomas et al., 2015). The SmTAL3 protein does not bind calcium ions despite the presence of two EF-hand-like structures (Francis and Bickle, 1992; Thomas et al., 2015). It is also the only member of this group about which we know at least a little regarding function; SmTAL3 interacts with dynein light chain as a part of a larger structure (Hoffmann and Strand, 1997). The fact that these proteins are unique in parasitic flatworms makes them interesting to researchers trying to find antischistosomal drugs. For that reason, the proteins have been tested for interaction with praziquantel (PZQ), thyamial, CaM antagonists – chlorpromazine, trifluoperazine, and W-7. SmTAL1 reacted with all of those earlier mentioned substances except for thyamial, SmTAL2 reacted with W-7 and SmTAL3 reacts with CaM antagonists and thyamial, but it did not react with PZQ (Thomas et al., 2015). The fact that SmTAL3 did not interact with PZQ indicates that the for function of PZQ the interaction with calcium is the key, not the possession of EF-hand-like-structure.

Homologous tegumental proteins have been identified: **SjTP22.6** in *S. japonicum* (Waine et al., 1994) and **Sh22.6** in *S. haematobium* (Fitzsimmons et al., 2004). Both are capable of binding calcium as they contain an EF-hand domain (Waine et al., 1994). Those proteins are targets to humane IgE response. Interestingly, the proportion of antigen response to those proteins grew with age. For example, the percentage of adults expressing IgE to Sh22.6 was 35.5% which is significantly higher than the proportion of expressed IgE in children, showing only 10.3% (Fitzsimmons et al., 2004). However, the proportion of response is likely not as related to age as it is to the infection intensity, which usually increases with hosts age in *S. mansoni* infections (Webster et al.,

1998). The fact that those proteins are considered homologous indicates that the *S. japonicum* and *S. haematobium* will probably express the same traits.

4.1.1.2. SjTP22.4 tegumental calcium binding protein

Another tegumental calcium binding protein of *S. japonicum,* **SjTP22.4**, is membrane anchored, single EF-hand protein. Immunofluorescence using antibodies against recombinant SjTP22.4 (rSjTP22.4) has shown that the protein is particularly expressed in tegument and intestinal epithelium of schistosomulum and adult worm with slightly increased expression in male tegument. However, the female showed more expression in parenchymatous tissues. In cercariae, the expression mainly occurred in ventral sucker and weakly in tegument (Zhang et al., 2012). SjTP22.4 participates in opening of Ca^{2+} channels and ion uptake. It was also discussed that if a vaccine consisting of cocktail composed of SjTP22.4 and other tegumental proteins was used in combination with PZQ, it is probable that PZQ's lethality to schistosomes would increase (Zhang et al., 2012). Mice immunised with rSjTP22.4 showed 41% reduction in egg granuloma ratio compared to mice that have been immunised with phosphate-buffered saline (PBS) control. Also, in the immunised mice a decrease in inflammatory reaction around the eggs was observed indicating they failed to develop into miracidia. Furthermore, immunised mice which were also treated with PZQ showed even more reduction in egg-granuloma ratio reaching to 53% in comparison with the control. These results are particularly interesting as they show that despite the fact that immunisation with rSjTP22.4 doesn't affect the egg burden, it affects the vigor of the eggs which is a more important factor as it reduces schistosome transmission (Zhang et al., 2012).

4.1.1.3. SjTP20.8 tegumental calcium binding protein

A 20.8-kDa protein has been found in *S. japonicum* tegument and was assigned a name **SjTP20.8.** It has two EF-hand domains, one transmembrane region and can bind calcium *in vitro.* It has been shown that the protein is expressed only in tegument and is absent in all other structures of adult worms. The protein is thought to be an important element of *S. japonicum* calcium intake process. Experiments with anti-SjTP20.8 antibodies have shown surprising results. The antibody has promoted survival of adult schistosomes in the portal system almost three times, but it inhibited deposits of eggs and reduced their counts by approximately 40% (Xu et al., 2014). The laid eggs in the immunised group also had smaller fibrotic lesions around them and more inflammatory cells in the proximity. This is possibly indicating that the adults were not able to produce fully viable

eggs and that their eggshells, which require calcium (Chennaiah et al., 2004), may be incomplete (Xu et al., 2014).

4.1.1.4. Annexin B22

Annexins are calcium-dependent phospholipid binding-proteins present in all eukaryotes, and according to the recent nomenclature those annexins of invertebrates are called annexin B. **Annexin B22** and its homologues are found in *S. mansoni* (Leow et al., 2014)*, S. bovis* (de la Torre-Escudero et al., 2012) and *S. japonicum* (Cantacessi et al., 2013). The annexins were found upregulated in tegument-covered life stages of schistosomes, the schistosomula and adults with slightly higher abundance in males. This corresponds with the opinion that annexin is responsible for membrane binding and plays a role in membrane fusion (Leow et al., 2014). It is also thought that annexin is involved in anchoring the outer membranes of tegument in schistosomes (Van Hellemond et al., 2006).

