

**Physiology and pharmacology of flatworm muscle**

by

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## ABBREVIATIONS

2-APB	Aminoethoxydiphenyl borate
AC	Adenylate cyclase
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CICR	Ca <sup>2+</sup> -induced Ca <sup>2+</sup> release
DAG	Diacylglycerol
DHP	Dihydropyridine
DMEM	Dulbecco Modified Eagle's Medium
DMI	Inorganic DMEM
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol- <i>bis</i> -( <i>b</i> -aminoethyl ether) <i>N, N, N'</i> <i>N'</i> -tetraacetic acid
FaRPs	FMRFamide-like peptides
FLPs	FMRFamide-like peptides
GPCR	G-protein coupled receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HVA	High voltage-activated
LVA	Low voltage-activated
NLPs	Neuropeptide-like proteins
NPFs	Neuropeptide Fs
PAAAs	Phenylalkylamines
PFK	Phosphofructokinase
PKA	Protein kinase A

PKC	Protein kinase C
PLC	Phospholipase C
RNAi	RNA interference
SERCA	Sarcoendoplasmic reticulum Ca <sup>2+</sup> ATPase
sFTX	Funnel-web toxin
SR	Sarcoendoplasmic reticulum
TRPs	Transient receptor potential channels
VOCCs	Voltage-operated Ca <sup>2+</sup> channels

## INTRODUCTION

### SCHISTOSOMIASIS AND CHEMOTHERAPY

Parasitic platyhelminths (flatworms), both flukes and tapeworms, infect a variety of vertebrates, including humans, pets and production animals. The number of existing anthelmintic drugs is few and there is an ongoing concern that parasites are developing resistance to available drugs, making the potential choices even fewer. The discovery of a new generation of anthelmintics able to fight infections caused by the blood fluke *Schistosoma mansoni* is very urgent and at least in part limited by the lack of our knowledge of basic parasite biology and physiology.

Human schistosomiasis is principally caused by one of the six species of parasitic worms, *Schistosoma mansoni*, *S. haematobium*, *S. intercalatum*, *S. malayensis*, *S. japonicum* and *S. mekongi*. Schistosomiasis caused by *S. mansoni* and *S. japonicum* is the most abundant. Schistosomes are blood flukes that belong to the phylum Platyhelminthes, class Trematoda, which require a vertebrate and a snail host for completion of their life cycle. Endemic areas and transmission of the disease are limited by available snail hosts. Currently there are more than 200 million people infected with human schistosomiasis in 74 countries worldwide, mostly in tropic regions of Africa, East Asia, Middle East, China, and South America. Mortality from the disease is mostly caused by “fibro-occlusive disease secondary to the immune stimulus of schistosome eggs and end organ damage” (Kheir *et al.*, 1999; Wynn *et al.*, 2004).

Treatment choices nowadays are confined to praziquantel (a pyrazinoquinolone). Praziquantel has proved to be very effective against five of six *Schistosoma* species. However, there have been ongoing reports suggesting increasing resistance of the parasite to it (Ismail *et al.*, 1999; William *et al.*, 2001), heightening the urgency for the development of new treatment options. It has been reported that anti-malarial drug artesunate has some efficacy against urinary schistosomiasis in humans (Inyang-Etoh *et al.*, 2004) and experimental *S. mansoni* infection in mice (Shaohong *et al.*, 2006), but its use raises a concern that widespread use of the drug against schistosomiasis can lead to resistance in plasmodium.

There is a widespread agreement in the parasitology and public health communities that new anti-schistosomal drugs are urgently needed. One of the most suitable places to look for new targets for anthelmintic drugs is the neuromuscular system.

Physiology and pharmacology of flatworm muscle can be studied using intact live animals, muscle strip preparations or dissociated muscle fiber preparations (Day *et al.*, 1994; Graham *et al.*, 2000; Blair *et al.*, 1994). With the development of an isolated muscle preparation (Blair *et al.*, 1991; Day *et al.*, 1994), where muscle fibers are deprived of any neural input, it became possible to directly test pharmacological agents' effects on the muscle, and to do patch-clamp studies on individual muscle fibers. In this preparation, individual muscle fibers are dispersed using a combination of enzymatic and mechanical disruption, yielding accessible contractile muscle fibers

for further study (Mendonça-Silva *et al.*, 2006; Miller *et al.*, 1996; Cobbett and Day, 2003; Day *et al.*, 1994; Moneypenny *et al.*, 2001).

The search for vulnerable targets for drug therapy in the flatworm has led scientists to investigate neurotransmission mediated by neuropeptides as a possible option for drug intervention (Day and Maule, 1999). Neuropeptides are abundant intercellular signaling molecules throughout the invertebrates, but many of these signaling molecules have no known structural correlates amongst mammals. Elucidation of the pathways employed by neuropeptides in the parasitic flatworm muscle can serve as a valuable tool in search of new drug target options. Since parasite maintenance in the laboratory setting can be very hazardous, laborious and expensive, model organisms can be used as a pilot study for developing appropriate experimental protocols.

## **SCHMIDTEA MEDITERRANEA AS A MODEL ORGANISM**

### ***Schmidtea mediterranea* as a model for regeneration**

Extensive studies of stem cell biology, regeneration and tissue homeostasis face inability of current model systems like *Drosophila melanogaster* and *Caenorhabditis elegans* to fully address these fundamental biological processes. Both models proved to be not fully suitable for these studies due to short lifespans, lack of tissue regeneration and adult somatic stem cells (Sánchez Alvarado 2004; Reddien *et al.*, 2005). Also they were found to be not completely amenable to modern RNA interference techniques (Sánchez Alvarado 2006). A new model invertebrate system needed to be introduced for more fulfilling studies. Regenerative



capacity of planarians was known for over a hundred years (Reddien *et al.*, 2005). The planarian *S. mediterranea* has emerged as an attractive model system since it meets major requirements for being a model organism. It is one of simplest metazoans with well-manifested properties of interest, primarily powerful regenerative properties. It is also easy to maintain and explore in a laboratory setting (Sánchez Alvarado 2004). Being a stable diploid ( $2n=8$ ) with a genome size of  $4.8 \times 10^8$  basepairs (Sánchez Alvarado 2006) makes *S. mediterranea* very advantageous among other planarian species, very few of which are stably diploid. Tissues of adult planarians undergo extensive turnover (Reddien *et al.*, 2005) and body parts lost to injury are regenerated as a result of neoblast division. Neoblasts are a population of totipotent adult somatic stem cells distributed all over the organism (Palakodeti *et al.*, 2006; Sánchez Alvarado 2004; Reddien *et al.*, 2005). Replacement of aged and damaged cells is poorly understood, but with advent of extensive genetic manipulation *S. mediterranea* can serve to determine the molecular and cell biology of regeneration in higher animals (Reddien *et al.*, 2005, Palakodeti *et al.*, 2006; Kiefer 2006), which cannot be addressed using current invertebrate models (Sánchez Alvarado 2006).

Tools for studying regeneration processes in *S. mediterranea* have been developed (Sánchez Alvarado and Newmark, 1999). Among them are neoblast labeling methods, the creation of cDNA collections and exploration of planarian genes by mutant screens and use of RNA interference to disrupt gene function. Recent sequencing and ongoing assembly of *S. mediterranea* genome resulted in the creation of *S. mediterranea* Genome Database (SmedGD) (Robb *et al.*, 2008).

Use of dsRNA technology in *S. mediterranea* provided insight into molecular activities responsible for biological function. Large scale gene inhibition experiments produced RNAi phenotypes of 240 genes that are responsible for many specific defects in regeneration, 85% of which are conserved in other organisms and encode significantly homologous proteins (Reddien *et al.*, 2005). Clear understanding of gene function related to regeneration in this animal will shed light on the biology of regeneration in higher animals.

### ***Schmidtea mediterranea* as a model for parasitic platyhelminths**

*S. mediterranea* as a tractable model for developmental biology can also be beneficial for studies done within the phylum Platyhelminthes itself. Elucidation of the *S. mediterranea* genome opened possibilities to discover critical components of neural signaling in these animals as well as in their parasitic counterparts.

Neurotransmission mediated by FMRFamide-like peptides (FaRPs or FLPs) has been proposed as a potential drug target for novel anthelmintics (Day and Maule, 1999). Neuropeptides are abundant intercellular signaling molecules throughout the invertebrates, and many of these signaling molecules have no known structural correlates amongst mammals. Some of the FLP sequences are known while lots more are still to be discovered. Search for new FLPs candidates besides isolation from species includes genomics approach, when FLPs in a particular species are predicted from their genome sequence based on genome comparison with other species with known FLPs.

Physiology of flatworm muscle and a role of the complement of voltage-operated channels in it can also be addressed using *S. mediterranea* as a model flatworm. Voltage-operated  $\text{Ca}^{2+}$  channels (VOCCs) are of interest since  $\text{Ca}^{2+}$  signaling is important in the physiology of muscle. Despite the fact that the presence of VOCCs in both free-living and parasitic flatworms has been evident from molecular data (Kohn *et al.*, 2001), it has been difficult in flatworms to record the inward currents carried by these channels, and even more difficult to perform their pharmacological characterization.

## **VOLTAGE-OPERATED $\text{Ca}^{2+}$ CHANNELS IN VERTEBRATES**

### **VOCCs structure and classification**

Voltage-operated  $\text{Ca}^{2+}$  channels (VOCCs) play important roles in excitable cells by coupling changes in the membrane potential to intracellular  $\text{Ca}^{2+}$ -activated events such as muscle contraction, secretion, neurotransmitter release, stimulation of second messenger systems and regulation of gene expression. Since different physiological responses are linked to different patterns of  $\text{Ca}^{2+}$  entry, a variety of channels exists, which depending on channel type and specific properties provide specific pattern of  $\text{Ca}^{2+}$  fluxes. In vertebrates, various classes of VOCCs are defined by: 1) molecular characterization of channel subunits; 2) electrophysiological criteria, such as the magnitude of depolarizing voltage step required for activation, the time course of inactivation, the magnitude of the conductance of a single calcium channel; and 3) pharmacological criteria based on their sensitivity to

dihydropyridines and  $\omega$ -toxins (Lipscombe *et al.*, 2004; Skeer *et al.*, 1992; Olivera *et al.*, 1994; Mintz *et al.*, 1992; Kasai and Neher, 1992).

Molecular data show that VOCCs consist of a pore-forming  $\alpha_1$  subunit, auxiliary  $\beta$  and  $\alpha_2\delta$ , as well as  $\gamma$  subunits (Witcher *et al.*, 1993; Curtis and Catterall, 1984). Most channel diversity arises from  $\alpha_1$  subunits. The  $\alpha_1$  subunit has been shown to be responsible for channel formation and pore selectivity (Takahashi *et al.*, 1987; Ertel *et al.*, 2000), sensing of transmembrane voltage (De Jongh *et al.*, 1989; Ertel *et al.*, 2000) and presence of sites of channel regulation by drugs, toxins and second messengers (Ertel *et al.*, 2000; Regulla *et al.*, 1991). While  $\alpha_1$  is crucial for pore selectivity, auxiliary subunits, mostly  $\beta$ , can determine the particular properties of  $\text{Ca}^{2+}$  channel, such as activation and inactivation kinetics, increased current amplitudes and the presence of specific sites allowing channel control by intracellular mechanisms (Varadi *et al.*, 1991; Arias 2006; Neely *et al.*, 1993; Reimer *et al.*, 2000; Hohaus *et al.*, 2000). The  $\gamma$ -subunit plays modulator roles of channel complex and is found in skeletal muscle and also in heart and in the brain (Ertel *et al.*, 2000). At least ten different genes encoding  $\alpha_1$  subunits have been identified, which set the grounds for a classification based on their sequence similarity, which within each group is represented by more than 70 % sequence similarity (Ertel *et al.*, 2000):  $\text{Ca}_v1$ ,  $\text{Ca}_v2$  and  $\text{Ca}_v3$  (Table 1).

Electrophysiologically, vertebrate VOCCs are divided into two major groups: high voltage-activated (HVA) and low voltage-activated (LVA). HVA ( $\text{Ca}_v1$  and  $\text{Ca}_v2$ )  $\text{Ca}^{2+}$  channels are slowly inactivating with larger conductances. They require

relatively strong depolarization for their activation, which limits their involvement to processes that are triggered by strong and sustained depolarizations. LVA ( $\text{Ca}_v3$ )  $\text{Ca}^{2+}$  channels are rapidly inactivating channels with smaller conductances and require less depolarization for activation.

Currents carried by HVA channels can be further subdivided into dihydropyridine (DHP)-sensitive (L-type) and DHP-insensitive (non-L-type). Non-L-type HVA channels are further classified based on their sensitivity to toxins (Table 1). The  $\omega$ -conotoxin GVIA blocks N-type channels in mammalian neurons (Kasai and Neher, 1992) and the synthetic analog of funnel-web toxin (sFTX) is a selective blocker of T-type channels in rat dorsal ganglion cells (Lin *et al.*, 1990).

Besides sensitivity to DHPs, vertebrate L-channels are blocked by phenylalkylamines (PAA) such as verapamil (Triggle and Janis, 1987) and are sensitive to  $\omega$ -Agallia toxin (Cohen *et al.*, 1992).

Subunits forming  $\text{Ca}^{2+}$  channels have tissue-specific expression (Angelotti and Hofmann, 1996; Reimer *et al.*, 2000). The  $\alpha_{1S}$  gene is expressed in the skeletal muscle and forms a channel which acts as a voltage sensor and couples depolarization to contraction by means of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) from the sarcoendoplasmic reticulum (SR) via ryanodine receptor activation (Tanabe *et al.*, 1988; Xu *et al.*, 2008; Ríos and Pizarro, 1991). The  $\alpha_{1C}$  gene is expressed in different tissues, including smooth muscle (Yokoyama *et al.*, 2006; Boyer *et al.*, 2001; Reimer *et al.*, 2000; Hohaus *et al.*, 2000), cardiac muscle (Hullin *et al.*, 1999; Schroder *et al.*, 2007) as well as in osteoblasts and neuronal tissue (Bergh *et al.*,

2003; Striessnig *et al.*, 2006). Here the  $\text{Ca}^{2+}$  channel operates as an ion channel and allows calcium influx, which triggers cardiac or smooth muscle contraction (Zhang *et al.*, 2007; Chen *et al.*, 2005). The  $\alpha_{1D}$  gene is co-expressed with the  $\alpha_{1C}$  in cardiovascular tissues (Marcantoni *et al.*, 2007) and also forms a channel responsible for synaptic transmission control in inner hair cells, adrenal chromaffin cells and photoreceptors (Johnson and Marcotti, 2008; Marcantoni *et al.*, 2007; Wu *et al.*, 2007). The  $\alpha_{1F}$  subunit has been shown to assemble the retinal specific L-type channel (Vigh and Lasater, 2004).

Within the mammalian system, the molecular-based classification is in good accordance to groups formed on similar physiological and pharmacological profiles: L-type ( $\text{Ca}_v1$ ); N-, P/Q-, R-type ( $\text{Ca}_v2$ ) and T-type ( $\text{Ca}_v3$ ). However channels from different groups have been shown to overlap in voltage dependence and inactivation kinetics (Regan *et al.*, 1991). The ability for activation at lower voltages should not be solely attributed to the family of T-type class  $\text{Ca}^{2+}$  channels, since studies in cochlea inner hair cells, pancreatic  $\beta$ -cells and in neuronal tissue (Koschak *et al.*, 2001; Scholze *et al.*, 2001; Xu and Lipscombe, 2001) have demonstrated presence of low-voltage-activated with fast kinetics neuronal L-type  $\text{Ca}^{2+}$  channels, belonging to  $\text{Ca}_v1.3$  family (Lipscombe *et al.*, 2004). The studied L-type  $\text{Ca}^{2+}$  channels formed by  $\alpha_{1D}$  subunits had produced currents that activated at more negative voltages as compared to the rest of the family, had different inactivation kinetics, and had the same or decreased sensitivity to DHPs.

**Table 1. Classification of vertebrate voltage-operated Ca<sup>2+</sup> channels.**

Table 1

Vertebrate Ca currents				
High-Voltage Activated (HVA)			Low-Voltage Activated (LVA)	
DHP-sensitive	DHP-insensitive			
L-type ( $Ca_v1$ ) (resistant to $\omega$ -toxins, sensitive to $\omega$ -Agallia toxin)	Non L-type ( $Ca_v2$ )			T-type ( $Ca_v3$ )
	Blocked by $\omega$ -conotoxin GVIA and $\omega$ -conotoxin MVIIA	Blocked by $\omega$ -conotoxin MVIIC + $\omega$ -agatoxin IVA	Toxin-resistant	
	N-type	P/Q-type	R-type	
<b><math>Ca_v1.1</math> (<math>\alpha_{1s}</math>)</b> Skeletal muscle <b><math>Ca_v1.2</math> (<math>\alpha_{1c}</math>)</b> Heart, smooth muscle, brain, pituitary, adrenal <b><math>Ca_v1.3</math> (<math>\alpha_{1d}</math>)</b> Brain, pancreas, kidney, ovary, cochlea <b><math>Ca_v1.4</math> (<math>\alpha_{1f}</math>)</b> Retina	<b><math>Ca_v2.2</math> (<math>\alpha_{1b}</math>)</b> Neurons (peripheral and CNS); Mediate neurotransmitter release at synaptic endings.	<b><math>Ca_v2.1</math> (<math>\alpha_{1a}</math>)</b> Central and peripheral neurons; synaptic terminals; Purkinje neuron cell bodies; Cochlea; Pituitary.	<b><math>Ca_v2.3</math> (<math>\alpha_{1e}</math>)</b> Brain (soma and proximal neuron dendrites); Retina; Heart; Cochlea; Pituitary.	<b><math>Ca_v3.1</math> (<math>\alpha_{1g}</math>)</b> Brain, nervous system <b><math>Ca_v3.2</math> (<math>\alpha_{1h}</math>)</b> Brain, heart, kidney, liver. <b><math>Ca_v3.3</math> (<math>\alpha_{1i}</math>)</b> Brain



## **VOCC mechanisms of regulation**

L-type channels may be controlled by a wide range of mechanisms. Regulation of L-type  $\text{Ca}^{2+}$  channels can be voltage-dependent and voltage-independent (Marcantoni *et al.*, 2007; Carbone *et al.*, 2001). Voltage-dependent modulation includes facilitation (by strong and long-lasting prepulses) and fast cAMP (cyclic adenosine monophosphate) / protein kinase A (PKA)-mediated phosphorylation favored by strong depolarizations.

Voltage-dependent facilitation of L-type channels results in increased current amplitudes due to long channel openings and high probability of being open in cardiac and skeletal muscle (Pietrobon and Hess, 1990; Ma *et al.*, 1996; Kleppisch *et al.*, 1994) as well as in neuronal and neuroendocrine tissues (Hoshi *et al.*, 1984; Kammermeier and Jones, 1998). In the case of N- and P-/Q-types of channels, voltage-dependent facilitation reduces the delayed transition caused by neurotransmitters from modified to a facilitated gating mode (Carabelli *et al.*, 1996) and involves dissociation of a  $\text{G}_o\beta\gamma$  subunit from the channel (Herlitze *et al.*, 1996; Boland and Bean, 1993). In ganglion and sympathetic neurons a phosphorylation mechanism involving protein kinase C (PKC) has been shown to prevent G-protein-mediated inhibition without any other effect (Barrett and Rittenhouse, 2000; Zhu and Ikeda, 1994).

The cAMP/PKA-mediated phosphorylation favored by strong depolarizations has been reported for neuroendocrine cells (Artalejo *et al.*, 1990; Artalejo *et al.*,

1991; Carbone *et al.*, 2001), skeletal muscle (Johnson *et al.*, 1997; Johnson *et al.*, 1994) and neurons (Sculptoreanu *et al.*, 1995).

Voltage-independent L-type  $\text{Ca}^{2+}$  channel regulation comprises potentiation by increased cAMP levels and inhibition by  $G_{i/o}$ . Inhibition by  $G_{i/o}$  is mainly confined to  $\alpha_{1D}$  and  $\alpha_{1C}$  subunits, expressed in neuronal and neuroendocrine cells, where current scale down with no change in the activation course is triggered by neurotransmitters and is direct membrane-delimited (Albillos *et al.*, 1996; Carbone *et al.*, 2001; Carabelli *et al.*, 1998; Hernández-Guijo *et al.*, 1999). On the contrary to L-type channels, inhibition by  $G_{i/o}$  of N- and P-/Q-type channels is voltage-dependent (Carbone *et al.*, 2001).

Cardiac muscle L-type  $\text{Ca}^{2+}$  channel potentiation by increased cAMP levels requires  $\beta_1$ -adrenergic stimulation or application of cAMP permeable analogs to activate protein kinase A (PKA), which phosphorylates the channel and increases its open probability resulting in increased current amplitude (Bean *et al.*, 1984).

Channel regulation may be possible due to the intrinsic property of  $\alpha_1$  subunit, or may require involvement of other subunits. Voltage-dependent facilitation in case of smooth and cardiac muscle  $\alpha_{1C}$  has been reported without the requirement of  $\beta$  subunits (Kleppisch *et al.*, 1994; Lee *et al.*, 2006), while in some cases their co-expression is obligatory for the proper channel regulation (Bourinet *et al.*, 1994; Kamp *et al.*, 2000; Platano *et al.*, 2000; Cens *et al.*, 1996). Out of four subunits ( $\beta_1$ ,  $\beta_2$ ,  $\beta_3$  and  $\beta_4$ ), only  $\beta_2$  is not permissive for  $\text{Ca}^{2+}$  channel facilitation (Cens *et al.*, 1996; Carbone *et al.*, 2001). Different regulatory outcomes seem to be attributed to

the ability of  $\beta$  subunits to affect the relationship of the gating charge movement and channel opening (Neely *et al.*, 1993; Olcese *et al.*, 1996).

## **VOLTAGE-OPERATED $\text{Ca}^{2+}$ CHANNELS IN INVERTEBRATES**

In invertebrates the tight link between molecular and pharmacological identity is not well maintained, thus making it hard to render a particular channel as being L- or non-L-type according to both molecular and pharmacological profiles. Amongst invertebrates both L- and non-L-type  $\alpha_1$  subunits have been cloned from flatworms *S. mansoni* (Kohn *et al.*, 2001) and *Bdelloura candida* (Greenberg *et al.*, 1995), the nematode *C. elegans* (Lee *et al.*, 1997; Schafer and Kenyon, 1995), the fruit fly *D. melanogaster* (Zheng *et al.*, 1995; Smith *et al.*, 1996), and a cockroach *Blatella germanica* (Inagaki *et al.*, 1998), amongst others. Only T-type  $\alpha_1$  subunit cloned so far is from *C. elegans* system (Jeziorski *et al.*, 2000). HVA  $\beta$  subunits have been cloned from *S. mansoni* (Kohn *et al.*, 2001b), the pearl oyster *Pinctada fucata* (Fan *et al.*, 2007), a squid *Loligo bleekeri* (Kimura and Kubo, 2003) and *C. elagans* (Jeziorski *et al.*, 2000).

Invertebrate  $\text{Ca}^{2+}$  channels have different pharmacological properties as compared to their vertebrate counterparts, with most of the difficulty arising in trying to define pharmacological separation between L- and N-types (Table 2). Different types of  $\text{Ca}^{2+}$  currents are determined by channel variation, which is defined by combination between  $\alpha$ ,  $\beta$ ,  $\alpha_2\delta$  and  $\gamma$  subunits isoforms. Due to a more limited number of genes encoding subunit sequences in invertebrates as compared to vertebrates, a variety of  $\text{Ca}^{2+}$  currents is made possible by post-translational

modification (e.g. presence of consensus sites for phosphorylation by protein kinases), alternate splicing of subunit transcripts (e.g. gene variation at key sites) and RNA editing (altered translation at certain sites). All these changes provide differences, such as channel kinetics and a presence or lack of motifs which can be intracellularly regulated. Since invertebrate  $\alpha_1$  subunits possess significant sequence similarity with their vertebrate counterparts, the channel they form is still referred to as a particular channel group according to the molecular properties, while pharmacology can be different (Table 2).

### **Ca<sup>2+</sup> signaling in invertebrates**

Ca<sup>2+</sup> signaling in both vertebrate and invertebrate muscle systems recruits both extracellular and intracellular Ca<sup>2+</sup> (Wray *et al.*, 2005; Greenberg, 2005). Intracellular Ca<sup>2+</sup> stores were identified ultrastructurally in jellyfish *Polyorchis penicillatus* (Lin and Spencer, 2001) and some other invertebrates. In flatworm muscle the presence of sarcoendoplasmic reticulum (SR) serves as an intracellular Ca<sup>2+</sup> reservoir, and has been proved by electron microscopy for turbellarians, trematodes and cestodes (MacRae, 1963; Silk and Spence, 1969; Lumsden and Byram III, 1967). In schistosomes, the presence of ryanodine receptors, which can mediate the release of Ca<sup>2+</sup> stored in the SR, has been demonstrated by [<sup>3</sup>H] ryanodine binding (Silva *et al.*, 1998). Flatworm muscle fiber SR has been shown to possess enough Ca<sup>2+</sup> to support muscle contraction elicited by ryanodine channel agonist caffeine. Dose-response curves have been provided for free-living flatworms *D. tigrina* and *P. littoralis*, and the trematode *S. mansoni* (Day *et al.*, 2000).

**Table 2. Differences in pharmacological separation between L-type and N-type voltage-operated Ca<sup>2+</sup> channels in vertebrates versus invertebrates.**

Table 2

	L-type	N-type
V E R T E B R A T E	<p><i>Sensory neurons:</i></p> <ul style="list-style-type: none"> <li>• <b>L-type</b> blocked by <math>\omega</math>-conotoxin GVIA<sup>1</sup></li> </ul> <p><i>Cardiac, skeletal or smooth muscle:</i></p> <ul style="list-style-type: none"> <li>• <b>L-type</b> not blocked by <math>\omega</math>-conotoxin GVIA<sup>1</sup></li> </ul>	<p><i>Chick brain:</i></p> <ul style="list-style-type: none"> <li>• <b>N-type</b> in synaptosomal membranes is blocked by <math>\omega</math>-conotoxin GVIA<sup>2</sup></li> </ul> <p><i>Frog muscle:</i></p> <ul style="list-style-type: none"> <li>• <b>N-type</b> in neuromuscular junction is blocked by <math>\omega</math>-conotoxin GVIA<sup>3</sup></li> </ul> <p><i>Rat brain:</i></p> <ul style="list-style-type: none"> <li>• <b>Non-L-type</b> in synaptosomal membranes is not blocked by <math>\omega</math>-conotoxin GVIA<sup>4</sup></li> </ul> <p><i>Mouse muscle:</i></p> <ul style="list-style-type: none"> <li>• <b>Non-L-type</b> in neuromuscular junction is not blocked by <math>\omega</math>-conotoxin GVIA<sup>5</sup></li> </ul>
		<p><i>Avian neurons:</i></p> <p><b>N-type?</b></p> <ul style="list-style-type: none"> <li>• Blocked by <math>\omega</math>-conotoxin GVIA<sup>6</sup></li> <li>• DHP-sensitive<sup>6</sup></li> </ul>
I N V E R T E B R A T E	<p><b>Gastropod <i>Aplysia californica</i></b></p> <p><i>Bag cell neurons:</i></p> <p><b>L-type?</b></p> <ul style="list-style-type: none"> <li>• HVA, inhibited by nifedipine<sup>7</sup></li> <li>• not blocked by <math>\omega</math>-conotoxin GVIA<sup>1</sup></li> <li>• Small channel conductance<sup>7</sup></li> <li>• Not stimulated by DHP-agonists<sup>8</sup></li> </ul>	<p><b>Squid</b></p> <p><i>Giant synapse:</i></p> <p><b>N- or P-type?</b></p> <ul style="list-style-type: none"> <li>• <math>\omega</math>-conotoxin GVIA has no effect<sup>9</sup></li> <li>• DHP-insensitive<sup>9</sup></li> <li>• Activated by small depolarizations<sup>9</sup></li> <li>• Blocked by FTX (funnel-web toxin)<sup>10</sup></li> </ul>
	<p><b>Cockroach <i>Periplaneta americana</i></b></p> <p><i>Skeletal muscle:</i></p> <ul style="list-style-type: none"> <li>• <b>L-type</b> PAA/DHP-sensitive<sup>11</sup></li> </ul> <p><i>Nervous system:</i></p> <p><b>L-type?</b></p> <ul style="list-style-type: none"> <li>• PAA-sensitive/DHP-insensitive<sup>11</sup></li> <li>• PAA-insensitive<sup>12</sup></li> </ul>	
	<p><b>Fruit fly <i>Drosophila melanogaster</i></b></p> <p><i>Brain membranes:</i></p> <p><b>L-type?</b></p> <ul style="list-style-type: none"> <li>• DHP- and PAA-binding sites are located on separate Ca channels<sup>13</sup></li> <li>• DHP/PAA-insensitive<sup>13</sup></li> <li>• PAA-sensitive/DHP-insensitive<sup>13</sup></li> <li>• DHP-sensitive/PAA-insensitive<sup>13</sup></li> </ul>	

**References:**

(1) McCleskey *et al.*, 1987; (2) Venema *et al.*, 1992; (3) Kerr and Yoshikami, 1984; (4) Suszkiw *et al.*, 1987; (5) Sano *et al.*, 1987; (6) Aosaki and Kasai, 1989; (7) Strong *et al.*, 1987; (8) Nerbonne and Curney, 1987; (9) Charlton and Augustine, 1990; (10) Llinas *et al.*, 1989b; (11) Skeer *et al.*, 1992; (12) Blagburn and Sattelle, 1987; (13) Pelzer *et al.*, 1989.

Caffeine turned out to be the most potent on *S. mansoni* muscle with EC<sub>50</sub> of 0.7 mM compared to 3.2 mM and 4.6 mM for *D. tigrina* and *P. littoralis*, respectively. The sarcoendoplasmic reticulum Ca<sup>2+</sup> ATPase (SERCA), a Ca<sup>2+</sup> pump which removes cytosolic Ca<sup>2+</sup> by sequestering it into SR has been characterized in the fruit fly *D. melanogaster* (Váradi *et al.*, 1989), in the crustaceans *Artemia franciscana* (Escalante and Sastre, 1993) and *Procambarus clarkii* (Zhang *et al.*, 2000), and in the flatworm *S. mansoni* (De Mendonça *et al.*, 1995).

The presence of voltage-operated Ca<sup>2+</sup> channels (VOCCs) in flatworm muscle has been demonstrated by contraction studies using depolarization with elevated extracellular K<sup>+</sup>. Specifically, depolarization elicits contraction of muscles from a number of different flatworms, and those depolarization-induced contractions are blocked by some classical VOCCs blockers (Mendonça-Silva *et al.*, 2006). Further, VOCCs currents have been measured using whole-cell patch clamp in both *G. tigrina* (Cobbett and Day, 2003) and *Bdelloura candida* (Blair and Anderson, 1993), and, very recently, in *S. mansoni* (Mendonça-Silva *et al.*, 2006). The presence of VOCCs in *S. mansoni* is also demonstrated by the identification of cDNAs encoding three different  $\alpha$  subunits and one  $\beta$  subunit of VOCCs (Kohn *et al.*, 2001; 2001b). Although the presence of VOCCs in both free-living and parasitic flatworms has been evident from molecular data, it has been difficult to record the inward currents carried by these channels, and even more difficult to perform experiments investigating the pharmacology of these important channels.

Experiments proposed to characterize the pharmacology of whole isolated flatworm muscle fiber membrane inward currents pose a challenge since these inward currents rapidly run down. As with VOCCs in all cells, there is often a rapid decrement in the amplitude of inward currents soon after the whole cell configuration is obtained. In these small muscle fibers of flatworms that rapid rundown is dramatic and presents a challenge to measuring the effects of putative blockers and thus their pharmacological characterization.

VOCCs in the skeletal muscle of crustaceans provide  $\text{Ca}^{2+}$  influx required for tension generation (Caputo and Dipolo, 1978). Some studies showed that  $\text{Ca}^{2+}$  influx is sufficient to account for tension development (Atwater *et al.*, 1974), while others say that  $\text{Ca}^{2+}$  influx mainly serves to trigger the release of  $\text{Ca}^{2+}$  from the SR intracellular stores that activates the contraction –  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) (Caputo and Dipolo, 1978; Mounier and Goblet, 1987). In crustacean skeletal muscle CICR is proposed to be the mechanism of excitation-contraction coupling (Györke and Palade, 1992). Unlike in other arthropod skeletal muscles, the superficial abdominal flexor muscle of the crustacean *Atya lanipes* is electrically unexcitable (Monterrubbio *et al.*, 2000; Bonilla *et al.*, 1992). When depolarized they do not develop graded potentials or  $\text{Ca}^{2+}$  spikes of all-or-none type, while they are dependent on some extracellular  $\text{Ca}^{2+}$  to trigger contraction. Even in the conditions that suppress outward conductance depolarization-induced  $\text{Ca}^{2+}$  currents are too small to be measured (no inward current detected with 138 mM  $\text{Ca}^{2+}$ ). Thus the extracellular  $\text{Ca}^{2+}$  requirement in the absence of inward voltage-operated  $\text{Ca}^{2+}$  currents can be explained by employing CICR. These channels were called “silent



Ca<sup>2+</sup> channels” and the currents with 138 mM Sr<sup>2+</sup> and Ba<sup>2+</sup> as charge carriers were sensitive to DHPs, the blockers of classical voltage-gated Ca<sup>2+</sup> channels.

### **Ca<sup>2+</sup> currents are regulated by neuropeptides**

Invertebrate nervous systems involve a number of different neurotransmitters, with regulatory neuropeptides being a major part of it. Neuropeptides are signaling molecules that control sensory and motor functions. They act through a variety of intracellular mechanisms and generalizations about the pathways they trigger cannot be made.

The first peptide neurotransmitter identified in invertebrates was myotropic pentapeptide proctolin RYLPT. It was identified in the hindgut of the cockroach *Periplaneta americana* (Brown and Starrat, 1975). In insects and crustaceans proctolin stimulates skeletal and visceral muscle and potentiates nerve-stimulated contractions of skeletal muscles (Facciponte *et al.*, 1996; Bartos *et al.*, 1994; Lange, 2002). It also potentiates depolarization-induced contractions of skeletal muscles under current clamp (Erxleben *et al.*, 1995) or in high-potassium solutions (Bishop *et al.*, 1987), as well as enhancing contractions triggered by caffeine (Wegener and Nässel, 2000). Pharmacological studies on the myotropic actions of proctolin in invertebrates showed the involvement of both VOCCs and non-voltage- dependent Ca<sup>2+</sup> channels (Lange *et al.*, 1987; Hertel and Penzlin 1986; Wilcox and Lange, 1995).

The L-type Ca<sup>2+</sup> current enhancement can be a good candidate to be a major determinant in the potentiation of contractions produced by modulatory

neurotransmitters. Studies on proctolin effects on the flexor neuromuscular system in the crayfish showed that it alone had no effect on plasma membrane  $\text{Ca}^{2+}$  channels of the tonic flexor muscle and thus did not induce muscle tension, but modulated their activity in response to depolarization and potentiated the depolarization-induced tension of the muscle (Bishop *et al.*, 1991). Proctolin enhanced influx of extracellular  $\text{Ca}^{2+}$  due to increased single-channel open probability determined by the reduction of the mean closed time and appeared to regulate  $\text{Ca}^{2+}$  channels via an intracellular messenger, possibly cAMP. In insect oviduct preparation (Holman and Cook, 1985) and cockroach hindgut (Cook and Holman, 1985) proctolin response however was relatively unaffected by removal of extracellular  $\text{Ca}^{2+}$  or addition of  $\text{Ca}^{2+}$  channel blockers. Thus plasma membrane  $\text{Ca}^{2+}$  channels may be not the only means by which proctolin influences muscle tension.

In the mollusk *Aplysia californica* the contraction amplitude and duration of accessory radula closer muscle can be regulated by small cardioactive peptides (SCPs), myomodulins and 5-HT (Březina *et al.*, 1994). In a voltage-clamp experimental setup, a DHP-sensitive L-type current was enhanced by 5-HT and SCPs as well as by elevation of cAMP. Inward current enhancement developed within seconds after modulator application, showed no desensitization, was blocked in a time-dependent manner by  $\text{Co}^{2+}$  and nifedipine (20-50  $\mu\text{M}$  completely eliminated the current, while 1  $\mu\text{M}$  affected the current at the end of the voltage step) and was practically irreversible.

In cockroach *Periplaneta americana* hyperneural muscle, the peptide seems to use mechanisms different from, or in addition to, membrane depolarization to induce  $\text{Ca}^{2+}$  influx since the VOCC blocker nifedipine only partially blocks the response to the peptide as compared to depolarization-evoked contractions (Wegener and Nässel, 2000; Lange *et al.*, 1987). It is still not clear to what extent proctolin-induced tonic insect muscle contraction is dependent on  $\text{Ca}^{2+}$  mobilized by inositol-1, 4, 5-trisphosphate ( $\text{IP}_3$ ) or CICR. Proctolin myoexcitatory effect was blocked by thapsigargin and ryanodine in a tonic insect muscle of *Periplaneta americana* (Wegener and Nässel, 2000). Proctolin has been shown to act through involvement of a PLC-activated pathway in foregut of the locust *Schistocerca gregaria* (Hinton and Osborne, 1995) and possibly might act through PKC activation (Wegener and Nässel, 2000). The myoexcitatory effect of proctolin seems to employ extracellular  $\text{Ca}^{2+}$  influx a prerequisite, while intracellular  $\text{Ca}^{2+}$  concentration increase appears largely to be due to intracellular  $\text{Ca}^{2+}$  release. Peptides induce  $\text{Ca}^{2+}$  influx by an activation or modulation of DHP-sensitive and voltage-independent sarcolemmal  $\text{Ca}^{2+}$  channels.

Another large group of invertebrate regulatory neuropeptides are FMRFamide-related peptides. These are short peptides, 4-20 amino acids long that have a common Arg-Phe-amide C-terminus (FMRFamide-related peptides – FaRPs or FLPs). FMRFamide was first identified in the clam *Macrocallista nimbosa* and was shown to be cardioexcitatory and to induce myoexcitatory responses in non-cardiac smooth muscle of mollusks (Price and Greenberg, 1977; Greenberg *et al.*, 1983). In insects FLPs have potent myotropic effect on both skeletal and visceral muscle

(Robb and Evans, 1994; Walther *et al.*, 1984). Besides arthropods and insects, FLPs are common in helminths, too. Four FLPs have been isolated and characterized biochemically from flatworm species: YIRFamide from *Bdelloura candida* (Johnston *et al.*, 1996), GYIRFamide from *Dugesia tigrina* and *Bdelloura candida* (Johnston *et al.*, 1995; Johnston *et al.*, 1996), RYIRFamide from *Arthurdendyus triangulates* (Maule *et al.*, 1994) and GNFFRFamide from the cestode *Moniezia expanza* (Maule *et al.*, 1993). Besides isolation from species themselves the search for new FLPs candidates includes a genomics approach, when FLPs in particular species are predicted from their genome sequence based on genome comparison with other species with known FLPs.

FLPergic neurons innervate almost all muscular systems in helminths, responsible for movement, feeding, reproduction and attachment within the host. Physiological data on FLPs in flatworms is presented by myoexcitatory effects on muscle preparations of turbellarian *Procerodes littoralis* (Money Penny *et al.*, 2001), trematodes *Fasciola hepatica* (Graham *et al.*, 1997; Graham *et al.*, 2000; Marks *et al.*, 1996) and *S. mansoni* (Day *et al.*, 1994); and cestode *Mesocestoides corti* (Hrčková *et al.*, 2002). Since musculature plays an important role in locomotion, attachment and feeding as well as in reproduction, it is very attractive as a potential target for the drug therapy. The dramatic effects on muscle systems make FLP systems potentially good target, but the mechanisms by which they produce these effects remain unknown.

YIRFamide is very potent in inducing individual muscle contractions in *S. mansoni*. YIRFamide causes contraction of individual muscle fibers in a concentration-dependent manner when it is microperfused onto the individual, dispersed muscle fibers. The peptide is very potent with  $EC_{50}$  0.1  $\mu$ M. YIRFamide-induced contractions are a bit different from those stimulated by depolarization with elevated extracellular  $K^+$ . The FLP-induced contractions are slightly slower at onset and lead to more overall smooth pattern of contraction.

Arthropod FMRFamide-related peptides belong to five subfamilies: myosuppressins, N-terminally extended-FLRFamides, -FMRFamides, -RFamides and sulfakinins (Aguilar *et al.*, 2004). Cross-phylum activity of some of them was tested in *Ascaris suum* body wall and ovijector muscles and on the dispersed muscle fibers turbellarian *Procerodes littoralis*. Almost all tested peptides modulate *A. suum* muscle in different ways (inhibitory, excitatory and biphasic effect on tension) and induced potent contractions of isolated *P. littoralis* muscle fibers (Mousley *et al.*, 2004).

Effects posed by native and non-native nematode FLPs can be subdivided into five types of responses, which might be indicative of at least five different FLP receptors on the muscle (Moffett *et al.*, 2003). Nematode FLPs showed to be myoexcitatory on the muscle activity of parasitic flatworm *Fasciola hepatica*, while flatworm FLPs failed to produce any visible effect on *A. suum* body wall and ovijector muscles (Marks *et al.*, 1997). Since peptides isolated from phylum Arthropoda have been shown to be potent on preparations obtained from Nematoda and

Platyhelminthes, it can be indicative of possible similarities in the ligand requirements for FLP receptors.