The fact that annexin is exposed in schistosomes makes it a potential vaccine target and experiments show that antibodies are responding to its signal, showing that it is an immunoreactive protein and that it can be recognized by host immune system (Leow et al., 2014).

4.1.1.5. Dysferlin

Another protein found in tegument of *S. masnsoni* is **dysferlin**. It has a C-terminal membrane anchor with multiple C2 cytoplasmic calcium-binding sites and may be responsible for calcium-dependent vesicle function with plasma membrane (Braschi et al., 2006; Fonseca et al., 2012).

4.1.2. Messenger proteins

4.1.2.1. Calmodulin

It has been concluded that *S. mansoni* contains two calmodulin genes. Both **SmCaM1** and **SmCaM2** show 99% identity and share 98% or greater identity with other flatworm, insect or mammalian CaMs, showing a high conservation of CaM among animals (Taft and Yoshino, 2011). SmCaM1 and SmCam2 are present in sporocysts of *S. mansoni* and their abundance differs. The SmCaM1 transcript abundance is lower in one-day sporocysts, relative to miracidia, but then gradually increases in 3- and 8-day sporocysts. SmCaM2 increases in 1- and 3-day sporocysts, relative to miracidia, however, its abundance decreases in the 8-day sporocysts to a steady level, which is lower than the miracidial level (Taft and Yoshino, 2011).

It has been concluded that CaM plays a role in sporocyst growth. The mechanism has been demonstrated on *in vitro* developing CaM ds-RNA treated sporocysts through RNA interference. Those have shown reduced CaM transcript and protein abundance, and at the same time reduced the average length (Taft and Yoshino, 2011). A decrease of 30% in SmCaM1 and 35% decrease in SmCaM2 transcript abundance have been shown as well as 35% decrease in SmCaMs protein levels. The authors also discussed that under *in vitro* conditions the consequences might not be as dramatic as if this decrease was caused in the *in vivo*. In primary sporocysts present in snails the consequences would probably be more serious, and such knockdown could be lethal for the parasite, as in the hostile host environment, fully functioning regulatory networks are vital for growth and development of the parasite (Taft and Yoshino, 2011).

Those findings are also interesting as SmCaM is primarily localised in the tegument of the sporocysts (Taft and Yoshino, 2011), which is mainly responsible for transporting nutrients as well as being the exclusive interface through which the parasite affects its host. Furthermore, treatment with CaM ds-RNA can cause the shorter appearance of primary sporocysts, and might be caused by impaired muscle function leading to the more rounded morphology of the parasite (Taft and Yoshino, 2011). The difference between CaM ds-RNA treated parasites and control are showed in Figure 7.

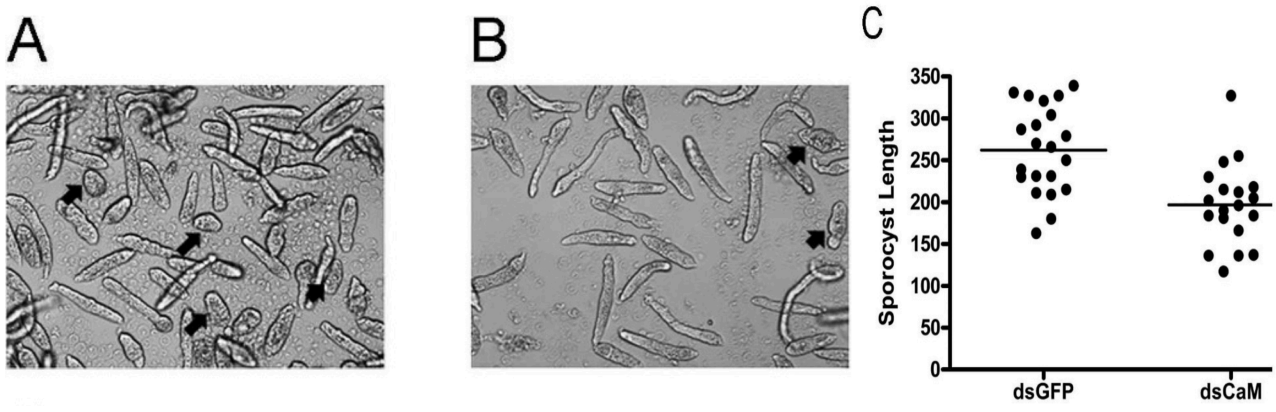

Figure 7 | **Difference of length in CaM ds-RNA treated and untreated sporocysts of *S. mansoni* after 7 days of exposure**
CaM ds-RNA treated sporocysts (A) show the significantly reduced length in comparison to control, of GFP ds-RNA treated (B) sporocysts. The difference is also visible in the graph(C), where measurements of sporocyst lengths are stated as well as the calculated median represented by horizontal lines.
Image adapted from Taft and Yoshino, 2011.