Using antisera raised against vertebrate neuropeptides, the nervous systems of flatworms have been shown to possess abundant neuropeptide immunoreactivities (Halton and Gustafsson, 1996). Besides FLPs, there are at least two more distinct families of neuropeptides in flatworms: peptides of similar size to FLPs that lack the carboxy-terminal Arg-Phe-amide (neuropeptide-like proteins – NLPs) and neuropeptide Y-like peptides of 36-40 amino acids (neuropeptide Fs – NPFs). NPFs structurally analogous to vertebrate neuropeptide Ys have been characterized from *Moniezia expanza* (Maule *et al.*, 1991), *Arthurdendyus triangulatus* (formely *Artioposthia triangulata*) (Curry *et al.*, 1992), *S. mansoni* and *S. japonicum* (Humphries *et al.*, 2004) and recently also discovered in the genome of a flatworm *S. mediterranea* (Day, unpublished).

Another group of arthropod neuropeptides, allatostatins, are potently myoactive in arthropods. Allatostatins induce dose-dependent contraction of flatworm *P. littoralis* isolated muscle fibers, while they were shown to be either inactive or inhibitory on *A. suum* body wall and ovjector muscle respectively (Mousley *et al.*, 2005).

### **Neuropeptides signaling mechanisms**

The signaling mechanism underlying FLPs effects in helminths is still unclear (Omar *et al.*, 2007). Many second-messenger systems transduce signals using G-proteins, which stimulate and inhibit enzymes like adenylate cyclase or

phospholipase C, leading to elevation or reduction of second messenger levels. To date the only identified FLP receptors are FMRFamide-gated sodium channel identified in the snail *Helix aspersa* (Linguegila *et al.*, 1995), orphan FLP GPCR in *Caenorhabditis elegans* (Mertens *et al.*, 2005) and the GPCR, characterized from the free-living flatworm *Girardia tigrina*, GtNPR-1 (Omar *et al.*, 2007). Upon GtNPR-1 identification, using chimerical G-proteins, the receptor has been shown to couple to either  $G\alpha_i$  or  $G\alpha_o$  pathway, along with a proposed possibility of  $G_i \beta\gamma$  dimers working through stimulation of phospholipase C  $\beta$  (PLC $\beta$ )-mediated cascade (Omar *et al.*, 2007). Genes encoding peptide receptors (GPCRs) in *C. elegans* genome are abundant counting up to 70 genes, with *npr-1* being homologous to *D. melanogaster* and the snail *Lymnaea stagnalis* genes (Geary *et al.*, 1999; McVeigh *et al.*, 2005). In nematodes the FLP AF2 is believed to act through a production of cAMP levels of which are believed to increase with muscle relaxation and decrease with muscle contraction (Geary *et al.*, 1999). In parasitic flatworms, liver fluke *Fasciola hepatica*, GYIRFamide-induced muscle contraction was shown to be PKC-dependent upon activation of PLC pathway (Graham *et al.*, 2000), which could be indicative of  $G\alpha_q$  involvement (Omar *et al.*, 2007).

Some other neuropeptides have been shown to cause inhibitory action on invertebrate muscle. Peptide sequence identical to SchistoFLRFamide was isolated and sequenced from the brain and retrocerebral complex of the locust (Peeff *et al.*, 1994). Studies on biological effects posed by the peptide on the locust oviduct visceral muscle have shown that the peptide has potent modulatory effect on the muscle. It has been shown to inhibit or significantly reduce spontaneous, proctolin-

or high-potassium-induced contractions (Wang *et al.*, 1995). The requirement of  $\text{Ca}^{2+}$  entry through plasma membrane via VOCCs and ligand-gated  $\text{Ca}^{2+}$  channels in these types of contractions may suggest these structures to be the point of ScistoFLRFamide action (Wang *et al.*, 1995; Lange *et al.*, 1991). Two membrane receptors associated with SchistoFLRFamide have been identified (Wang *et al.*, 1994). The variety of stimuli that can be altered by SchistoFLRFamide implies that it can act on excitation-contraction coupling pathway. The exact site of its action is far from being clear.



## SPECIFIC AIMS

Parasitic platyhelminths, both flukes and tapeworms, infect a variety of vertebrates, including humans and animals. *Schistosoma mansoni* is a blood fluke that inflicts huge morbidity and mortality rates in humans worldwide. The selection of existing anthelmintics is quite narrow and there is an ongoing concern that parasites are developing resistance to available drugs, making the potential choices even fewer. The discovery of a new generation of anthelmintics is at least in part limited by the lack of our knowledge of parasite biology and physiology. The platyhelminth nervous system is very complex, especially in terms of neurotransmission.

Neurotransmission mediated by FMRFamide-like peptides (FLPs) has been proposed as a potential drug target for novel anthelmintics. Functional data on flatworm FLPs is thus far confined to myoexcitatory action in both free-living and parasitic flatworms. Since musculature is very important for locomotion, feeding and reproduction, it is reasonable to hypothesize that interfering with FLP signaling would compromise the survival of the worms.

The overall specific aim of the current project is to broaden our knowledge of the physiology and pharmacology of flatworm muscle, including both free-living and parasitic species, especially in relation to neuromuscular excitation mediated by FLPs. A clear demonstration of the biology and signaling mechanisms involved in FLP signaling will validate further studies on FLP receptors and the neuropeptides themselves as appropriate drug targets. This research project has three specific aims to examine.

**SPECIFIC AIM 1: TEST THE HYPOTHESIS THAT EXTRACELLULAR  $\text{Ca}^{2+}$  IS REQUIRED IN MEDIATING FLP-INDUCED RESPONSE IN ISOLATED S.**

**MANSONI MUSCLE**

Different pharmacological agents can elicit myoexcitatory responses in flatworm muscle. It is supposed that muscle contraction is dependent on a rise in cytoplasmic  $\text{Ca}^{2+}$ , and that the source of this  $\text{Ca}^{2+}$  can be either intracellular (Wray *et al.*, 2005) or extracellular (Greenberg, 2005). Flatworm FLPs have been shown to potently and specifically induce muscle contraction in flatworms (Day *et al.*, 1994). Though FLPs play important roles in the physiology of flatworm muscle, their mechanism of action is not understood. Here we propose to test the hypothesis that extracellular  $\text{Ca}^{2+}$  is required to support FLP-induced contractions. If so, we will try to determine the nature of ion channels responsible for the  $\text{Ca}^{2+}$  fluxes across the sarcolemma by characterizing the pharmacology of the FLP-induced contractions.

- 1.1 Test the hypothesis that FLP-induced contractions are dependent on extracellular  $\text{Ca}^{2+}$
- 1.2 Test the hypothesis that VOCCs are an important conduit for FLP induced  $\text{Ca}^{2+}$  influx
  - 1.2.1 *FLP-induced contractions in the presence of VOCCs blockers*
  - 1.2.2 *Voltage-gated inward whole cell plasma membrane currents in the presence of VOCCs blockers*

1.2.3 FLP-induced changes in voltage-gated inward whole-cell plasma membrane currents.

**SPECIFIC AIM 2: TEST THE HYPOTHESIS THAT IN *S. MANSONI* MUSCLE FLPs TRIGGER  $\text{Ca}^{2+}$  INFLUX THROUGH INTRACELLULAR SIGNALLING CASCADE**

Functional data on flatworm FLPs are confined to myoexcitatory effects in the trematodes *S. mansoni* (Day *et al.*, 1994), *Fasciola hepatica* (Graham *et al.*, 2000) the monogenean *Diclidophora merlangi* (Moneypenny *et al.*, 1997), and the turbellarians *Procerodes littoralis* (Moneypenny *et al.*, 2001) and *Bdelloura candida* (Johnston *et al.*, 1995). Preliminary studies completed using *F. hepatica* muscle strips (Graham *et al.*, 2000) and *P. littoralis* isolated muscle fiber assay (Totten, unpublished) provided evidence for the involvement of a G-protein coupled receptor, and possibly second messenger pathways involving cyclic adenosine monophosphate (cAMP), phospholipase C (PLC), diacylglycerol (DAG) and protein kinase C (PKC). The next series of experiments are proposed to characterize possible involvement of second messenger pathways between activation of FLP receptors and final muscle contraction response. We will try to determine if PLC, PKC or/and protein kinase A (PKA) pathways are involved in mediating the response to FLPs in *S. mansoni* muscle.

We will test how the contraction response and changes in  $\text{Ca}^{2+}$  fluxes across the sarcolemma are altered in the presence of different inhibitors of intracellular signaling cascades enzymes. Upon completion of these sets of experiments we will be able to explore the diversity of FLP-activated signaling pathways.

- 2.1 Test the hypothesis that FLP-induced contractions are mediated by phospholipase C (PLC) activation
- 2.2 Test the hypothesis that FLP-contractions are mediated by PKC activation
- 2.3 Test the hypothesis that protein kinase A (PKA) is involved in mediating FLP-induced contractions.

**SPECIFIC AIM 3: TEST THE HYPOTHESIS THAT A FREE-LIVING FLATWORM, *SCHMIDTEA MEDITERRANEA*, CAN BE USED AS AN AMENABLE MODEL TO STUDY *S. MANSONI* MUSCLE PHYSIOLOGY AND PHARMACOLOGY**

*S. mediterranea* is a free-living flatworm that has become very popular for studies focused on developmental and regeneration biology due to the worms' remarkable regenerative capacity. Further, the worms are very amenable to gene silencing by RNA interference. Another advantage is that there is ongoing genome project which reveals the presence of functional sequences involved in intracellular signaling. Preliminary comparisons between the genomes of *S. mansoni* and *S. mediterranea* suggest a conservation of many of the elements of muscle biology (Zamanian *et al.*, 2007).

Our objective is to see if *S. mediterranea* is similar to its parasitic counterpart with respect to the general nature of the voltage-gated ion channels present in somatic musculature. Here we propose to do a number of electrophysiological experiments to see if *S. mediterranea* possesses a complement of voltage-operated ion channels similar to those in *S. mansoni* in physiological and pharmacological

respects. This will validate further use of this much more tractable species for experiments elucidating flatworm muscle physiology.

The presence of voltage-gated outward and inward currents has been demonstrated in muscle fibers of *Bdelloura candida* (Blair and Anderson, 1996), *Girardia tigrina* (Cobbett and Day, 2003) and *S. mansoni* (Mendonça-Silva *et al.*, 2006). In all of these cases it was very difficult to perform experiments investigating the pharmacology of the inward currents due to the rapid rundown of those currents. We have been successful in developing a protocol for examining the effects of pharmacological agents on inward currents of flatworms, as is detailed in Specific Aim 1. This will allow us to include in this Specific Aim a comparison of the pharmacology of the inward currents in the two worms.

- 3.1 Test the hypothesis that *S. mediterranea* muscle possesses outward current profile and pharmacology similar to that of *S. mansoni*
- 3.2 Test the hypothesis that *S. mediterranea* muscle possesses inward current profile and pharmacology similar to that of *S. mansoni*.

## MATERIALS AND METHODS

### Worms

*S. mediterranea* worms were maintained in the dark in fresh autoclaved ultrapure water supplemented with planarian salts (mM): NaCl 1.6; CaCl<sub>2</sub> 1.0; MgSO<sub>4</sub> 1.0; MgCl<sub>2</sub> 0.1; KCl 0.1; NaHCO<sub>3</sub> 1.2 (pH 7.0).

*S. mansoni* worms were maintained in the mouse host until ready to harvest from mouse mesenteric vein 45-60 days post-infection.

### Muscle cell preparation

Isolated muscle fibers were obtained from adult *S. mansoni* and *S. mediterranea* worms using muscle cell digestion protocol daily.

The isolation medium used during the procedure was based on a modified Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, USA) to which the following chemicals were added (mM): CaCl<sub>2</sub> 2.2 (5.0 in case of *S. mediterranea*); MgSO<sub>4</sub> 2.7; glucose 61.1; NaCl 25.7; dithiothreitol 1.0; gentamicin solution (Roche Applied Science, USA) 1% (v/v) and serotonin 1 μM (15 μM in case of *S. mediterranea*) (pH 7.4).

The dissociation medium was isolation medium with added 1 mM ethyleneglycol-*bis*-(*b*-aminoethyl ether) *N, N, N', N'*-tetraacetic acid (EGTA), 1mM ethylenediamine tetraacetic acid (EDTA) and bovine serum albumin (BSA) 0.1% (w/v).

The final medium was BSA-free dissociation medium to which 1 mg/ml enzyme papain was added (Roche Applied Science, USA).

*S. mansoni* muscle cell isolation was done at 37 °C. *S. mediterranea* muscle cell isolation was done at room temperature.

Live medium sized worms (15-20 pairs of *S. mansoni* or 5-6 *S. mediterranea*) were rinsed (4x) with isolation medium, and chopped 35-40 times in two directions with a razor blade. Resultant worm pieces after rinsing with the same medium (4x) were incubated for 30 minutes on shaker table with papain-containing medium (in three installments). After this medium was removed and worm pieces washed (2x) with isolation medium, they were incubated for an additional 10 minutes in dissociation medium. After final wash with isolation medium (2x) worm pieces were broken using a Pasteur pipette by forcing them back and forth through the pipette orifice. Cell suspension was plated into plastic 35 mm Petri dishes (Corning Inc., USA) in the same isolation medium and allowed to settle for one hour at 19 °C. Cells were used for contraction studies and electrophysiological recording within six hours after plating. Morphological type of isolated *S. mansoni* muscle fibers previously described as frayed (Day *et al.*, 1993) was used in contraction and electrophysiological studies.

### ***S. mansoni* isolated muscle fiber contraction studies**

Observation of isolated muscle fiber contraction was carried out using inverted microscope (Nikon Eclipse TS100) attached to a monitor. A TC-324B single-channel heater controller (Warner Instrument Corp., USA) was used to

preheat Petri dishes with plated fibers 10 minutes before testing and to keep them in warm environment throughout the experiment at 37 °C.

All tested agonists were delivered using a glass micropipette in the close proximity to a cell. Muscle fiber was considered contracted if shortened to less than 50% of its original length in 20 seconds.

For each plate 25-40 muscle fibers were randomly selected and agonists to be tested were pressure-ejected through the micropipette. The bathing medium was used as a negative control. Only fibers that were not spontaneously active were used in experiments.

### **Drug preparation and application**

Chemicals used were purchased from Sigma, except Calphostin C, chelerythrine chloride and Ro 31-8220 which were obtained from BIOMOL (BIOMOL International, L.P., USA). YIRFamide was obtained from Research Genetics.

#### *Contraction studies*

Verapamil, methoxyverapamil, Ro 31-8220 and calphostin C stock solutions were made in DMSO. Diltiazem, H-89, MDL-12,330A and chelerythrine chloride stock solutions were prepared in water. After further dilution of stock solutions DMSO concentration was never higher than 0.1 %.

Some experiments required isolation DMEM to be replaced with inorganic DMEM (DMI), which had the following composition (mM): KCl 4.1; HEPES 15.0; NaCl 82.5; glucose 79.9; CaCl<sub>2</sub> 3.6; MgCl<sub>2</sub> 3.3 (pH 7.4).



Depolarization-induced contractions were elicited by high potassium concentration solution (25 mM K<sup>+</sup>), which had the following chemicals added to distilled water (mM): potassium gluconate 25.0; HEPES 15.0; NaCl 2.6; sodium gluconate 79.5; glucose 40.0; CaCl<sub>2</sub> 3.6; MgCl<sub>2</sub> 3.3 (pH 7.4).

### *Electrophysiology*

The standard extracellular bathing solution for electrophysiological recordings was (mM): NaCl 130.0; KCl 5.0; CaCl<sub>2</sub> 2.0; glucose 10.0; MgCl<sub>2</sub> 1.0; HEPES 20.0 (pH 7.3). The standard electrode solution composition was (mM): KCl 135.0; HEPES 10.0; glucose 10.0; EGTA 2.0 (pH 7.3).

To record inward currents both Ca<sup>2+</sup> and Ba<sup>2+</sup> were simultaneously used as charge carriers. To isolate  $I_{Ca/Ba}$  the standard extracellular bathing solution composition was (mM): HEPES 20.0; MgCl<sub>2</sub> 1.0; glucose 10.0; NaCl 112.0; KCl 5.0; CaCl<sub>2</sub> 2.0; BaCl<sub>2</sub> 15.0 (pH 7.3). In inward current pharmacology experiments this solution was further referred as 'control solution'. It was also used as a vehicle when drugs needed to be introduced. The electrode solution contained (mM): HEPES 10.0; MgCl<sub>2</sub> 2.0; glucose 10.0; CsMethaneSulfonate 130.0; CsCl 20.0; EGTA 10.0 (pH 7.3).

All solutions used for electrophysiology were filtered (0.2 µm; Millipore Millex-GN). About 10 minutes before recording isolation medium was exchanged for extracellular bathing solution.

### *Inward currents pharmacology*

For extracellular application of control solution and drug-containing solutions a SF-77B Perfusion Fast-Step (Warner Instrument Corp, Hamden, CT, USA) system was used. Syringes with solutions were mounted approximately 14 inches over the preparation to give the flow rate of approximately 0.5 ml/min.

VOCCs antagonists (verapamil and diltiazem) were perfused onto patched cells. Verapamil and diltiazem were dissolved in distilled water to make stock solutions (0.1 M or 0.01M) and diluted in vehicle to obtain the final concentration of 10 or 100  $\mu$ M. YIRFamide stock solution (0.01M) was made in distilled water and stored at -20 °C. Aliquots were further diluted in the extracellular bathing solution to the final concentration of 1  $\mu$ M.

To record  $I_{Ca/Ba}$  high-resistance seals and whole cell access were obtained in cells being constantly perfused with the control solution. In control group cells remained under the perfusion for the whole length of the trial. In test groups after the inward current reached a steady state (1.5 min after the start of the trial) the lines supplying perfusion system were switched from control to drug-containing bathing solution for the duration of 2.5 minutes followed by washout with control solution.

### **Whole cell recording**

Whole-cell recordings were performed using Axopatch 200B (Axon Instruments, Inc.) patch clamp amplifier. Experiments were controlled and data recorded using pCLAMP software (pCLAMP 8.0) and Digidata 1320 analog-to-digital

interface (Axon Instruments, Inc.). Currents were amplified, low-pass filtered at 1 kHz and digitized at 11.111 kHz and stored on a personal computer.

Patch-clamp pipettes were double-pulled from borosilicate glass capillaries (outer diameter 1.2 mm, inner diameter 0.68 mm; World Precision Instruments, Inc., USA) using PC-10 Micropipette Puller (Narishige CO., LTD, Japan). Since isolated *S. mansoni* fibers are relatively small (15-20  $\mu\text{M}$ ), successful seals and whole-cell configuration were obtained using patch electrodes with resistances that usually ranged from 15 to 20 M $\Omega$ . Because recorded inward  $I_{\text{Ca/Ba}}$  from isolated *S. mansoni* muscle were relatively small (less than 100 pA), series resistance compensation was not usually used. Series resistances yielded after break-in were typically less than 50 M $\Omega$ , giving rise to an error in command potential less than 5 mV for 100 pA current. In case of *S. mediterranea* isolated muscle fibers patch electrodes had resistances 5-15 M $\Omega$ .

35 mm plastic Petri dishes were used as a recording chamber. *S. mansoni* cells used for the experiments had frayed morphology (as previously described by Day *et al*, 1993) and gigaohm seal formation was most easily obtained with contracted fibers.

All electrophysiological experiments in both *S. mansoni* and *S. mediterranea* isolated muscle fibers were carried out at room temperature (22-25 °C).

To record mixed outward currents and tail currents in *S. mediterranea* muscle fibers were voltage-clamped at the holding potential ( $V_h$ ) of -40 mV followed by a corresponding test pulse protocol.

To record  $I_{Ca/Ba}$  the main whole-cell voltage protocol was as follows. Cells were depolarized in 10 mV increments from  $V_h$  of -70 mV to +60 mV for 200 ms. Traces obtained at each voltage were the average of three runs. To perform inward currents pharmacology experiments muscle fibers were voltage-clamped at  $V_h$  of -70 mV and depolarized to +20 mV for 200 ms twice every 30 s.

Leak and capacity currents were assessed and subtracted online from test currents using P/3 protocol for outward currents and P/2 protocol for inward currents in pCLAMP software. All subsequent data analysis employed averaged leak subtracted current values.

### **Data analysis**

All contraction data are expressed as mean percentage of fibers contracting  $\pm$  SEM with n equal to a number of plates tested on at least three different experimental days. Dose-response data were analyzed using nonlinear regression provided by Prism 4.03 software (GraphPad Software Inc., USA).

For the plotting of graphs, individual peak and sustained leak subtracted values from each cell were first normalized with respect to the peak and sustained current values at 1.5 min after the start of the trial for this particular cell. Then graphs showing baseline current amplitude change during application and after washout of drugs were made from the normalized data.