Another experiment has shown that a known CaM antagonist, trifluoperazine, can be efficient in inhibiting the transformation of miracidia in a concentration-dependent manner. After 18 hours of culture in 2μM trifluoperazine, 68% miracidia were transformed. However, when exposed to 10μM trifluoperazine, only 20% transformation occurred (Taft and Yoshino, 2011).

It has also been shown that CaM is present in the sensory papillae of miracidia and that it might be responsible for transduction of calcium signals during egg hatching. However, the efforts to inhibit the hatching of eggs using Cam dsRNA have been unsuccessful (Taft and Yoshino, 2011). Although it was discussed whether calcium can affect hatching of *S. mansoni* eggs, the hypothesis was first neither confirmed nor completely rejected (Katsumata et al., 1988). In a study published by the same author a year after, the hypothesis was confirmed. It showed that the hatching was negatively affected by calcium channel blocker diltiazem and by CaM antagonist W-7, revealing that hatching of *S. mansoni* is a calcium-dependent process (Katsumata et al., 1989). The inhibition was proved not to be caused by diltiazem toxicity, as removal of the compound allowed the eggs to hatch normally (Taft and Yoshino, 2011). CaM is, however, present in all life stages of schistosomes as we must realise that it is one of the fundamental proteins in calcium related processes (McCammick et al., 2016).

4.1.3. Chaperones

4.1.3.1. Calreticulin

Calreticulin is a versatile lectin-like chaperone, which is influencing calcium homoeostasis by affecting Ca^{2+} content in the ER. It is also participating during synthesis of many molecules, such as ion channels, receptors or transporters (Michalak et al., 1999). It is present in all life stages of *S. mansoni* (Khalife et al., 1994) and cercarial secretions (Knudsen et al., 2005). As a matter of fact, calreticulin is present in every cell of higher organisms except for erythrocytes (Ferreira et al., 2004). It is thought that the protection against encapsulation by haemocytes in snail the host might be one of the reasons for the presence of calreticulin in *S. mansoni* primary sporocysts (Taft et al., 2009). This hypothesis is supported by an interesting detail from organism. In then endoparasitic wasp *Cotesia rubecula*, calreticulin-like protein is injected into the host and prevents encapsulation of the developing parasitoid by inhibition of haemocyte spreading (Zhang et al., 2006). Calreticulin is also present in penetration glands of *S. mansoni* cercariae suggesting its role in regulating the proteases/elastase and migration of the cercariae (Kasper et al., 2001, as cited in Ferreira et al., 2004).

4.1.3.2. Smp_054240 or TCTP chaperone-like protein

Proteomic study of *S. mansoni* showed that a chaperone-like protein containing calcium binding domain, **Smp_054240**, a homologue to a human translationally controlled tumour protein (**TCTP**) is present, yet its function is unknown (Mathieson and Alan, 2009). It has also been categorised as a novel heat shock protein as it binds to native proteins and protects them from thermal denaturation (Gnanasekar et al., 2009). Another study (Rao et al., 2002) discussed a *S. mansoni* TCTP (SmTCTP) and it is almost certain that the two proteins – Smp_054240 and SmTCTP are one and the same. However, proper nomenclature is yet to be established. Although the transcripts of SmTCTP are present in all life stages of *S. mansoni,* only schistosomula and adult worms, the stages present in vertebrate hosts, express high levels of SmTCTP. Interestingly, SmTCTP can induce histamine release from basophilic cells in a dose-dependent manner in cell cultures with antigens from eggs, cercariae and adults of *S. mansoni* and thus regulate the immune response of the host. Another important feature of the histamine release has been shown which indicates that as histamine induces vasodilatation in the host, its release probably facilitates easier migration of the parasite into the blood vessels (Ercoli et al., 1985, as cited in El-Ansary and Al-Daihan, 2005). SmTCTP shows 58% identity and 78% similarity with *S. japonicum* TCTP (Rao et al., 2002). A study of TCTP in *Trichinella spiralis* has shown that temperature increase causes large increase in expression of TCTP up to 5.7 fold (Mak et al., 2001). The possible effect on cercarial TCTP transferring from poikilothermic intermediate host to its definitive, homoeothermic host could also explain the increase in expression (El-Ansary and Al-Daihan, 2005).

4.1.3.3. SmIrV1 and SjIrV1 – calcium binding proteins similar to calnexin

SmIrV1 protein (***S****. **m**ansoni* **Ir**radiated **v**accine) was originally identified as one of the schistosomal antigens strongly or uniquely recognised by mice that were protectively vaccinated with irradiated cercariae. A homolog protein, **SjIrV1** of *S. japonicum* shares 84% amino acid identity with SmIrV1. The amino acid sequence of SmIrV1 is also similar to the one of chaperone-like protein calnexin which is similar to calreticulin (Hooker and Brindley, 1999). It is expressed in cercariae and tegument of adult worms and schistosomules (Hawn and Strand, 1994). It can be divided into three regions; neutral N-terminal sequence, proline- and tryptophan- rich P region and a highly acidic C region (Hawn et al., 1993). The protein is highly expressed during the transformation of cercaria into schistosomula and is one of the major phosphoproteins of adult worms (Hawn and Strand, 1994).

The fact that these proteins have fundamental functions in the cell makes them also vaccine candidates against schistosomiasis (Hawn and Strand, 1994).

4.1.4 Proteases

4.1.4.1. Calpain

Calpain, a calcium-activated cysteine protease, was found in of *S. mansoni* and its most researched large subunit can be found under the assignation **Sm-p80** (Molehin et al., 2016)**.** In *S. japonicum* there is an ortholog showing 99.1% identity in nucleic acid sequence (Ohta et al., 2004). Calpain is present in acetabular glands of cercariae and also in cercarial secretions (Knudsen et al., 2005), but can be expressed in various stages throughout the life cycle of the parasite (Kumagai et al., 2005). In *S. japonicum* cercariae it is present in penetration glands and secretions (Ohta et al., 2004). Its presence in cercarial secretions has been found thanks to the "footprint" the cercariae leave on as surface they touch with their secretory glands, to which the antibodies against *S. japonicum* calpain bound strongly (Kumagai et al., 2005). In related avian schistosome *Trichobilharzia regenti* calpain might be responsible for surface membrane synthesis regulation as it is in *S. mansoni*. However, the hypothesis has not been confirmed yet (Leontovyč et al., 2016). It plays a key role as a mediator of the surface membrane synthesis (Siddiqui et al., 1991) of *S. mansoni*. In *S. mansoni,* it was also found in the musculature and surface syncytium of both males and females (Siddiqui et al., 1991). In the mechanically transformed schistosomules the tail region was stained with a monoclonal antibody, indicating the presence of calpain (Ohta et al., 2004). Also, the Sm-p80 vaccine shows cross species protection against *S. haematobium* (Karmakar et al., 2014) and *S. japonicum* (Zhang et al., 2001).

When mice were immunised with recombinant *S. japonicum* calpain, an impressive effect on worm burden (reduction of 41.1% when compared to control), egg production (around 50% reduction in immunised mice) and reduction of liver granuloma formation was observed (Ohta et al., 2004). This and the fact that calpain is expressed in all life stages of schistosomes makes it an excellent potential target for vaccines as the antibodies could recognise the parasite as soon as the cercariae enter the host body (Kumagai et al., 2005).

Based on those properties of calpain, Sm-p80 based vaccine is currently in the current good manufacturing practices testing production in preparation for phase I/II human clinical trials (Molehin et al., 2016).

4.1.5. Other calcium binding proteins

4.1.5.1. SjE16.7 and SmE16 calcium binding egg specific protein

SjE16.7 is a secretory protein synthesised in egg subshell region (Ashton et al., 2001) and can be recognised by hosts immune system. *S. japonicum* produces a 16.7-kDa egg specific calcium binding protein **SjE16.7** (Hu et al., 2003). It possesses 2 EF-hand domains that are composed

of α-helices separated by a loop, and have a specific shape (helix-loop-helix motif) that shows certain resemblance with the human hand, thus getting its name. The loop can bind Ca^{2+} and Mg^{2+} ions which activate the protein (Wu et al., 2014). This protein is present only in eggs and not in adult *S. japonicum* (Wu et al., 2014).

Cloning of this protein and studying the immune response to it resulted in an interesting discovery that the SjE16.7 promotes neutrophil chemotaxis through Rac GTPase pathway. Neutrophils are the cause of necrotic lesions or granulomatous pathology. Thus, they allow the egg migration through the gut tissue and initiate liver pathology (Wu et al., 2014). Also, SjE16.7 is a macrophage activator, inducing the macrophage chemotaxis and promoting cytokine production in macrophages, amplifying inflammation in the acute stage (Fang et al., 2015).

This immune reaction to the eggs in the tissue can cause disorders such as urinary bladder cancer, hepatosplenomegaly or hepatic fibrosis (Ross et al., 2002). Specific antibodies against Sje16.7 are present in animals six weeks after infection, however the authors do not mention its possible use in diagnostics (Wu et al., 2014).