Patch-clamp data of pharmacology experiments are presented as mean normalized current  $\pm$  SEM with n equal to a number of cells tested during a particular

experiment. Prism software was also used to plot normalized currents data in electrophysiology experiments. For statistical analysis of VOCCs blockers and YIRFamide effect Mann-Whitney test was performed ( $P$  value  $<0.05$ ) using the same program package.

Peak current values used corresponded to peak amplitudes within first 50 ms of the 200 ms test pulse by 5 point smoothing. Using this approach applies a highpass boxcar filter with a specified lower and upper cutoff range. The specified number of smoothing points is used to smooth superfluous aberrations in the data before plotting. The sustained current values corresponded to mean amplitudes within the last 50 ms of the 200 ms test pulse.

## RESULTS

### I. FLPs ARE MYOEXCITATORY IN ISOLATED FLATWORM MUSCLE

#### A. CONTRACTION STUDIES

Physiological data on FLPs in flatworms is presented by myoexcitatory effects on muscle preparations of turbellarian *Procerodes littoralis* (Money Penny *et al.*, 2001), trematodes *Fasciola hepatica* (Graham *et al.*, 1997; Graham *et al.*, 2000; Marks *et al.*, 1996) and *S. mansoni* (Day *et al.*, 1994); and cestode *Mesocestoides corti* (Hrčková *et al.*, 2002). Since FLPs seem to play an important role in the physiology of the flatworm, the search for new possible FLP candidates is ongoing. Besides biochemical isolation from species the search includes genomics approach, when FLPs in particular species are predicted from their genome sequence based on genome comparison with other species with known FLPs. A number of FLPs have been predicted from genome sequence of flatworms (Table 3). All of the neuropeptides listed in Table 3 were tested for their ability to induce contraction of isolated *S. mansoni* muscle fibers (Figure 1).

YIRFamide, as previously described, induced individual muscle contractions in *S. mansoni* in a concentration-dependent fashion (Figure 2). When it was microperfused onto the individual, dispersed muscle fibers, the peptide was very potent with  $EC_{50}$  0.1  $\mu$ M. Since YIRFamide was the most potent of the peptides tested on muscle contraction, it was used for further studies at 1  $\mu$ M concentration. We could also reproduce depolarization-induced contractions using perfusion with

elevated extracellular  $K^+$ . YIRFamide-induced contractions were a bit different from those stimulated by depolarization with elevated extracellular  $K^+$ . The FLP-induced contractions were slower at onset and lead to more overall smooth pattern of contraction.

## **II. EXTRACELLULAR $Ca^{2+}$ IS REQUIRED IN MEDIATING FLP-INDUCED RESPONSE IN *S. MANSONI* MUSCLE**

### **A. CONTRACTION EXPERIMENTS**

#### **FLP-induced contractions are dependent on extracellular $Ca^{2+}$**

Our approach was to examine the effects of various  $Ca^{2+}$  conditions on the FLP-induced contractions using the dispersed muscle fiber contraction assay.

Three different experimental conditions were used (Figure 3):

- 1) Bathing solution as well as perfusion solution with dissolved drugs (YIRFa and caffeine) contained inorganic DMEM (DMI) with  $Ca^{2+}$ . Ion composition for DMI was the following (mM): KCl 4.1; HEPES 15.0; NaCl 82.5; glucose 79.9;  $CaCl_2$  3.6;  $MgCl_2$  3.3 (pH 7.4);
- 2)  $Ca^{2+}$  was omitted from both tip and bathing solutions;
- 3)  $Ca^{2+}$  chelator EGTA was added to the bathing solution to the final concentration of 0.5 mM.

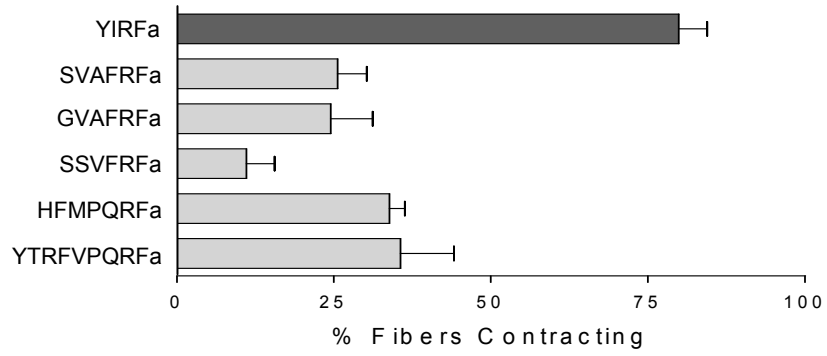
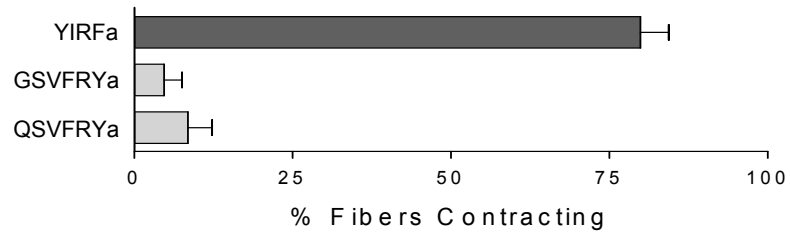
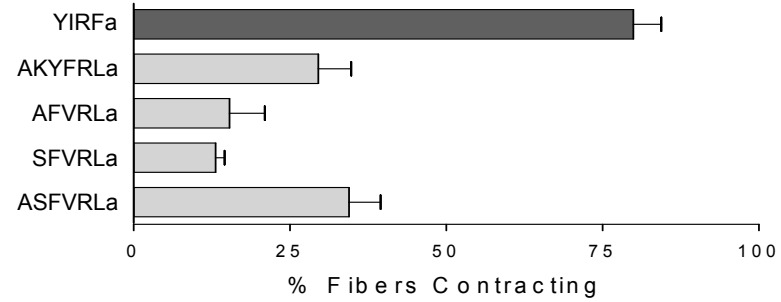
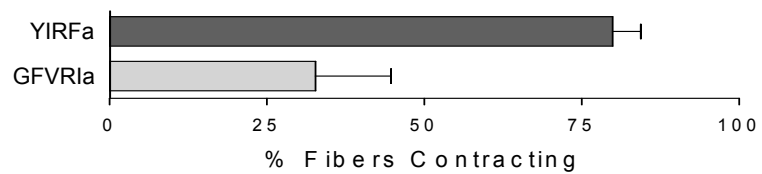
**Table 3. Some of flatworm neuropeptides predicted from genome sequence are used in contractions studies.** Flatworm neuropeptides presented here are predicted from genome sequence. The peptide is denoted in standard single letter aa annotation, and the C-terminal “a” represents an amide group. The peptides with names beginning “sm” are from *S. mansoni*, and those beginning “smed” are from *S. mediterranea*.



**Table 3**

<b>Flatworm Neuropeptide</b>	<b>Peptide name</b>
HFMPQRFa	sm-flp-1a
YTRFVPQRFa	sm-flp-1b
GFVRLa	sm-nlp-1a
AFVRLa	sm-nlp-1b
SSVFRFa	smed-flp-1a
SVAFRFa	smed-flp-1b
GVAFRFa	smed-flp-1c
GSVFRYa	smed-flp-1d
QSVFRYa	smed-flp-1e
AKYFRLa	smed-npl-2
SFVRLa	smed-nlp-1a
ASFVRLa	smed-nlp-1b

**Figure 1. Structure-activity relationship of flatworm neuropeptides.** Flatworm neuropeptides predicted from genome sequence of *S. mansoni* and *S. mediterranea* were tested at 1  $\mu$ M on their ability to contract isolated *S. mansoni* muscle fibers. Peptides are denoted in standard single letter aa annotation, and the C-terminal “a” represents an amide group. The peptides names are listed in the Table 1.

**Figure 1****A***'R Fa' Peptides***B***'R Ya' Peptides***C***'R La' Peptides***D***'R Ia' Peptides*

When  $\text{Ca}^{2+}$  was not present in the medium, depolarization-induced contractions were reduced from  $68 \pm 1$  % ( $n=10$ ) in control group to  $8 \pm 5$  % ( $n=4$ ) in test group. A similar picture was observed with FLP-induced contractions, where they decreased to  $17 \pm 4$  % ( $n=9$ ) from  $74 \pm 1$  % ( $n=14$ ). Caffeine (10 mM)-induced contractions were reduced too, but to a smaller extent  $77 \pm 2$  % ( $n=6$ ) compared to controls  $95 \pm 1$  % ( $n=3$ ).

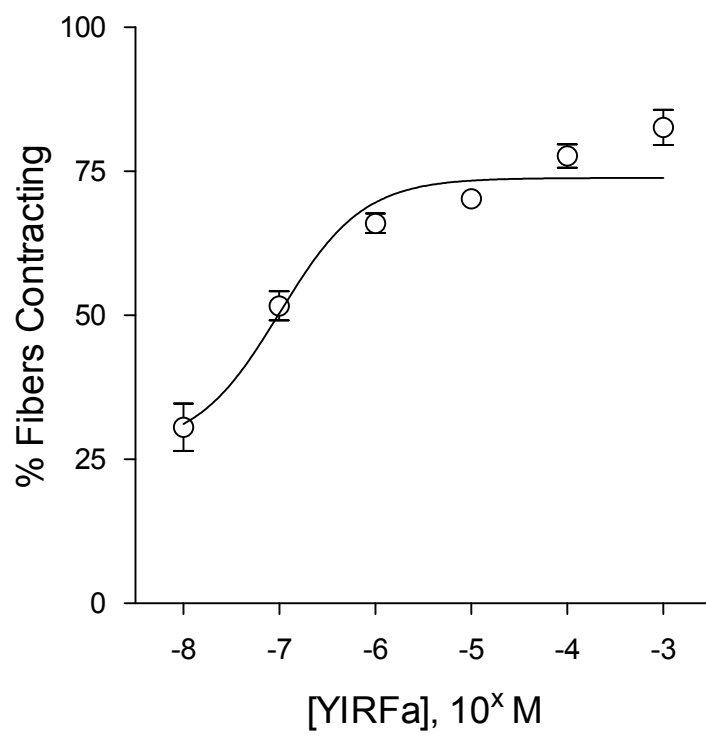
When the  $\text{Ca}^{2+}$  chelator EGTA was added, depolarization- and YIRFamide-induced contractions were totally abrogated, while caffeine was still able to stimulate contractile activity of the muscles to  $67 \pm 2$  % ( $n=8$ ). Thus a conclusion can be made that cells required extracellular  $\text{Ca}^{2+}$  to support muscle contraction in response to the peptide, since in the presence of EGTA intracellular  $\text{Ca}^{2+}$  stores were available, and were still sufficient to produce a contraction response to caffeine.

### **Voltage-operated $\text{Ca}^{2+}$ channels are possibly involved in mediating FLP-triggered $\text{Ca}^{2+}$ influx**

Since  $\text{Ca}^{2+}$  fluxes through the plasma membrane are involved in mediating the response to FLPs, we tried to determine the nature of the ion channels carrying those fluxes and their pharmacology. Our initial hypothesis was that VOCCs are the conduit for the  $\text{Ca}^{2+}$  influx. We tested this hypothesis using two different approaches. Firstly, we examined the ability of exogenous VOCC blockers to inhibit the FLP-induced contractions and secondly, we examined the ability of VOCC blockers to block voltage-gated  $\text{Ca}^{2+}$  currents in the muscle fibers using whole cell patch clamping.

**Figure 2. Dose-response curve for the FLP YIRFamide-induced contractions of isolated *S. mansoni* muscle fibers.** Isolation DMEM was used as the bathing medium and to make dilutions of the FLP to specified concentrations. YIRFamide was perfused onto individual fibers. Each data point is the mean  $\pm$  SEM, with  $n \geq 3$  plates tested (at least 30 fibers per plate) at each data point.

Figure 2



The effect of exogenous VOCC blockers on the ability of FLPs to produce contractions of individual muscle fibers was examined. The following inhibitors were tested:

- 1) the phenylalkylamine inhibitors, verapamil and methoxyverapamil;
- 2) the dihydropyridine inhibitors, nifedipine and nifedipine;
- 3) the benzothiazepine inhibitor, diltiazem.

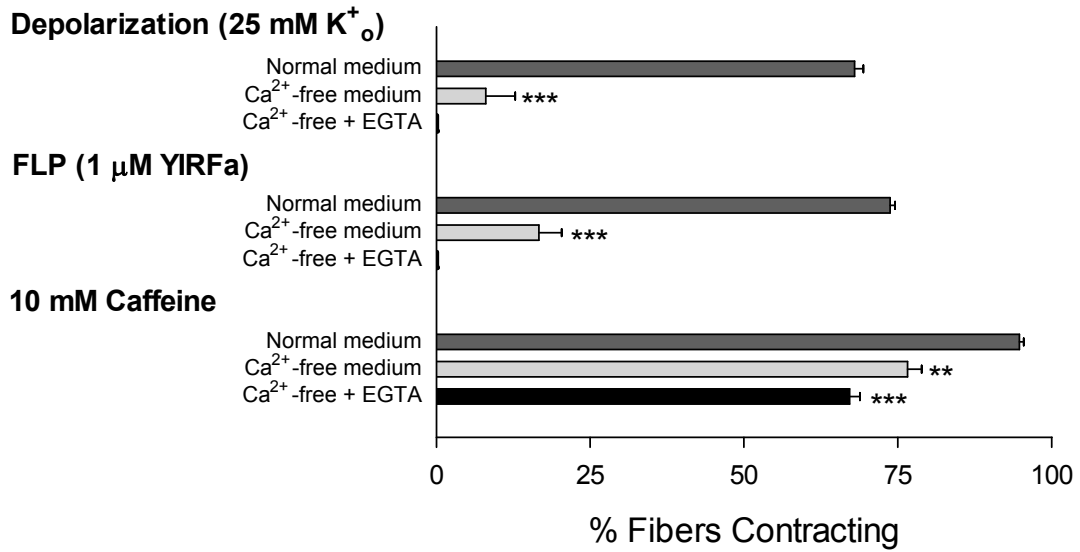
We performed contraction studies using 1  $\mu\text{M}$  YIRFamide as the stimulant. In these contraction studies, DMI was used as the extracellular medium. 15-20 minutes prior to stimulation with the FLP, 10  $\mu\text{M}$  or 100  $\mu\text{M}$  of various VOCC blockers were added to the DMI incubation medium (Figure 4). While not all VOCC blockers inhibited the FLP-induced contractions, some of them did.

The least potent of the inhibitors tested was diltiazem. Percentage of the YIRFa-induced contractions in the control group ( $77 \pm 2\%$ ,  $n=18$ ) was not significantly reduced by diltiazem at 10  $\mu\text{M}$  ( $69 \pm 2$ ,  $n=6$ ). Its ability to inhibit the contractions was more significant at 100  $\mu\text{M}$ , where the contractions were significantly inhibited to  $63 \pm 4\%$  ( $n=5$ ). Both verapamil and methoxyverapamil were significantly inhibitory at 10  $\mu\text{M}$ , where they reduced the percentage of fibers contracting ( $60 \pm 3\%$ ,  $n=6$ ; and  $62 \pm 3\%$ ,  $n=5$ , respectively). At 100  $\mu\text{M}$  they both further significantly decreased percentage of contracting muscles ( $16 \pm 3\%$  for verapamil,  $n=5$ ; and  $14 \pm 3\%$  for methoxyverapamil,  $n=5$ ). Nifedipine at 10  $\mu\text{M}$  was the most potent of the inhibitors tested, where it significantly reduced the percentage of contracting fibers

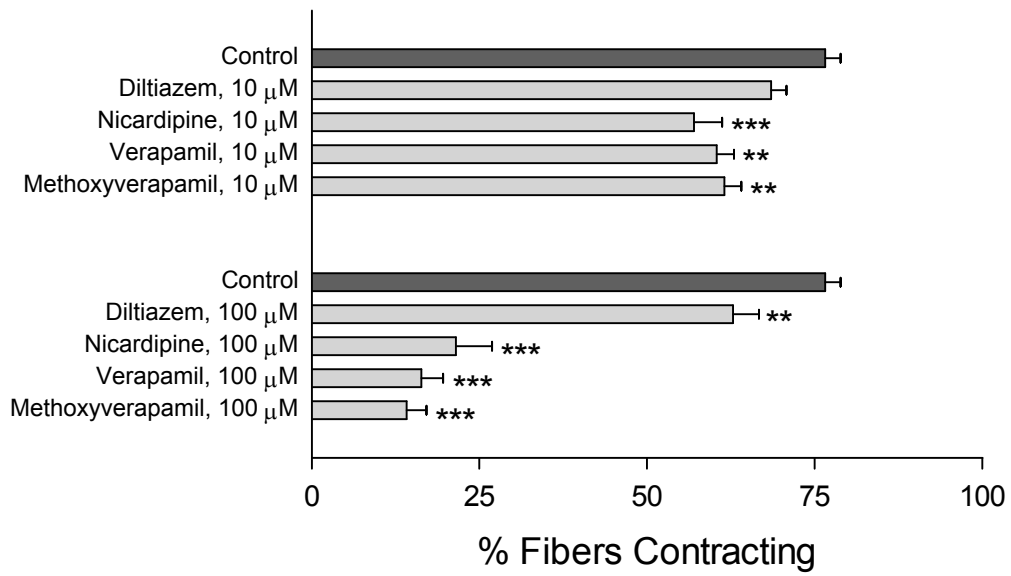
**Figure 3. The FLP-induced contractions are dependent on the presence of extracellular  $\text{Ca}^{2+}$ .** Both depolarization- and FLP-induced contractions are abolished in the presence of 0.5 mM extracellular EGTA. Only caffeine, which recruits the release of intracellular stores  $\text{Ca}^{2+}$ , is still capable of contracting isolated muscle fibers. Data points for each graph are presented as mean  $\pm$  SEM % fibers contracting with  $n \geq 3$  tested on at least three different experimental days. Here and throughout the text: asterisks denote the statistically significant difference at  $P < 0.05$  for Student's  $t$  test.



Figure 3



**Figure 4. The sensitivity of YIRFamide-induced contractions to voltage operated  $\text{Ca}^{2+}$  channel (VOCC) blockers.** Each data point is the mean  $\pm$  SEM, with  $n \geq 5$  for each agent. Drugs were added 15-25 min before testing with 1  $\mu\text{M}$  YIRFamide. YIRFamide dilutions were made in DMI. The bathing and dilution medium for the drugs was DMI. The ability of VOCC blockers to reduce the myoexcitatory response to YIRFamide suggests involvement of VOCCs in mediating  $\text{Ca}^{2+}$  influx.  $P < 0.05$  for Student's  $t$  test (asterisks of the same pattern are related to contractions obtained for a particular stimulant in different conditions as compared to the control obtained with the same stimulant).

**Figure 4**

( $57 \pm 4$ ,  $n=9$ ). At 100  $\mu\text{M}$  nifedipine reduced the percentage of contractions to  $22 \pm 5$  ( $n=7$ ). The ability of these blockers to inhibit the contractions produced by YIRFamide implicates VOCCs as the pathway for extracellular  $\text{Ca}^{2+}$  entry in the FLP-induced contractions.

The next series of experiments were proposed to characterize the pharmacology of the whole isolated muscle fiber membrane currents and possible involvement of channels carrying them in mediating the cellular response to the stimulation with YIRFamide.

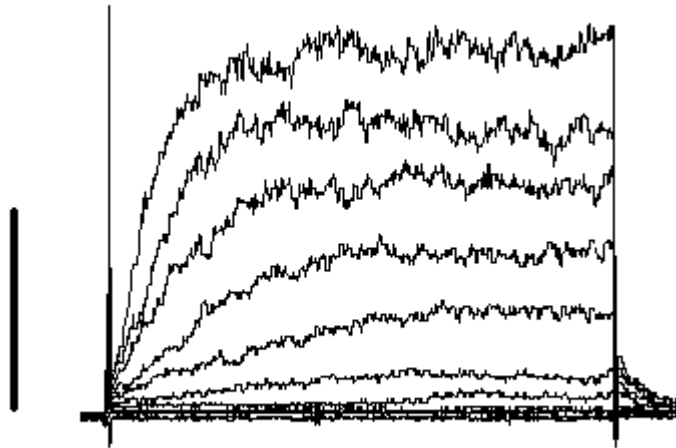
## **B. VOLTAGE-CLAMP EXPERIMENTS**

The profile of voltage-gated currents activated by the depolarization of the plasma membrane using whole cell voltage-clamp technique and electrode solutions having  $\text{K}^+$  as a major cation was shown to be dominated by outward current (Figure 5A). Voltage-step protocol applied to muscles clamped at  $-40$  mV and stepped from  $-60$  mV to  $+50$  mV in  $10$  mV increments produced an outward current present at potentials positive to  $-30$  mV. Outward current was relatively moderate in amplitude and did not show much inactivation within the duration of  $200$  ms test pulse (Figure 5B). Maximum currents were observed at depolarization to  $+50$  mV ( $320 \pm 25$  pA for peak currents and  $310 \pm 23$  pA for sustained currents,  $n=6$ ). In such conditions no inward currents were observed. After changing the holding potential ( $V_h$ ) of clamped muscles from  $-40$  mV to  $-70$  mV along with replacing electrode  $\text{K}^+$  with  $\text{Cs}^+$  and addition of  $\text{Ba}^{2+}$  to the solution it was possible to record whole cell inward currents.