S. mansoni eggs produce a very similar 16-kDa egg specific calcium binding protein, **SmE16** (Moser et al., 1992), which shares 70% sequence identity with SjE16.7 and homologies with calmodulin and troponin C, mostly around the calcium binding sites. The detection of SmE16 antibodies in host sera points out to the molecule being immunogenic, but to this day the function of SmE16 remains unclear (Moser et al., 1992; Wu et al., 2014).

4.1.5.2. SjCa8 and SmCa8 or CaBP calcium binding proteins

Cercarial 8-kDa calcium binding protein, **SjCa8**, has been found in *S. japonicum* cercariae and skin stage schistosomules, but in no other life stages of *S. japonicum*. It shares a high degree of identity with *S. mansoni* 8-kDa protein, **SmCaBP** (Lv et al., 2008), which is primarily expressed in cercariae of *S. mansoni*. Its gene expression is turned on within 50 minutes from the moment when cercariae enter the water from the snail and rapidly shut off once they transform into schistosomula (Ram et al., 1989). It shares more than 30% homology with calmodulin (CaM) of other species with the most similarities around the calcium binding loops (Hu et al., 2008). More studies have been conducted on SjCa8 and considering the high relatedness of the two proteins, we can assume the SmCaBP's properties will be similar/same.

SjCa8 contains one pair of Ca^{2+} binding motifs, unlike CaM that contains two pairs (Hu et al., 2008). Its calcium binding properties seem to be the key to its effectivity. SjCa8 was found in different compartments in cercariae such as tegument, head gland, and body-tail junction (Ram et

al., 1994) but not in the tail (Lv et al., 2008). In those locations it might be responsible for tegument repairs and modifications, which are much needed in order to adapt during the transformation to schistosomula (Gerasimenko et al., 2001; Reddy et al., 2001). Also, SjCa8 is present in cercarial penetration glands and is secreted from cercariae as a part of cercarial secretory products (Lv et al., 2008). Furthermore, it has been found that rSjCa8 inhibits macrophage migration in a dose-dependent manner, and through this mechanism, it allows the cercariae that penetrated into host skin to evade and down-regulate immune response. The protein also down-regulates the expression of NO (nitric oxide) in RAW254.7 macrophages. The authors of these findings suggest that this effect may be a result of lowered intracellular calcium concentrations in macrophages caused by rSjCa8 protein (Liu et al., 2015).

There are also multiple reasons to consider SjCa8 as a potential vaccine target; when mice were vaccinated with recombinant SjCa8 and adjuvant, a surprisingly strong antibody response was measured. The reduction rate of 50.39% in worms was observed, and even higher reduction of intestinal and liver egg deposits was measured (50.63% and 54.16%, respectively), mediated by induced Th1-type immune response through high IgG2a and IgG2b antibodies presence in infected mice (Lv et al., 2008).

4.1.5.3. Calponin

Calponin, an ATPase activity inhibiting 38.3-kDa calcium binding protein, has been found in all *S. japonicum* life stages. Its main effect is believed to be in stages of schistosomes that actively use muscles (especially adults, whose muscles are strikingly similar to smooth muscles of vertebrates) (Gobert et al., 2009; Yang et al., 1999) as it is mainly related to smooth musculature of vertebrates (Jones et al., 2001). This and the fact that it was localised in the musculature of cercariae and adults indicates that its main purpose is in muscular processes, specifically contraction (Jones et al., 2001). A proteomic study of four life stages of *S. mansoni* (eggs, cercariae, lung schistosomula and adult worms) has shown the presence of calponin (Curwen et al., 2004) and this protein was also found in cercarial secretions by proteomic studies (Knudsen et al., 2005). The stated information about calponin indicates that it is probably present in all schistosomes as it plays a key role in their life.

4.2. Calcium affected aspects of schistosomes

Calcium binding proteins are by far not the only way in which calcium affects the schistosome life. One of the most known and discussed effects of calcium is the regulation of cercarial elastases in preacetabular glands and calcium-dependent channels in schistosomes. The the effect of calcium on infectivity and other processes will be specified below.

4.2.1. Calcium content of preacetabular glands

Cercariae are the invasive life stages that infect the vertebrate hosts. For that reason, finding out the mechanism of their entry is one of the primary aims of many studies. The first observations regarding calcium content in cercariae have been made as early as in the 1950s and concerned the calcium content in preacetabular glands of *S. mansoni* cercariae (Gordon and Griffiths, 1951, as cited in Dresden and Asch, 1977).

Throughout the time, more specific results have been presented regarding this issue. Those studies showed that the glands most likely contain **calcium carbonate** (Dresden and Asch, 1977). It has been shown that the proteases stored in preacetabular glands of cercariae of *S. mansoni* are calcium-dependent and, interestingly, that Ca^{2+} can both inhibit and stimulate the protease activity, respective to its concentration. Higher concentrations of Ca^{2+} (10mM) acted as an inhibitor while lower concentrations (below 10mM) stimulated the protease activity (Dresden and Edlin, 1974).