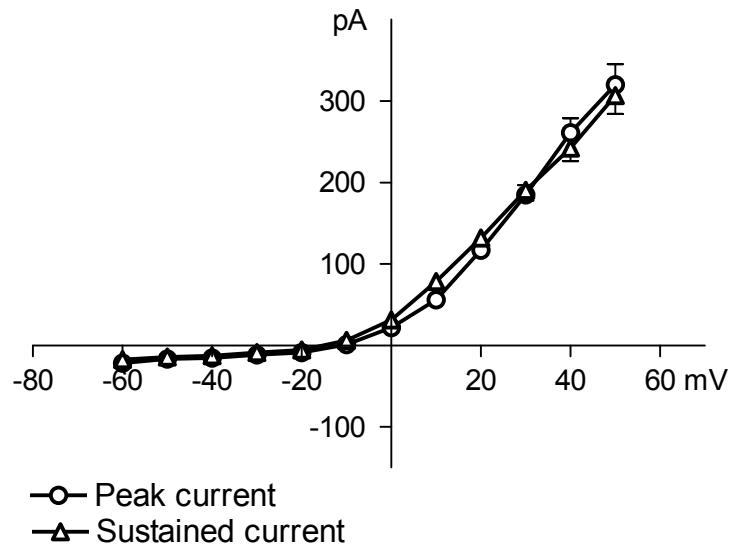
**Figure 5. Voltage-dependent outward currents mask the inward currents in muscle fibers of the parasitic flatworm *S. mansoni*.** (A) An example of traces recorded from *S. mansoni* muscle fibers held at -40 mV using a 200 ms pulse step protocol from -60 mV to +50 mV in 10 mV increments (in this figure, traces represent non leak-subtracted data). The scale bar is 200 pA. (B) Current-voltage relationship for outward currents recorded from *S. mansoni* muscle. Values for the I-V plot are the average of three replicates at each voltage value. Peak current measurements were taken by five point smoothing for the 50 ms time span after the start of the test pulse and the sustained current values are the mean current amplitude obtained within the last 50 ms before the end of the test pulse (in this figure, I-V relationship for the outward currents is done for non leak-subtracted currents).

Figure 5

A



B



We have been able to record whole-cell inward cationic currents in the presence of  $\text{Ca}^{2+}/\text{Ba}^{2+}$  cationic mixture, using whole-cell configuration of the patch-clamp technique in single-electrode voltage clamp mode. Inward voltage-activated current was recorded under conditions that suppressed outward  $\text{K}^+$  conductance by the replacement of internal  $\text{K}^+$  with  $\text{Cs}^+$  and enhanced with 15.0 mM  $\text{Ba}^{2+}$  as a charge carrier in addition to 2.0 mM  $\text{Ca}^{2+}$  ( $I_{\text{Ca/Ba}}$ ). A voltage step protocol of 200 ms duration was executed on individual muscle cells held at -70 mV. Current signals were amplified (Axopatch 200B), filtered, digitized and transferred to a personal computer. Step depolarizations in 10 mV increments revealed inward currents of relatively small size. The inward currents did not show evidence of inactivation within the period of the 200 ms depolarizing pulse.

Pharmacological characterization of VOCCs in both free-living and parasitic flatworms has been hindered since the currents carried by these channels are small in amplitude and rundown quickly. While the presence of VOCCs in the parasite *S. mansoni* has been evident from molecular data and supported by physiological data, their pharmacological characterization has not been performed.

Under the specified conditions patched cells exhibited the following profiles (%): no currents recorded 38; outward currents profile 6; inward currents profile 56. Relatively small proportion of cells that showed inward currents profile had currents of the amplitudes amenable to pharmacological characterization using common VOCC blockers and neuropeptides – 41 %. The dramatic rundown of these inward currents soon after the whole cell configuration had been obtained posed a

challenge for completing pharmacology experiments using putative blockers and neuropeptides.

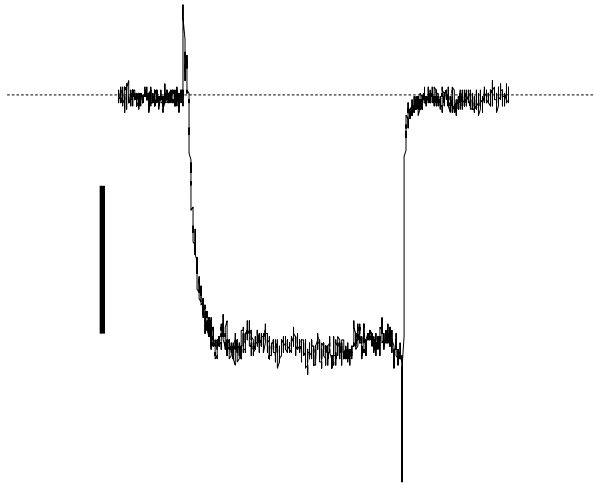
To complete this set of experiments the number of depolarizing sweeps in the standard voltage-step protocol was reduced to one sweep at the voltage level where the largest current amplitude has been typically recorded. Otherwise prolonged full-scale protocol would have added to acceleration of the current rundown. Since the largest current amplitudes were observed at +20 mV, a new protocol with a single depolarizing step from a holding potential of -70 mV to +20 mV was used. To obtain the relationship between the current amplitude and time, voltage steps were executed every 30 seconds. Currents stimulated by the protocol generally reached their peak values within the first 1.5 minutes after the start of the trial, then remained relatively stable for the next 2.0 - 2.5 minutes, followed by a gradual decline in the current amplitude over the next 6.0 - 7.0 minutes (Figure 6). Thus the trial time was reduced to cover the 6.5 minutes after whole cell access, a time period in which the currents' behaviour was pretty predictable. Since before running down currents remained at the relatively stable level for some time it was possible to apply VOCC inhibitors to observe their effect on the channels. By comparing inward currents in muscles in which the putative blockers were applied to muscles in which the control vehicle was applied it was possible to perform some of the pharmacological characterizations of these important channels for the first time.



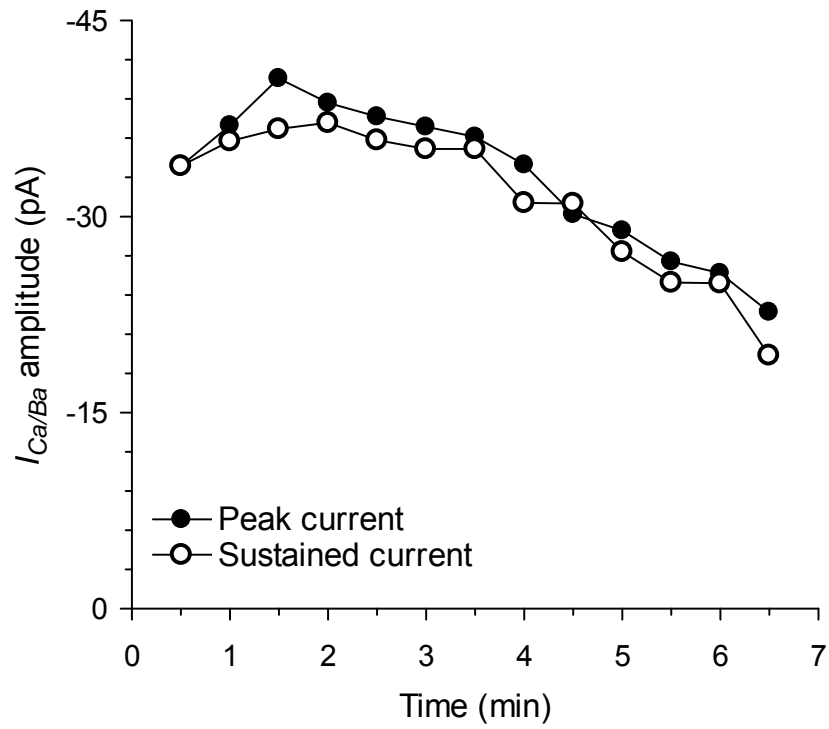
**Figure 6. Whole cell voltage-gated  $I_{Ca/Ba}$  currents of *S. mansoni* isolated muscle fibers evoked by 200 ms depolarizing step to +20 mV from the holding potential  $V_h$  of -70 mV. (A) A representative  $I_{Ca/Ba}$  current trace. Scale bar is 20 pA. (B)  $I_{Ca/Ba}$  runs down with time. On average currents reached peak amplitude 1.5 minutes after the start of the trial and maintained a steady state within next 2.0-3.0 minutes followed by gradual decline in amplitude. Peak and sustained current values were plotted versus time for a typical recording presented in (A).**

Figure 6

A



B



The panel of possible inhibitors to be tested was reduced by their ability to remain soluble in the solution containing high concentrations of divalent cations, presence of which allows recording the inward currents. One of the inhibitors which did not pose such a problem was verapamil, which could be dissolved at 10  $\mu\text{M}$  and 100  $\mu\text{M}$  in the bathing solution.

For extracellular application of control and drug-containing solutions a SF-77B Perfusion Fast-Step system (Warner Instruments) was used. Obtaining seals and whole cell access were performed with the fibers being constantly perfused with the control solution. Control group fibers remained under perfusion for the whole length of the trial. In test groups after the inward current reached a steady state (1.5 minutes after the start of the trial) the lines supplying perfusion system were switched from control to drug-containing bathing solution for the duration of 2.5 minutes followed by washout with the control solution.

#### **Effect of $\text{Ca}^{2+}$ channel blocker verapamil on $I_{\text{Ca}/\text{Ba}}$**

After being able to routinely record inward currents in the presence of the  $\text{Ca}^{2+}/\text{Ba}^{2+}$  cationic mixture it was possible to perform some pharmacological experiments using the phenylalkylamine VOCC blocker verapamil. Effects of the drug at 10  $\mu\text{M}$  and 100  $\mu\text{M}$  were tested on its ability to inhibit peak and sustained currents. Peak current values were determined by five points smoothing within the first 50 ms of the test pulse and sustained current values were obtained as mean values within the last 50 ms of the test pulse. For every cell tested peak and sustained current values were normalized to those values obtained at 1.5 minutes

after the start of the trial, when the inward current typically reached a maximum and right before solutions switch. Normalized values were used to compose graphs.

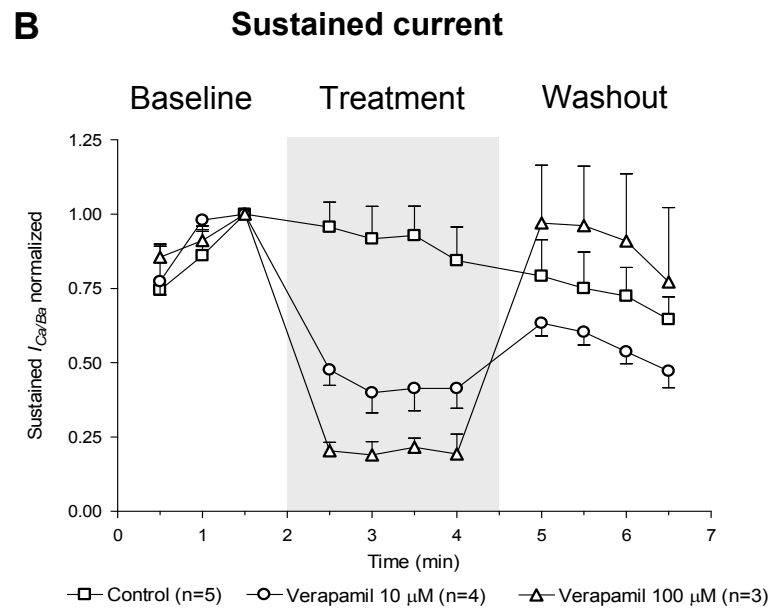
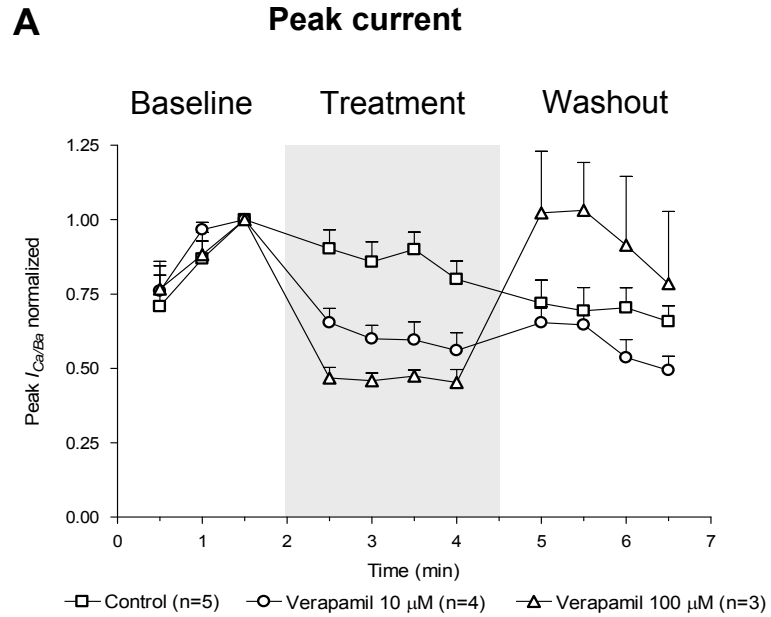
Verapamil showed to be more potent on sustained than peak currents at both concentrations tested (Figure 7). Normalized sustained current values at the 2.5 minute time point of the trial were significantly reduced to  $48\pm 5\%$  with verapamil tested at  $10\ \mu\text{M}$  ( $n=4$ ) and to  $20\pm 3\%$  when tested at  $100\ \mu\text{M}$  ( $n=3$ ) ( $P<0.05$ , Mann-Whitney test) as compared to the control group ( $n=5$ ) where the amplitude was reduced to  $96\pm 8\%$  as a result of rundown (Figure 7B).

Peak normalized currents values were not significantly reduced by application of  $10\ \mu\text{M}$  verapamil ( $65\pm 5\%$ ,  $P<0.05$ , Mann-Whitney test), while at  $100\ \mu\text{M}$  current inhibition was statistically significant to  $47\pm 4\%$  ( $P<0.05$ , Mann-Whitney test) as compared to the control group where normalized current was reduced to  $90\pm 6\%$  (Figure 7A).

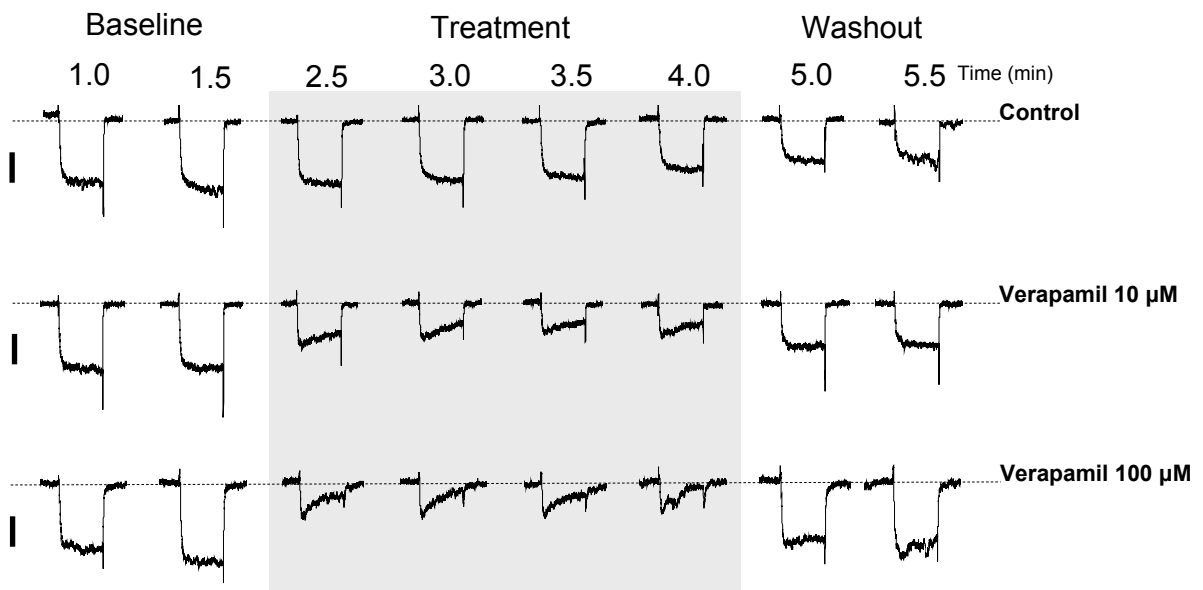
The inhibitory effect of verapamil was reversible. It was very important to wash the drug out, since due to the labile nature of  $I_{Ca/Ba}$ , it could have been confusing to distinguish the effect of the inhibitor from the natural rundown. Individual current traces for control and test group recordings are shown in Figure 8.

**Figure 7. Phenylalkylamine  $\text{Ca}^{2+}$  channel blocker verapamil reversibly inhibits  $I_{\text{Ca/Ba}}$  in *S. mansoni* isolated muscle fibers.** (A) Effect of verapamil on peak  $I_{\text{Ca/Ba}}$ ; (B) Effect of verapamil on sustained  $I_{\text{Ca/Ba}}$ . For each cell tested, in control and test groups, peak and sustained currents were normalized to corresponding current values obtained at 1.5 minute after the start of the trial. Data were plotted as mean  $\pm$  SEM. Seals and break-in were performed with cells constantly perfused with control solution. Cells remained under the perfusion for the whole length of the trial. Shaded box indicates time points where cells were exposed to the drug-containing bathing solution (for the test groups) or control solution itself (for the control group).

Figure 7



**Figure 8. Representative traces of  $I_{Ca/Ba}$  activated by depolarizing *S. mansoni* muscle fibers from the holding potential  $V_h$  of -70 mV to +20 mV by 200 ms test pulse in the absence or presence of verapamil (10  $\mu$ M or 100  $\mu$ M). Dotted line denotes zero current level and the scale bar is 20 pA (traces from different fibers are scaled equally). The time scale of the experiment is shown above traces.**

**Figure 8**



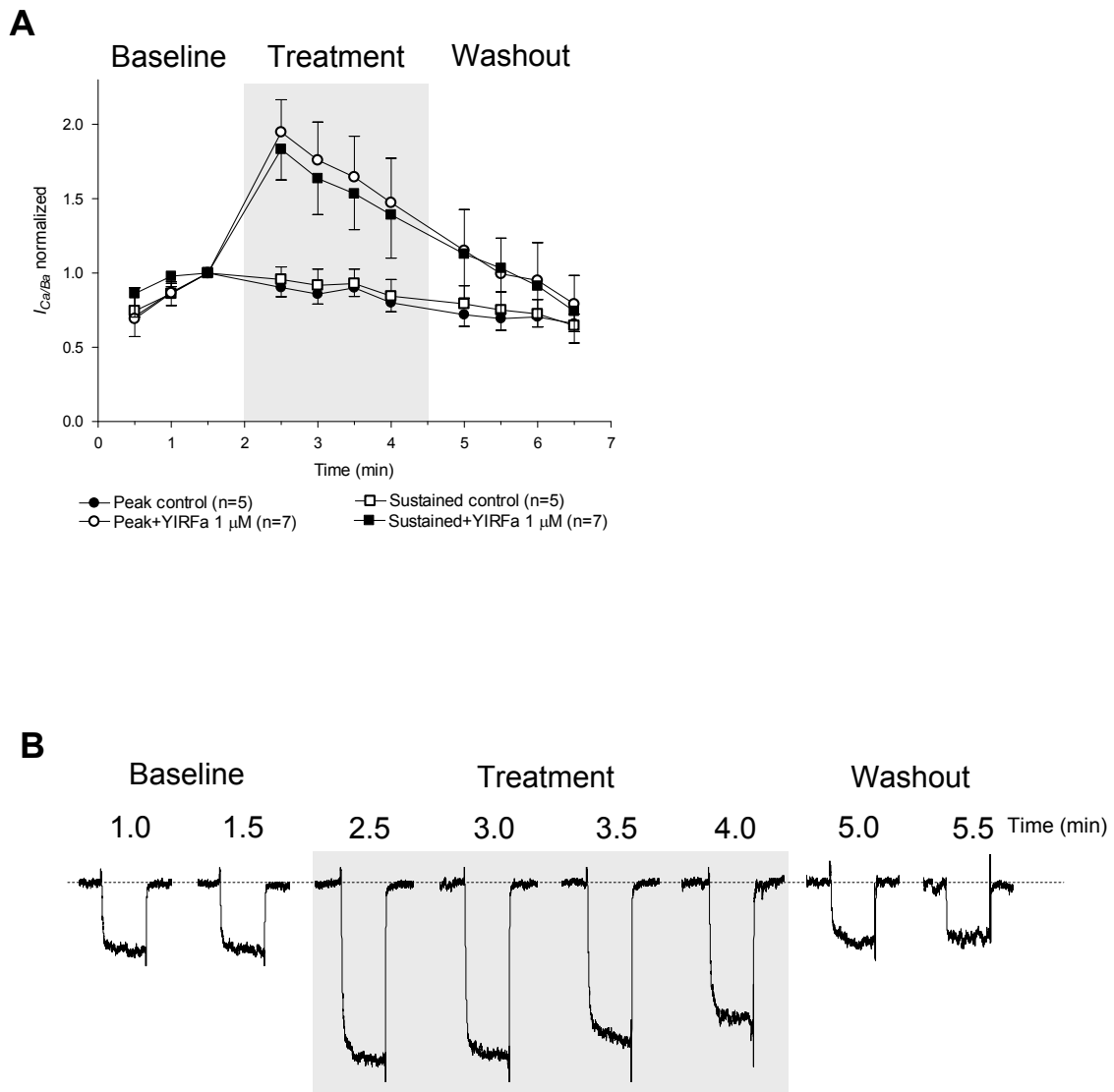
**YIRFamide enhances  $I_{Ca/Ba}$  in isolated *S. mansoni* muscle fibers**

The FLP YIRFamide significantly increased the amplitude of both peak and sustained currents when tested at 1  $\mu\text{M}$  ( $P < 0.05$ , Mann-Whitney test) (Figure 9A). At 2.5 minute time point of the trial in the test group normalized peak and sustained currents increased to  $190 \pm 22\%$  and  $180 \pm 21\%$  ( $n=7$ ) correspondingly as compared to peak and sustained values in the control group ( $90 \pm 6\%$  and  $96 \pm 8\%$ ,  $n=5$ ). The effect of the neuropeptide was reversible. Individual current traces showing current amplitude enhancement by the FLP are shown in Figure 9B.

In order to confirm that the verapamil sensitive current was the same one amplified by YIRFamide, we tested the effect of co-application. While YIRFamide significantly increased current amplitude, its potentiating effect was reduced with verapamil being simultaneously present in the perfusion solution at 100  $\mu\text{M}$  (Figure 10). The normalized peak current amplitude in the group simultaneously treated with YIRFamide and verapamil was not significantly different from the control group ( $130 \pm 15\%$ ,  $n=4$  as compared to  $90 \pm 6\%$ ,  $n=5$ , correspondingly, Figure 10A). While there was still some increase in the current amplitude it was not found significantly different ( $P < 0.05$ , Mann-Whitney test). The normalized sustained current values (Figure 10B) were not significantly different from the values in control group ( $79 \pm 10\%$  and  $96 \pm 8\%$ ,  $P < 0.05$ , Mann-Whitney test). The traces for the cells simultaneously treated with 1  $\mu\text{M}$  YIRFamide and 100  $\mu\text{M}$  verapamil are shown in Figure 10C.

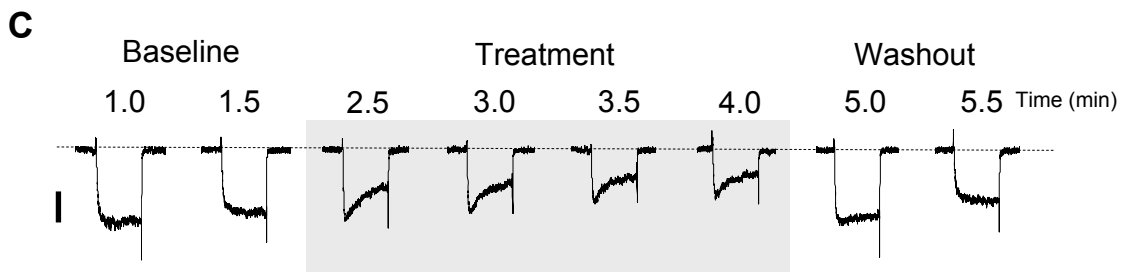
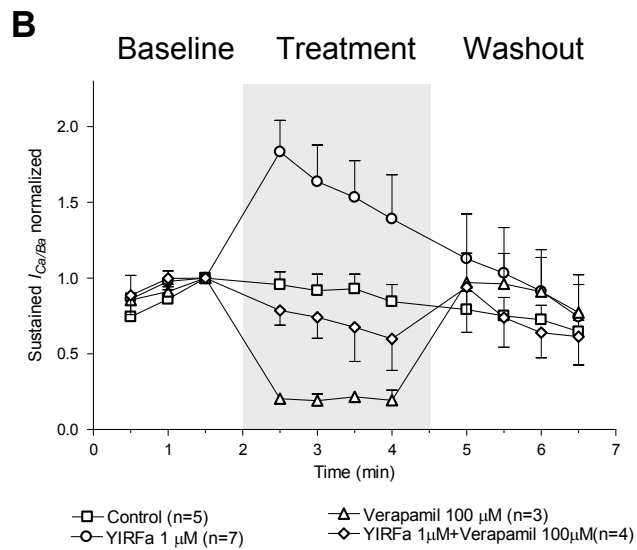
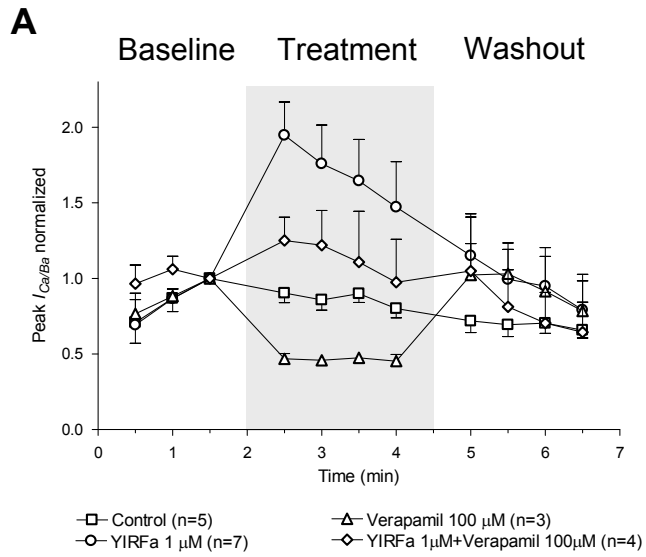
**Figure 9. YIRFamide enhances whole cell voltage-gated  $I_{Ca/Ba}$  elicited by 200 ms depolarizing test pulse from the holding potential  $V_h$  of -70 mV to +20 mV in isolated *S. mansoni* muscle fibers.** (A) Both peak and sustained  $I_{Ca/Ba}$  increased in amplitude upon treatment with YIRFamide (1  $\mu$ M). YIRFamide was applied as indicated with the shaded box. Data were normalized as described in Figure 7 and plotted as mean  $\pm$  SEM; (B) Representative traces of  $I_{Ca/Ba}$  before, during and after application of the peptide. Dotted line denotes zero current level and the scale bar is 20 pA. The time scale of the experiment is shown above traces.

Figure 9



**Figure 10. YIRFamide-mediated increase of  $I_{Ca/Ba}$  in *S. mansoni* isolated muscle fibers was reduced during treatment with the  $Ca^{2+}$  channel blocker verapamil.** (A) Verapamil (100  $\mu$ M) reduced the increase of peak  $I_{Ca/Ba}$  produced by 1  $\mu$ M YIRFamide; (B) The increase in sustained  $I_{Ca/Ba}$  produced by 1  $\mu$ M YIRFamide was reduced to a greater extent by 100  $\mu$ M verapamil. (C) Representative traces of  $I_{Ca/Ba}$  before, during and after treatment with YIRFamide and verapamil. The experimental format is identical to that in Figure 7. Data were plotted as mean  $\pm$  SEM. Both verapamil and YIRFamide were dissolved in extracellular bathing solution and delivered simultaneously during the time span indicated by a shaded box. Dotted line denotes zero current level and scale bar is 20 pA. Time scale of the experiment is shown above traces.

Figure 10



The inability of the neuropeptide to elevate current levels in the presence of verapamil to the levels obtained without the blocker confirms VOCCs involvement in carrying  $\text{Ca}^{2+}$  influx triggered by the FLP.

### **III. FLPs TRIGGER $\text{Ca}^{2+}$ INFLUX THROUGH INTRACELLULAR SIGNALLING CASCADE**

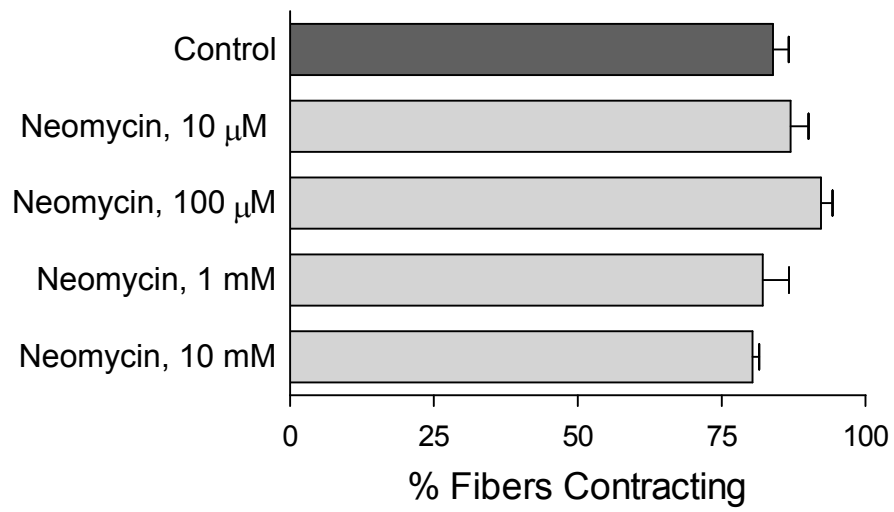
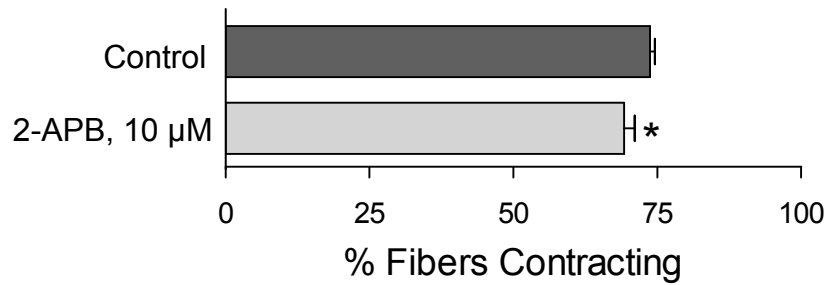
#### **A. CONTRACTION STUDIES**

##### **Effect of phospholipase C (PLC) inhibitors**

In these studies, the effect of putative blockers of PLC and  $\text{InsP}_3$ -sensitive stores blockers on the ability of FLPs to induce individual muscle contraction was examined. YIRFamide at 1  $\mu\text{M}$  was used to produce muscle contraction. The DMI medium was used as bathing solution to which the drugs were added 20-40 minutes before the application of YIRFamide. PLC inhibitor antibiotic neomycin was used in the range of the following concentrations: 10  $\mu\text{M}$ , 100  $\mu\text{M}$ , 1 mM and 10 mM (Figure 11A). Within the range of concentrations tested it did not produce inhibitory effect on YIRFamide ability to induce contractions of individual muscles.

$\text{InsP}_3$ -sensitive blocker aminoethoxydiphenyl borate (2-APB) was tested at 10  $\mu\text{M}$  against 1  $\mu\text{M}$  YIRFamide (Figure 11B). 2-APB was not very effective at inhibiting contractions, the response was reduced to  $69 \pm 2\%$  ( $n=6$ ) as compared to the control group  $74 \pm 1\%$  ( $n=14$ ), though this difference was found to be statistically significant (Student's  $t$  test,  $P < 0.05$ ).

**Figure 11. Effects of phospholipase C (PLC) inhibitor antibiotic neomycin and InsP<sub>3</sub>-sensitive store blocker aminoethoxydiphenyl borate (2-APB) on YIRFamide-induced contractions.** Contractions were evoked by 1  $\mu$ M YIRFamide. DMI was used as extracellular and dilution medium. Drugs were added 30 minutes prior microperfusion. Each data point is mean  $\pm$  SEM, with  $n \geq 4$ .

**Figure 11****A****B**



### **Effect of protein kinase C (PKC) inhibitors**

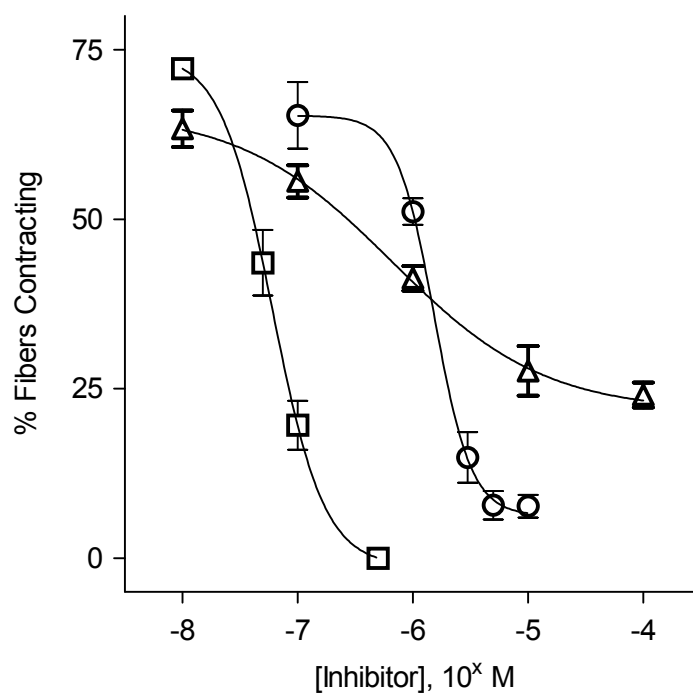
YIRFamide myoexcitation at 1  $\mu\text{M}$  was tested in the presence of PKC inhibitors. A panel of inhibitors was used: chelerythrine chloride, Ro 31-8220 and calphostin C (Figure 12). DMI was used as the extracellular medium to which inhibitors were added 20-40 minutes prior microperfusion with the FLP.

The concentration-response curve for chelerythrine chloride, a potent and specific inhibitor of PKC that interacts with the enzyme catalytic subunit with  $\text{IC}_{50}=0.7 \mu\text{M}$  (Herbert *et al.*, 1990), was obtained in series of contraction experiments performed with  $\text{IC}_{50}$  at 0.66  $\mu\text{M}$ , which fell within the concentration range specific for PKC inhibition.

Ro 31-8220 which is known as a competitive, selective inhibitor for PKC ( $\text{IC}_{50}=10 \text{ nM}$ ) over PKA ( $\text{IC}_{50}=900 \text{ nM}$ ) and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase ( $\text{IC}_{50}=17 \mu\text{M}$ , BIOMOL International), did inhibit peptide-induced contractions, but required a higher concentration. The  $\text{IC}_{50}$  for Ro 31-8220 in experiments with peptide-induced contractions was 1.54  $\mu\text{M}$ .

Calphostin C turned out to be the most potent inhibitor of the peptide-induced contractions, with  $\text{IC}_{50}$  60.6 nM. The 95 % confidence interval for the obtained curve  $\text{IC}_{50}$  fell within the range of concentrations known to be specific for PKC inhibition (50 nM) in other preparations (BIOMOL International).

**Figure 12. Various protein kinase C (PKC) inhibitors showed different pattern of concentration-dependent inhibition of YIRFamide-elicited contractions in *S. mansoni* muscle fibers.** Isolated muscle fibers contraction response to 1  $\mu$ M YIRFamide was tested in the presence of cell permeable PKC inhibitors calphostin C, chelerythrine chloride and Ro 31-8220. Cells were bathed in isolation DMEM. Inhibitors were added 20-40 min prior the experiment. Each data point is mean  $\pm$  SEM, with  $n \geq 3$ .

**Figure 12**

- Calphostin C
- △ Chelerythrine chloride
- Ro 31-8220

PKC involvement in mediating the muscle contraction response upon stimulation with YIRFamide is suggested by the obtained results with PKC inhibitors. Three of them showed inhibitory effect with the most potent inhibitor calphostin C being active within the specific range of concentrations for PKC inhibition suggest PKC involvement in mediating the response triggered by the FLP.

### **Effects of adenylate cyclase (AC) and protein kinase A (PKA) inhibitors**

The activity of cAMP-dependent PKA has been shown to be the major regulator of  $\text{Ca}^{2+}$  influx through some VOCCs. Transmembrane receptors of many hormones and neurotransmitters are coupled to AC via G-proteins. Upon receptor binding with an agonist the activated G-protein acts on AC by activating or inhibiting its enzymatic activity, depending on whether the G protein is stimulatory or inhibitory ( $G_s$  and  $G_i$ , respectively).  $G_s$  stimulation of AC results in the conversion of ATP to cAMP, which in turn acts on a variety of second effectors, one of which is PKA. Phosphorylation by activated PKA can be linked to L-type  $\text{Ca}^{2+}$  channels opening (Naguro *et al.*, 2004).

The PKA pathway involvement in mediating peptide-induced contractions was tested using two AC inhibitors (MDL 12.330A and SQ 22.536) and two PKA inhibitors (H-89 and myristoylated PKA inhibitor, amide 14-22). Responses to 1  $\mu\text{M}$  YIRFamide were obtained in the presence of each of the chemicals tested within a certain concentration range.

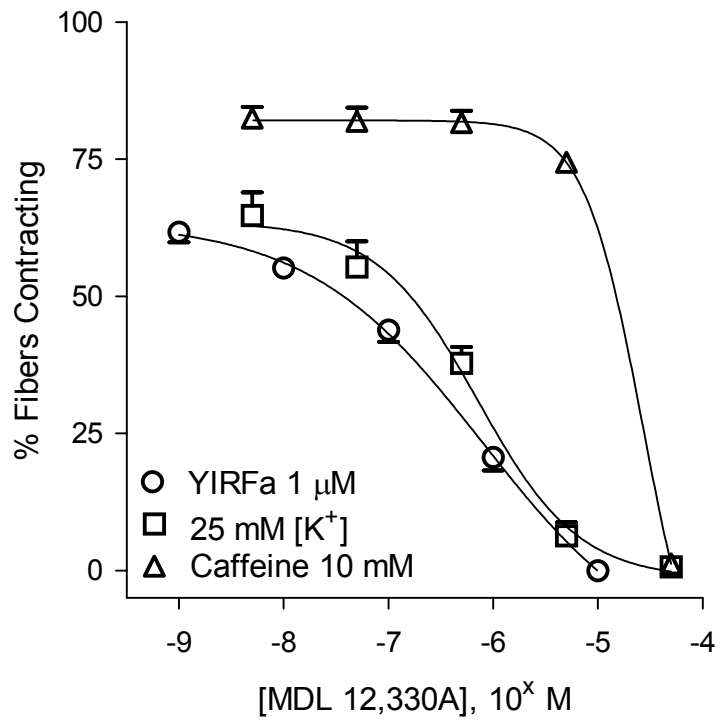
MDL 12.330A showed concentration-dependent inhibition of contractions to YIRFamide (Figure 13). It showed very high potency, gradually inhibiting

contractions and totally abolishing them at 10  $\mu\text{M}$ . The  $\text{IC}_{50}$  for MDL 12.330A applied against peptide-induced contractions was 0.80  $\mu\text{M}$ .

The response to 1  $\mu\text{M}$  YIRFa in the presence of SQ 22.536 was significantly inhibited ( $P < 0.05$ , Student's  $t$  test) (Figure 14). As compared to MDL 12.330A it has been reported to have more specific activity towards AC since it is deprived of effect on cAMP phosphodiesterase, the action that could mask the inhibitory effect of the drug on AC (Lippe and Ardizzone, 1991). The inhibition produced by SQ 22.536 within the range of concentrations tested (5 nM-50  $\mu\text{M}$ ) was somewhat similar in extent, which could be ascribed to some non-specific action of the inhibitor in this particular case. Thus it was not clear whether AC was involved in mediating the final contraction response. In the literature MDL 12.330A has been reported to exert some other actions besides AC inhibition, especially those connected with slow type VOCCs (Grupp *et al.*, 1980). Since concentration-dependent inhibition of YIRFamide-induced contractions was observed with this non-specific AC inhibitor MDL 12.330A, it was tested against depolarization- and caffeine-elicited contractions too (Figure 13). MDL 12.330A was an effective inhibitor of depolarization-induced contractions with a concentration-dependent inhibition, similar to that obtained with the peptide. The  $\text{IC}_{50}$  in this experiment was 0.71  $\mu\text{M}$ .