Preacetabular glands contain unusually high Ca^{2+} levels equivalent to **8-12M** (Dresden and Edlin, 1975). The calcium optimum of serine proteinases, specifically **elastase**, of *S. mansoni* was found at 2mM and primary target of proteolytic activity was established to be elastin. However, the enzyme degrades also other proteins such as keratin, laminin or type IV collagen (McKerrow et al., 1985). These molecules are components of host skin and therefore it is more than likely that the content of acetabular glands is used to disrupt the integrity of skin and allow successful invasion of cercariae. It was also shown that calcium is 1000 to 2000 times more abundant in preacetabular glands than in tail region, which was selected as a control (Dresden and Edlin, 1975), proving that the core region of calcium occurrence in cercariae are the acetabular glands. Elastase was shown to be chymotrypsin-like serine protease and can commonly be found in the literature under the abbreviation **SmCE**. The true elastases are rather rare in animals, however SmCE has shown its capability of degrading the molecule of elastin (Salter et al., 2000).

S. haematobium preacetabular glands posess orthologs to elastase of *S. mansoni* (Salter et al., 2002). For some time, it was thought that *S. japonicum* does not posses those human-like elastases and the mechanism of disruption of the hosts skin rely on **cathepsin B** ortholog as a

primary proteinase (Ingram et al., 2011) this information was also supported by the fact that the cathepsin B was 40 fold more active than cathepsin B in *S. mansoni* (Dvořák et al., 2008). Later studies have however identified an ortholog of *S.mansoni* cercarial elastase (CE) **SmCE-2b** in *S. japonicum* but unlike *S. mansoni* and *S. haematobium* only one ortholog gene was found in *S. japonicum* (Zhou et al., 2009).

Another schistosome, the avian neurotrophic *Trichobilharzia regenti* is capable of invading the human skin and causing cercarial dermatitis, also known as swimmers itch (Horák et al., 1999). The targeted hosts of *T. regenti* are usually birds and their neural tissue, however occasionally the schistosomule can get further than skin causing neurologic problems in mammals (Dolečková et al., 2009). Much like in other schistosomes, the calcium content in preacetabular and of *T. regenti* and *T. szidati* is unusually high (shown by staining using potassium oxalate) and probably regulates the content of preacetabular glands and might serve as cross-linker to the mucopolysacharides in the latter (Mikeš et al., 2005). Postacetabular glands of *T. regenti* contain cathepsin B2 (**TRCB2**) with slightly acidic optimum pH that serves as a functional substitute to the elastase of *S. mansoni* and being able to degrade skin and neural tissues (Dolečková et al., 2009).

4.2.2. Calcium in tail shedding by cercariae

The tail shedding of schistosomes is one of the defined steps required to undergo by cercariae in order to consider them schistosomules (Stirewalt et al., 1966). The other steps are emptying of acetabular glands, change of surface that leads to altered permeability and loss of specific cercarial shape. The cercarial tail loss in *S. mansoni* is also a calcium-dependent process, being affected by chelating agents such as an ethylendiaminotetraacetic acid (**EDTA**) and ethylenglycotetraacidic acid (**EGTA**) that are known to bind calcium ions (Howells et al., 1974). Also, the tail loss is induced by calcium ionophore **A23187** the effect was even stronger when combined with 0.3mM **linoleate**, an unsaturated fatty acid, suggesting that those acids enhance Ca^{2+} influx into cercariae and assist in the tail loss (Hara et al., 1993).

4.2.3. Effects of calcium on successful invasion to definitive host

Once cercariae of *Schistosoma* emerge from the snail, they swim to seek their definitive host. During this period, they tend to lose the calcium content of preacetabular glands to the surroundings, if there is a low level of Ca^{2+} in the water (Fusco et al., 1991). This loss can significantly

decrease the effectivity of penetration and infectivity. For example, calcium chelators such as EDTA decrease the infectivity and reduce the penetration into the final host (Lewert et al., 1966). Calcium also affects removal of the glycocalyx once cercariae have successfully penetrated the host using proteases released from the preacetabular glands (Fusco et al., 1991). Through this mechanism, they eliminate the risk of exposition to complement-mediated immune response (Ruppel et al., 1983, as cited in Modha et al., 1998).

Calcium may also play various other roles such as stimulating blood clotting in order to seal ruptured capilaries during penetration to the mammalian host, binding phospholipids and preventing membrane damages. Calcium also plays role in preventing glycocalyx dispersal and presentation by antigen-presenting cells or have effect on damaging the parasites membrane if the concentration of Ca^{2+} is too high (Modha et al., 1998).

5. Praziquantel as a trematocide and its relation to calcium

Praziquantel (PZQ) is a current drug of choice against human schistosomiasis as stated by WHO and was developed in the 1970s (Maule and Marks, 2006). PZQ is a pyrazinoisoquinoline antihelminthic and possesses two enantiomers. It is administered as a racemate. However, only the "leavo" or R-form has schistosomicidal properties (Fenwick et al., 2003). Its molecular formula is $C_{19}H_{24}N_2O_2$ see FIG. 8 for illustration. Thanks to its major effectivity (PZQ is effective against all human species of schistosomes and shows little side effects) and low cost – 0.30 USD per adult for single treatment (Fenwick et al., 2003) – it has become the sole therapy of schistosomiasis practically. The efficiency of therapy can be easily illustrated with results from Burkina Faso where one round of treatment marked 92.8% overall reduction and 87.1% reduction in the prevalence of *S. haematobium* in over two years (Touré et al., 2008).

However, growing concerns are emerging regarding possible PZQ drug resistance (Maule and Marks, 2006). The resistance has already been induced under laboratory conditions (Fenwick et al., 2003; Doenhoff et al., 2002; Greenberg, 2013) and reports from field are emerging as well from Egypt and Senegal (Doenhoff et al., 2002).

Praziquantel and its mode of action and resistance against it are very broad topics and it is not the objective of this work to discuss all of those. The following sub-chapters should shed some light onto those topics in context of calcium influence.

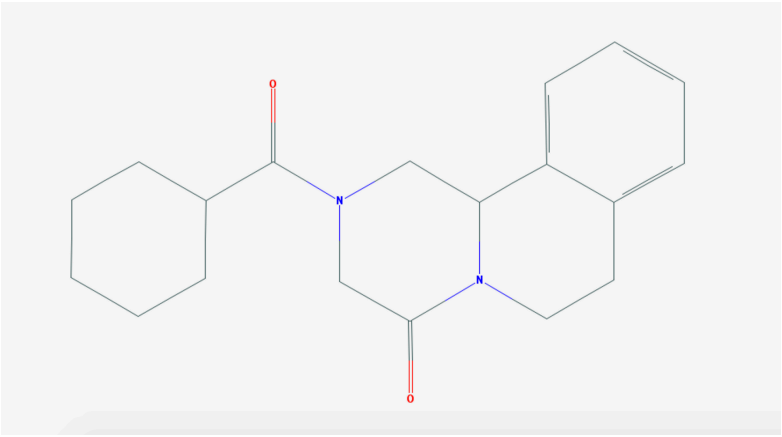

Figure 8 | **Molecule of Praziquantel**
Image adapted from: Pubchem (https://pubchem.ncbi.nlm.nih.gov/compound/praziquantel)

5.1. Mechanism of action

Even though the general knowledge on PZQ action such as induction of **rapid calcium influx** (Pax et al., 1978) and **degeneration of tegument**, bubble formation and **contraction of the worms** are known (Becker et al., 1980) (see FIG 9 for more detail). The exact mechanism is however still yet to be defined, despite decades of active use. PZQ is also responsible for changing the antigenicity of schistosomes through tegumental disruption, leading to **immune reactions** (Day et al., 1992).

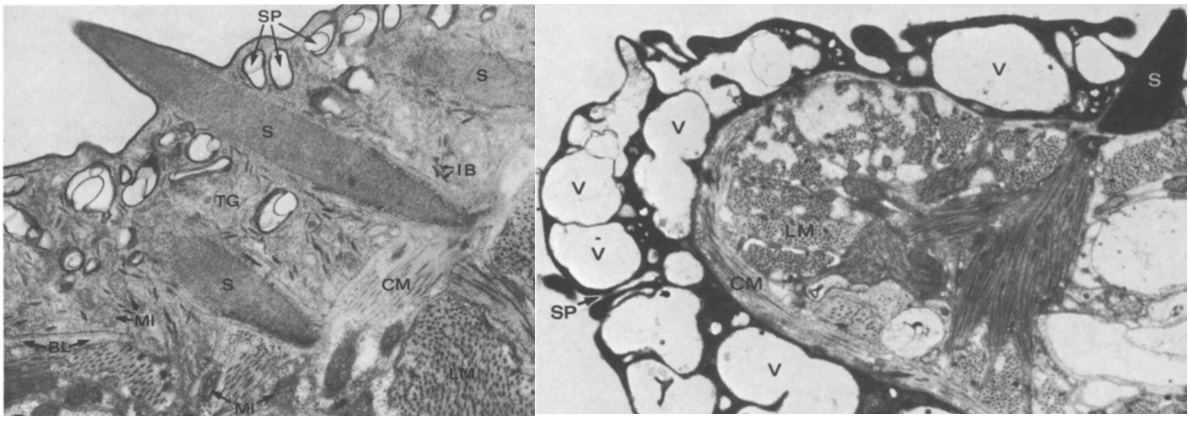

Figure 9 | **Comparison of thin sections of male *S. mansoni* control (left) and treated with 100µg PZQ/ml for 60 minutes. 15 000 magnified.**

On the left picture, control thin section of male S. *mansoni* showing the usual organisation of syncytial tegument (TG), surface pits (SP), spines (S), basal layer (BL) and longitudinal (LM) and circular muscles (CM). The picture on the right shows degeneration of tegument with extreme vacuolization (V).
Image adapted from Becker et al., 1980.