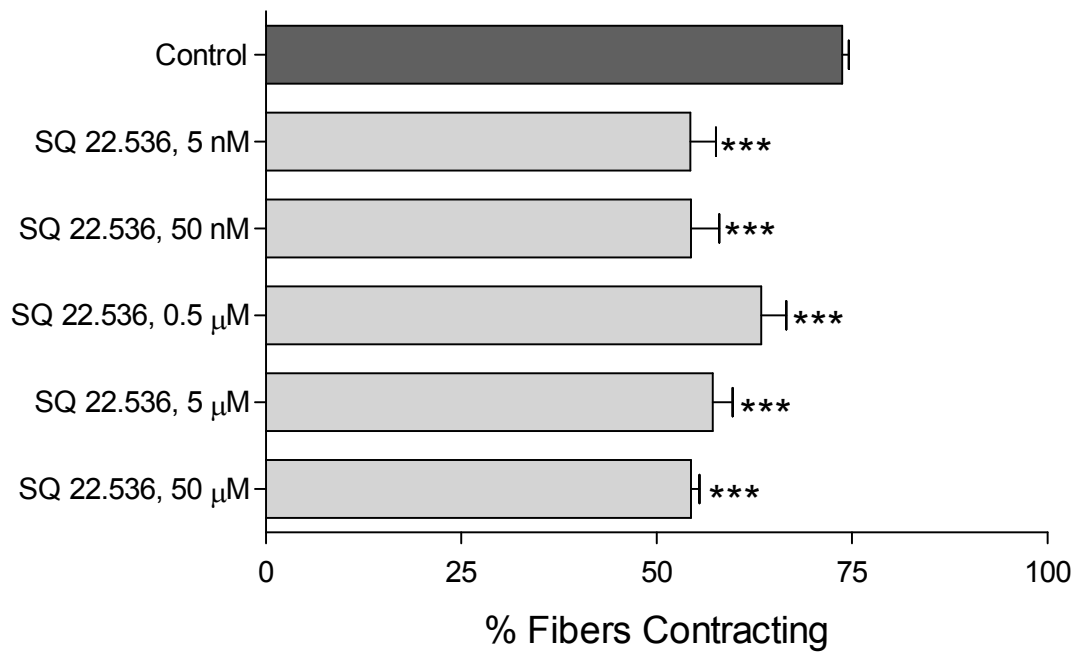
Caffeine-induced contractions were not blocked by MDL 12.330A within the concentration range that was inhibitory for depolarization-induced contractions, even though at 50  $\mu\text{M}$  it did totally abolished the caffeine-induced contractions.

**Figure 13. YIRFamide-, depolarization- and caffeine-induced contractions in the presence of adenylate cyclase inhibitor MDL 12.330A.** Contractions to 1  $\mu$ M YIRFamide and 25 mM  $[K^+]$  were inhibited by MDL 12.330A in concentration-dependent manner, while caffeine-induced response was abolished at higher concentrations (50 mM). Cells were bathed in isolation medium (DMEM). The inhibitor was added 10-25 min before testing. YIRFamide and caffeine were dissolved in isolation DMEM and in DMI correspondingly. Each data point is mean  $\pm$  SEM ( $n \geq 4$  plates prepared on at least three different experimental days).

**Figure 13**

**Figure 14. Effect of specific adenylate cyclase (AC) inhibitor SQ 22.536 on YIRFamide-induced contractions.** Isolation DMEM was used as bathing medium to which the drug was added 30-40 prior microperfusion with 1  $\mu$ M YIRFamide. YIRFamide was dissolved in isolation DMEM. Each data point is mean  $\pm$  SEM, with  $n \geq 5$ .



**Figure 14**

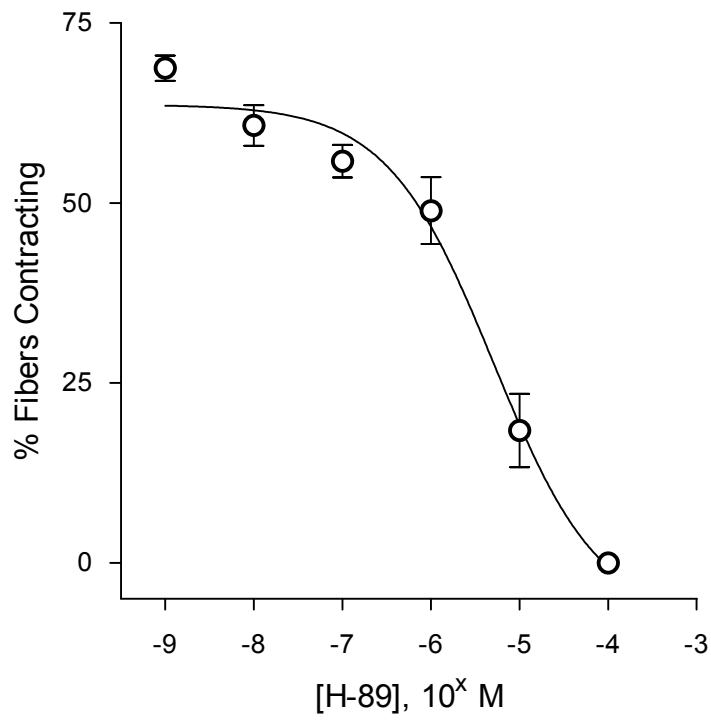
The obtained data with the inhibitors were not sufficient to judge on the AC being stimulated upon the neuropeptide application. Since the AC inhibitor blocked both peptide- and depolarization-induced contractions with similar concentration-dependence, its action may not be a specific inhibition of the specific pathway linking the FLP to the VOCCs. Rather it may be a less specific inhibitor of the energetic pathways utilized in contractions in general, and not the FLP-induced contractions in specific.

A common PKA inhibitor H-89 provided concentration-dependent inhibition of peptide-induced contractions (Figure 15), which were totally blocked at 100 $\mu$ M, which is consistent with the results obtained with contractions, produced by solutions with elevated [K<sup>+</sup>] (Day *et al.*, 1994). Here the IC<sub>50</sub> was 5.08  $\mu$ M.

In spite of the fact that H-89 has become very popular in signal transduction studies there are some limitations of the drug reported in the literature. In a study on specificity of different protein kinase inhibitors H-89 has been shown to inhibit eight protein kinases at 10  $\mu$ M, three of which (MSK1, S6K1 and ROCK-II) were inhibited with a similar or a greater potency than that for PKA (Davies *et al.*, 2000). To further test the involvement of PKA, highly selective PKA inhibitor, cell-permeable myristoylated PKI (14-22) amide was used.

PKI (cAMP-dependent PKA inhibitor heat-stable protein) selectively inhibits the free catalytic subunit of the enzyme (Ashby and Walsh, 1972; Glass *et al.*, 1989), within nanomolar range in mammalian systems and acts competitively with respect to substrate (Whitehouse and Walsh, 1983).

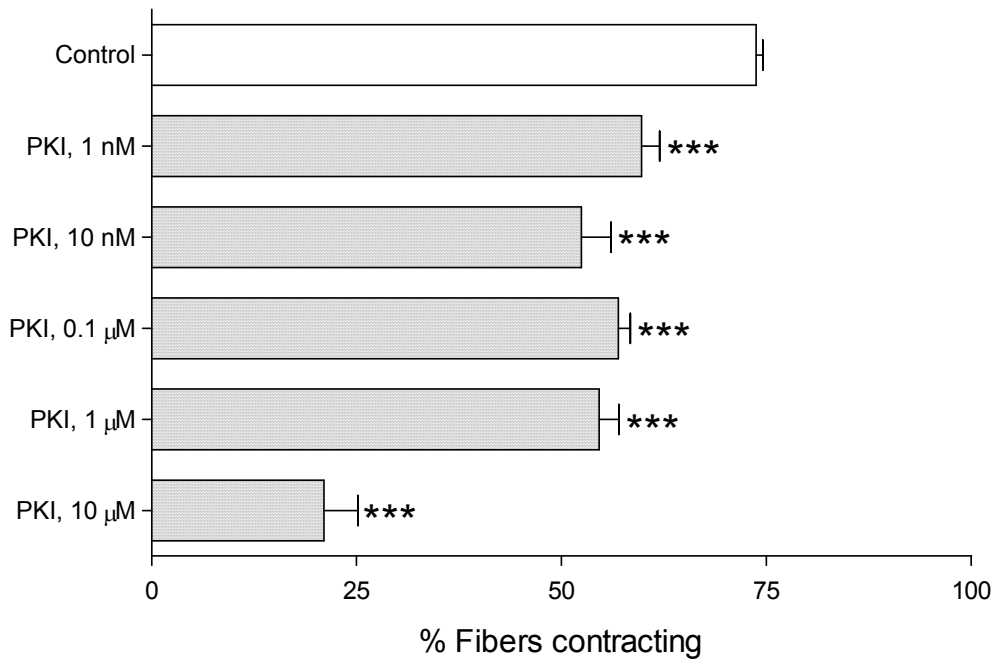
**Figure 15. Common protein kinase A inhibitor H-89 reduced the percentage of *S. mansoni* muscles contracting in response to YIRFamide (1  $\mu$ M) in dose-dependent manner.** Cells were bathed and YIRFamide dilutions were made in isolation DMEM. The inhibitor was added 20-30 min before testing. Each data point is mean  $\pm$  SEM (n $\geq$ 4 plates prepared on at least three different experimental days).

**Figure 15**

PKI significantly inhibited YIRFamide-induced contractions at 10  $\mu\text{M}$  ( $21 \pm 4$ ,  $n=8$ ) as compared to percentage of muscles contracted in control group ( $74 \pm 1$ ,  $n=14$ ), but this is a concentration too high to be attributed to specific PKA inhibition. At 1 nM - 1  $\mu\text{M}$  range the degree of inhibition was pretty similar for different concentrations tested. This result shows that in the range from nanomolar to micromolar concentrations PKI does not inhibit YIRFamide-induced contractions to a large extent (Figure 16).

In total, the results with AC and PKA inhibitors are equivocal about the role of the cAMP pathway in the YIRFamide-induced contractions. While some AC inhibitors do inhibit the FLP-induced contractions, they also inhibit depolarization-induced contractions. So their effect may not be specific. It would be easy to attribute that effect to general inhibition of energy production and not as a specific link between the FLP and VOCC. Further the inability of PKA inhibitors to produce concentration-dependent inhibition as well as similar inhibitory extent obtained for a wide range of concentrations can be explained by some other possible effects. It can be proposed that elevated levels of cAMP are needed to stimulate energy production necessary to support muscle contraction to any of stimulatory agents. To test this hypothesis a non-selective PKA inhibitor H-89 was tested on 10 mM caffeine-induced contractions (Figure 17). H-89 was tested in the 10 nM–100  $\mu\text{M}$  range. It reduced the percentage of caffeine-induced contractions beginning at 0.1  $\mu\text{M}$  ( $79 \pm 2$ ,  $n=3$ ) as compared to  $91 \pm 2$  in the control group, ( $n=7$ ) and was relatively potent at 100  $\mu\text{M}$  ( $69 \pm 5$  % contracting,  $n=4$ ). Thus a conclusion regarding non-specific effect of the H-89 inhibitor can be made.

**Figure 16. Effect of highly selective specific protein kinase A (PKA) inhibitor cell-permeable myristoylated PKI (14-22) amide on YIRFamide-stimulated contractions.** Isolation DMEM was used as bathing medium to which the drug was added 30-40 min prior microperfusion with 1  $\mu$ M YIRFamide. YIRFamide was dissolved in isolation DMEM. Each data point is mean  $\pm$  SEM, with  $n \geq 5$ .

**Figure 16**

It's inhibitory effect on both YIRFamide-induced contractions, as well as on caffeine-stimulated contractions, even though different in extent, can serve as an indication that indeed it acts on energy producing machine thus depriving the muscles of the energy necessary to support the contraction.

#### **IV. SCHMIDTEA MEDITERRANEA AS A MODEL FLATWORM**

##### **A. MORPHOLOGY**

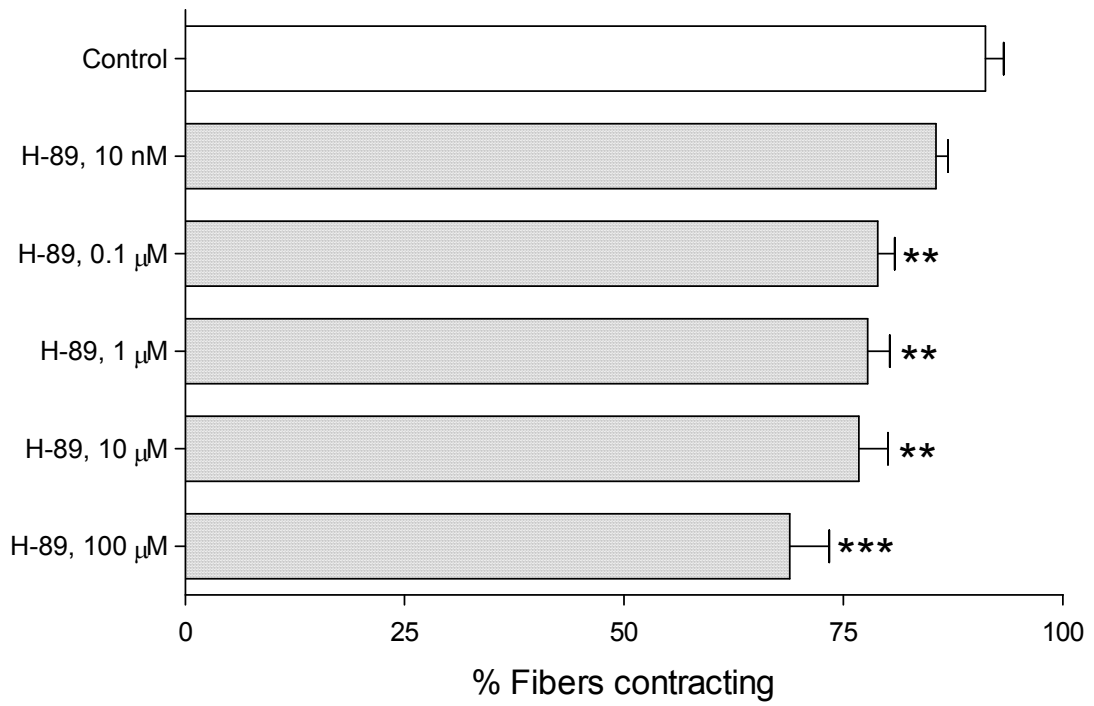
The isolation procedure yielded numerous muscle fibers amenable for electrophysiological studies. Two morphologically distinguished types of fibers were present in the preparation (Figure 18). The first type (Figure 18A) to some extent resembled crescent type of muscle fibers isolated from *S. mansoni* (Day *et al.*, 1993), which had elongated morphology and were relatively large in size (up to 50-100  $\mu\text{m}$ ). This type of fibers was the most abundant in the preparation. The second fiber type had very pronounced 'stick' morphology and ranged 30-60  $\mu\text{m}$  in size (Figure 18B). 'Stick' fibers were relatively less common, averaging to less than one third of the total cell population.

##### **B. VOLTAGE-CLAMP STUDIES**

To determine the nature of voltage-gated currents which are activated by the depolarization of the muscle fibers, the whole cell voltage-clamp technique was employed using an electrode solution which approximates the ionic composition of standard intracellular solution. Depolarizing test pulses following a 1s prepulse to -70 mV from a holding potential of -40 mV induced large outward current which



**Figure 17. Effect of protein kinase A inhibitor H-89 on caffeine-induced contractions.** Isolated muscle fibers contraction ability to 10 mM caffeine was tested in the presence of PKA inhibitor H-89. The extracellular medium as well as the medium to make caffeine dilution was DMI. The drug was added to the medium 15-20 minutes before testing. Each data point is mean  $\pm$  SEM, with  $n \geq 5$ .

**Figure 17**

activated around -30 mV (Figure 19B). Peak outward current showed signs of inactivation within the duration of the 500 ms test pulse, while the sustained current did not.

The maximum current amplitude was observed at depolarization to +50 mV ( $1100 \pm 110$  pA for peak and  $300 \pm 32$  pA for sustained currents,  $n=8$ ) (Figure 19A). In these conditions no inward currents were observed.

The selectivity of this outward current was determined using a fairly standard protocol analyzing tail currents (Figure 20). A big depolarizing test pulse followed by varied repolarizing post-pulse potentials allows visualization of tail currents as channels close upon the repolarization. Tail currents give an opportunity to study the direction of current flow at the potentials where channels are normally closed and thus to see the point where current changes in direction (reversal or equilibrium potential,  $E_{rev}$ ). Considering selective permeability of the muscle fiber membrane to  $K^+$  or  $Cl^-$  ions we calculated the  $E_{rev}$  in the standard conditions (5 mM  $K^+$  and 141 mM  $Cl^-$  in the extracellular medium) and in the experimental conditions, when outside  $K^+$  and  $Cl^-$  concentrations were elevated to 34 mM and 170 mM respectively (Table 4). Provided the membrane is selective for  $K^+$  ions, the calculated equilibrium potential  $E_{rev}$  changes from -84 mV in standard conditions to -35 mV in experimental conditions. If the membrane was selectively permeable for  $Cl^-$  ions, then the calculated  $E_{rev}$  shifts from -1 mV to -6 mV. By plotting tail currents values observed at different repolarizing potentials in standard and experimental conditions for each cell it was possible to obtain values for the observed  $E_{rev}$  of the outward current

**Figure 18. Photographs of isolated *S. mediterranea* muscle fibers.** (A) An example of isolated fibers with elongate morphology. This type of fibers was the most abundant in the preparation. (B) Photograph of isolated fibers with 'stick' morphology. Muscle fiber cyton, a cell body which consists of the nucleus and cytoplasm, most of the time stayed intact after the isolation procedure.

**Figure 18**

**A**

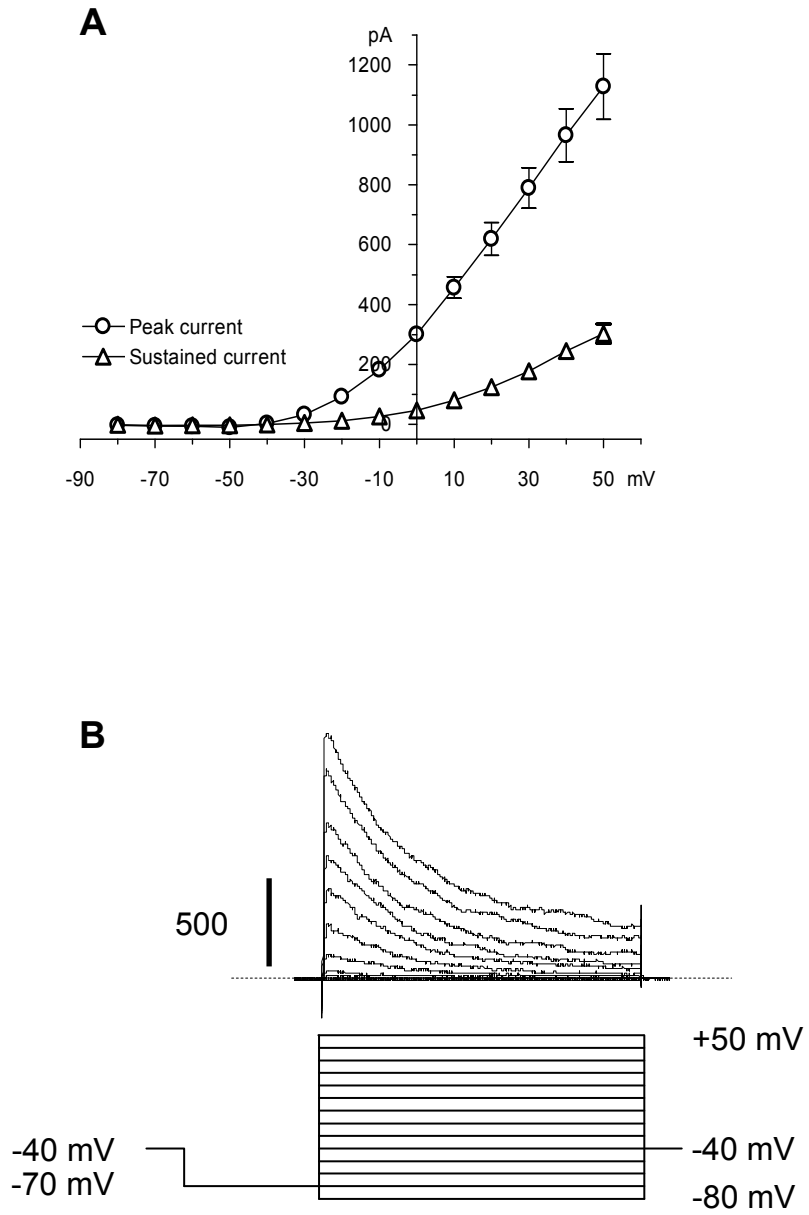


**B**



**Figure 19. Voltage-dependent outward currents are present in muscle fibers of the free-living flatworm *S. mediterranea*.** (A) Current-voltage relationship for outward currents using a depolarizing step pulse protocol of 500 ms duration. Test voltages ranged from -80 to +50 mV in 10 mV increments. The holding potential was -40 mV followed by 1 s prepulse to -70 mV. Traces for *I-V* plot were obtained as the average of three runs at each voltage value. Peak current measurements were determined by five points smoothing (see Materials and methods section) for the 50 ms time period after the start of the test pulse and sustained current values were the mean current values of the 50 ms time span before the end of the test pulse. (B) An example of recorded traces from an isolated muscle fiber. The scale bar is 500 pA.