The **voltage-gated Ca^{2+} channels** (VGCC), particularly their ion channel β-subunits are probable molecular targets of PZQ (MendonÇa-silva et al., 2006). In schistosomes, two types of

β-subunits are present. One is similar to those of other animals and one is specific for schistosomes. Those structures vary from other animal's known β-subunits in *S. mansoni* and *S. japonicum* (Aragon et al., 2009). The coexpression of those with mammalian α-subunits results in the channels sensitivity to PZQ (Cioli et al., 2008). The VGCCs have a crucial role in regulating the intracellular calcium levels and also regulate some of the most important processes in schistosomes such as gene expression, muscle contraction or neurotransmitter release (Salvador-Recatalà and Greenberg, 2012).

When the VGCCs of schistosomes are exposed to praziquantel, a massive calcium influx occurs and results in a disruption in parasite calcium homoeostasis. This results in worm contraction, vacuolisation and disruption of tegument that is lethal for the worms (Aragon et al., 2009). The calcium theory has been supported by the fact that the addition of nifedipine, a VGCC inhibitor, results in only 50% mortality of worms in a concentration of PZQ, which would normally be 100% lethal (Pica-Mattoccia et al., 2008).

However, **nifedipine** shows some other interesting results when combined with PZQ, or alone. It reduced the viability of *S. mansoni* and tegumental injuries were apparent. Also, the fecundity was affected, resulting in **zero** eggs being produced by female worms (Silva-Moraes et al., 2013). This is an extremely important observation as the major pathologies caused by schistosomes are caused by produced eggs, not to mention their importance for spreading (Bonn, 2004).

A study concluding that PZQ doesn't show lethal effects on immature worms has been published and also discusses that the PZQ action itself might not be the key aspect to affect the schistosomicidal effect *in vitro* and that the immune action may be an important factor to the parasite death *in vivo* has been published in 2008 (Pica-Mattoccia et al., 2008; Doenhoff et al., 1987).

Also, effects of PZQ are partially inhibited by cytochalasin D (CyD), a disruptor of actin cytoskelet and inhibitor of VGCCs. The exposure to CyD allowed adult worms to survive the PZQ exposition despite the massive calcium influx, showing that the high calcium levels are one of the initial steps in schistosomicide that requires other conditions, such as the integrity of actin cytoskelet (Pica-Mattoccia et al., 2008). The results of various studies indicate that even though we have been using PZQ for decades, the precise effect of PZQ is yet to be shown and that a long and challenging journey to understanding its precise effects is still ahead of us.

6. Conclusions

Calcium plays a key role in all living organisms, whether plants or animals. This work has mapped basic calcium functioning in living organisms with special emphasis on blood flukes of genus *Schistosoma* and their calcium binding proteins and function thereof.

Calcium is one of the most important elements in crucial molecular processes of *S. mansoni*, *S. japonicum* and *S. haematobium* and in other schistosomes as well. Calcium dependent-proteins facilitate some of the crucial processes in schistosomal lives, being responsible for successful hatching of eggs, host invasion, feeding, evasion of immune system of the final host or excretion of schistosomes eggs that cause serious pathologies to their hosts.

The current drug of first choice, praziquantel is the only schistosomicidal agens that is used for treatment of schistosomiasis at the moment. It causes rapid calcium influx into the parasite, causing spasms and tegumental degradation that results in its death. However, unsettling reports of praziqzuantel resistance are emerging and potential successors of this drug could be other calcium influencing agens such as nifedipine, a calcium channel blocker.

Calcium binding proteins are also one of the main targets of antischistosomal vaccines as they are often expressed in tegument and engage in host-parasite interactions. As a matter of fact, a Sm-p80 based vaccine is currently being very close to the first human trials.

All of those facts make calcium and calcium-dependent proteins a very interesting and important feature of schistosomes that could perhaps one day help us effectively fight those debilitating parasites of humans and other homoeothermic vertebrates.

In my future studies, I would be particularly interested in assembling a calcium-dependent proteins based vaccine cocktail that would make the treatment of mammalian and avian schistosomes such as *Trichobilharzia* or *Schistosoma* easier or unnecessary thanks to immunisation of definitive hosts.

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- Pubchem (https://pubchem.ncbi.nlm.nih.gov/compound/praziquantel) in Figure 8, accessed 2016-05-08 11:43:37
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