Figure 19



in the two conditions (Figure 20). Average observed  $E_{rev}$  in the standard condition of -95 mV shifted upon increasing extracellular  $K^+$  and  $Cl^-$  concentrations to -15 mV at 25 °C.

The observed  $E_{rev}$  change was similar to predicted  $E_{rev}$  change in case the membrane was solely selective for  $K^+$  ions thus giving an indication that the current was mostly carried by  $K^+$ . This selectivity implies possession of a complement of  $K^+$  channels by *S. mediterranea* muscle.

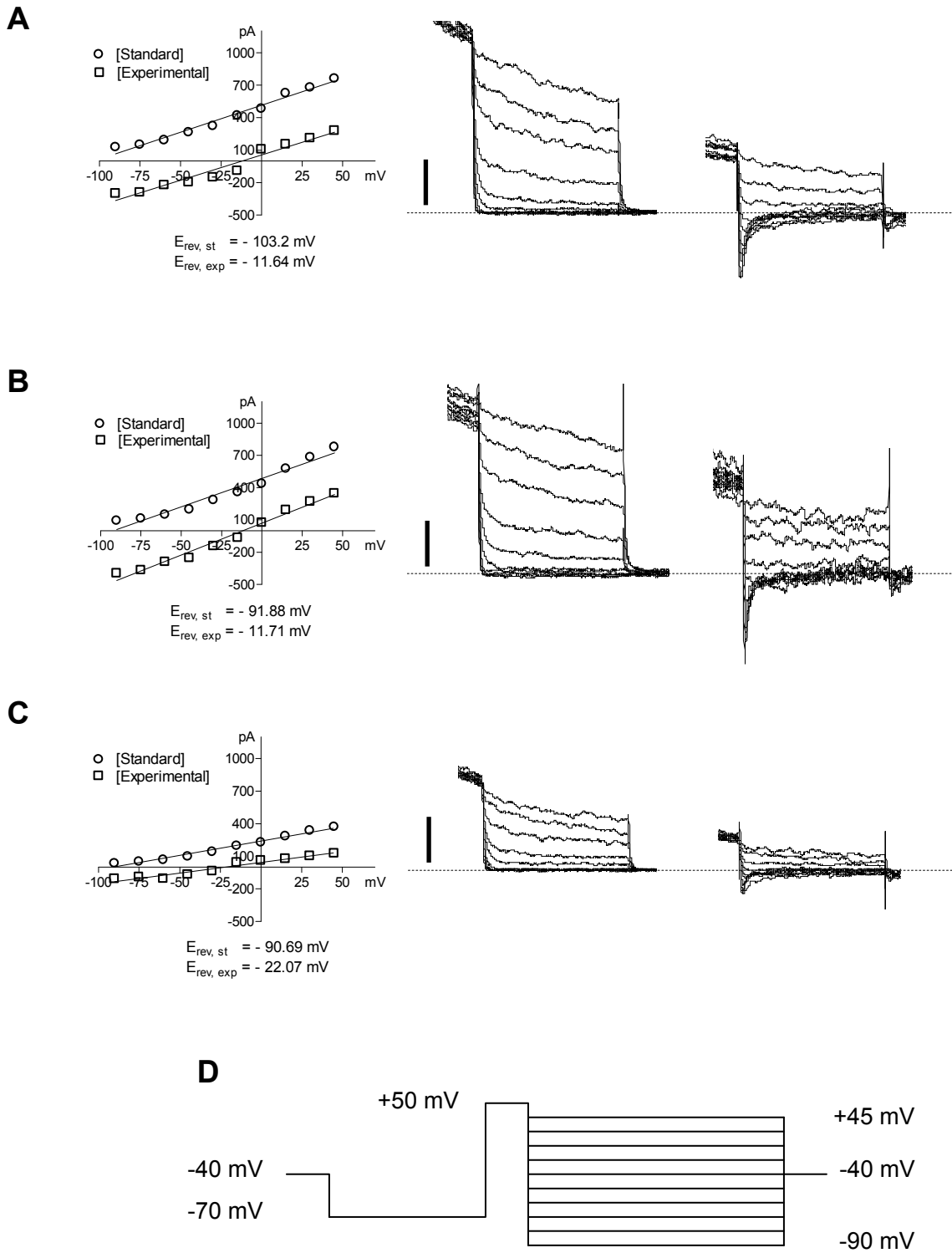
### **Voltage-gated inward currents**

Recording of voltage-activated inward current was possible in conditions that suppressed outward  $K^+$  conductance by internal  $K^+$  replacement with  $Cs^+$  and with enhancement with 15.0 mM  $Ba^{2+}$  as a charge carrier in addition to 2.0 mM  $Ca^{2+}$ . Using depolarizing in 10 mV increments voltage-step protocol from  $V_h$  of -70 mV to +60 mV mixed inward  $I_{Ca/Ba}$  current was isolated (Figure 21A). This inward current did not show evidence of inactivation within the period of the 200 ms depolarizing pulse and was small in amplitude (Figure 21B). Peak current values were determined by five points smoothing within the first 50 ms of the test pulse and sustained current values were obtained as mean values within the last 50 ms of the test pulse. With depolarization, amplitude of evoked currents was increasing with maximum current recorded at +20 mV ( $-53 \pm 7$  pA for peak and  $-35 \pm 5$  pA for sustained current respectively,  $n=9$ ) followed by amplitude decrease with further depolarization. These inward currents were very labile running down within first ten



**Figure 20. Reversal potential  $E_{rev}$  for the outward current in *S. mediterranea* muscle fibers is dependent on extracellular  $[K^+]$ .** (A)-(C) The relationship between the tail current values and voltages and examples of tail current traces obtained with standard solutions composition (trace on the left) and after switching to experimental conditions (Table 4) for three individual cells. Current values plotted against voltage were obtained by 5 points smoothing (see Materials and methods) for the 20 ms period 2 ms after the cessation of the depolarizing test pulse. Graph straight lines denote linear regression line through all the points. The point where it crosses the X-axis represents the current reversal potential, which is altered by changes in  $[K^+]$  and  $[Cl^-]$ . Current traces shown were activated by different after-pulse potentials of 150 ms duration following 200 ms depolarizing pulse to +50 mV. The holding potential was -40 mV. Dotted line denotes zero current level. The scale bar is 200 pA. (D) The full tail protocol.

Figure 20



**Table 4. Equilibrium potential  $E_{rev}$  (mV) of the outward current recorded from isolated *S. mediterranea* muscle depends on the selective permeability of the muscle to ions and their extracellular and intracellular concentrations.**

**Table 4**

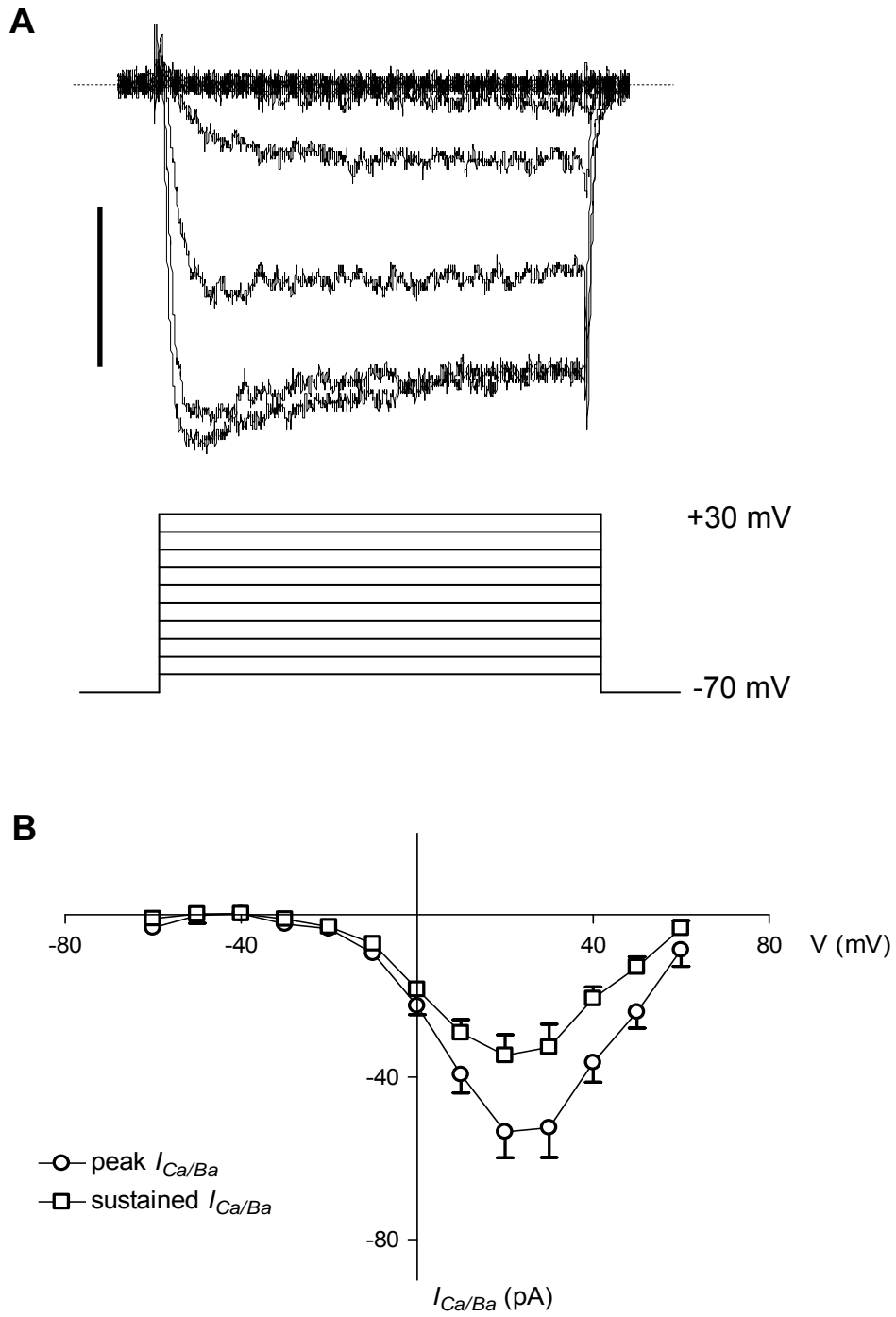
Chemicals	Standard solution composition, (mM)		Experimental solution composition, (mM)	
	Extracellular	Intracellular	Extracellular	Intracellular
NaCl	130	-	130	-
KCl	5	135	34	135
CaCl <sub>2</sub>	2	-	2	-
Glucose	10	10	10	10
MgCl <sub>2</sub>	1	-	1	-
HEPES	20	10	20	10
EGTA	-	2	-	2

Ion	Standard concentrations (mM)		Experimental concentrations (mM)		$[\text{ion}]_{\text{extracel}}/[\text{ion}]_{\text{intracel}}$		2.303RT/zF (mV) at 25 °C	Equilibrium potential (mV)	
	Extracel	Intracel	Extracel	Intracel	Standard	Experiment		Standard	Experiment
K <sup>+</sup>	5	135	34	135	0.037	0.252	+59.16	-84.71	-35.41
Cl <sup>-</sup>	141	135	170	135	1.044	1.259	-59.16	-1.11	-5.92

Equilibrium potential, E <sub>rev</sub> (mV) at 25 °C	Standard concentrations	Experimental concentrations
Observed E <sub>rev</sub> (mV)	-95.26	-15.14

**Figure 21. Voltage-dependent inward currents are present in the isolated *S. mediterranea* muscle fibers.** Currents were evoked by a 200 ms depolarizing pulse protocol from the holding potential of -70 mV to +60 mV in the presence of 2 mM CaCl<sub>2</sub> and 15 mM BaCl<sub>2</sub>. (A) Individual example of mixed  $I_{Ca/Ba}$  currents present in the fibers. Depolarizing voltages were applied in 10 mV increments. Larger depolarization produced larger currents. Dotted line denotes zero current level. Scale bar is 40 pA. (B)  $I$ - $V$  plot for recorded mixed  $I_{Ca/Ba}$ . Peak current values were determined by five points smoothing within the first 50 ms of the test pulse and sustained current values were obtained as mean values within the last 20 ms of the test pulse. Current traces obtained at each voltage for each cell are the average of three runs.

Figure 21



minutes of the recording protocol, thus making experiments on their pharmacology very difficult.

After reducing the main depolarizing protocol to just one step depolarizations from  $V_h$  to +20 mV which were executed every 30 seconds it was possible to generate recordings to determine the inward current time course (Figure 22A) as well as perform some pharmacological experiments. Peak as well as sustained currents gradually ran down with time, reached their maximum on the 1.5 minute after start of the trail, remained relatively stable in amplitude within next 90-150 seconds and were almost gone by the end of 8.0 minute time span. Thus the trial length for performing pharmacology experiments was reduced to have 6.5 minute time course.

### ***Effect of $Ca^{2+}$ voltage-operated channels (VOCC) blockers on inward currents***

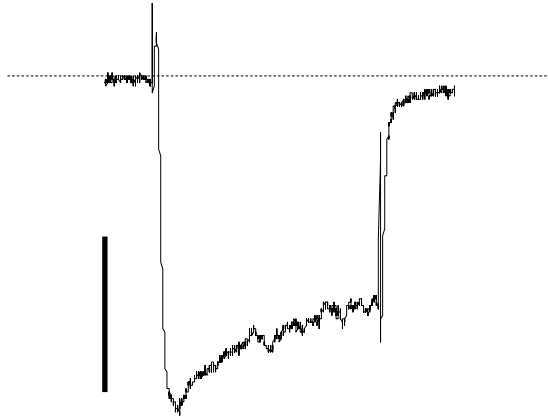
After being able to routinely record inward currents in the presence of  $Ca^{2+}/Ba^{2+}$  cationic mixture it was possible to perform some pharmacological experiments. Special perfusion system setup was used to deliver drugs on patched cells in whole cell configuration. Seals and break-in were obtained with cells being constantly perfused with the standard  $Ca^{2+}/Ba^{2+}$  extracellular (control) solution. This solution was used while obtaining steady baseline current recording and washouts, as well as a vehicle for drugs tested. The variety of VOCCs blockers was limited by their water solubility, since hydrophobic drugs were not compatible with the vehicle.

**Figure 22. Whole cell voltage-gated  $I_{Ca/Ba}$  currents of *S. mediterranea* isolated muscle fibers evoked by 200 ms depolarizing step to +20 mV from the holding potential ( $V_h$ ) of -70 mV. (A) Representative current trace. Scale bar is 40 pA. (B) Inward mixed  $I_{Ca/Ba}$  gradually runs down over time. Step depolarization from  $V_h$  of -70 mV to +20 mV was performed every 30 seconds. Peak and sustained current values were plotted versus time for a typical recording. On average currents reached their maximum on a fourth run 1.5 minutes after the start of the trail and by the end of the 8<sup>th</sup> minute were almost gone. Current trace at each time point is the average of two runs.**

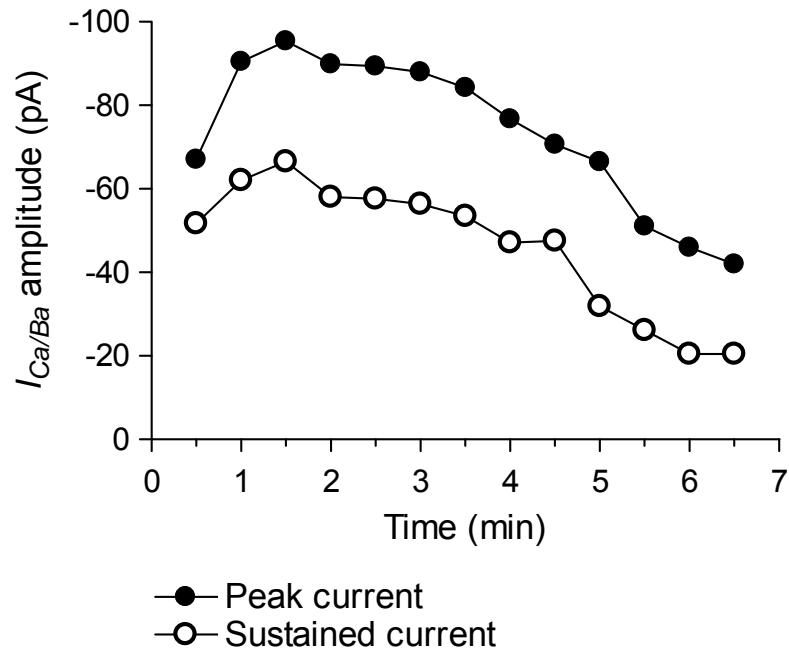


Figure 22

A



B



Phenylalkylamine VOCCs blocker verapamil and benzothiazepine VOCCs blocker diltiazem were used at 10  $\mu$ M concentration. Effects of the drugs on peak and sustained currents, their time course and individual traces were obtained (Figures 23 and 24). For every cell tested peak and sustained current values were normalized to those values obtained at 1.5 minute time point after the start of the trial, right before solutions switch.

Normalized peak current amplitudes were neither significantly reduced in the group treated with verapamil ( $91\pm 4$  %,  $n=4$ ), nor in diltiazem treatment group ( $100\pm 9$  %,  $n=4$ ) as compared to control group ( $99\pm 3$  %,  $n=5$ ) at 2.5 minute time point after the start of the trial ( $P<0.05$  Mann-Whitney test).

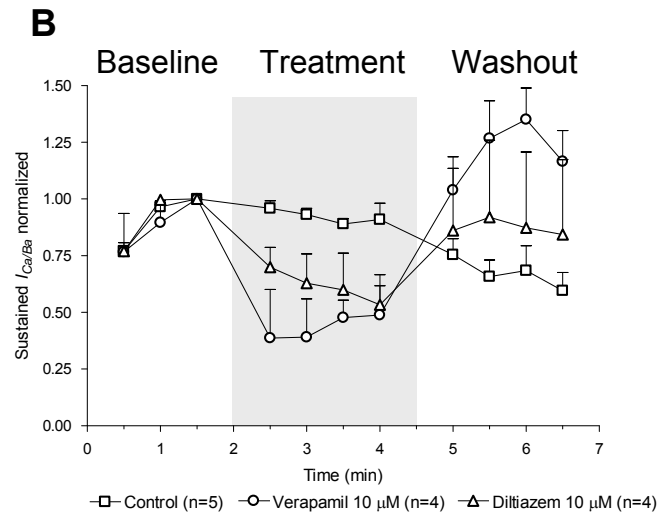
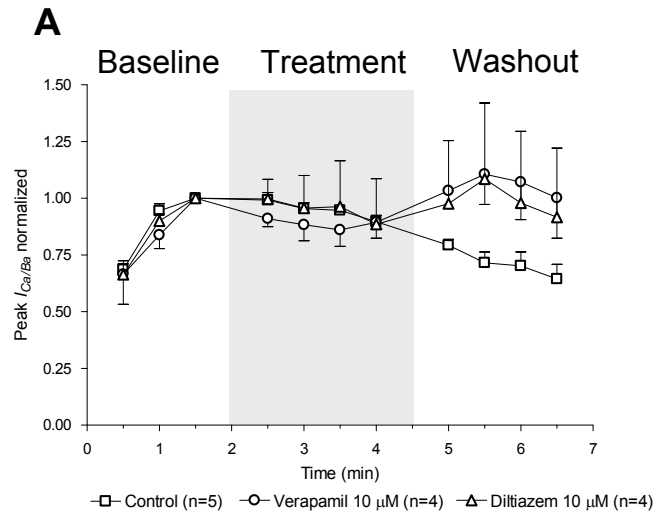
Verapamil showed to be potent on sustained currents ( $P<0.05$ , Mann-Whitney test). It reduced normalized sustained current amplitude at 2.5 minute time point to  $39\pm 21$  % ( $n=4$ ) as compared to control group normalized currents measurements ( $96\pm 3$  %,  $n=5$ ). Diltiazem did not produce significant inhibition of sustained normalized currents, reducing them to  $70\pm 9$  % ( $n=4$ ) at 2.5 minute trial time point.

As well as in the case of *S. mansoni* inward currents pharmacology characterization, using VOCCs blockers, it was very important to observe currents resuming after drug washout to be able to ascribe the current amplitude lowering to the action of drugs. In both drug treatments the effect was fully reversible.

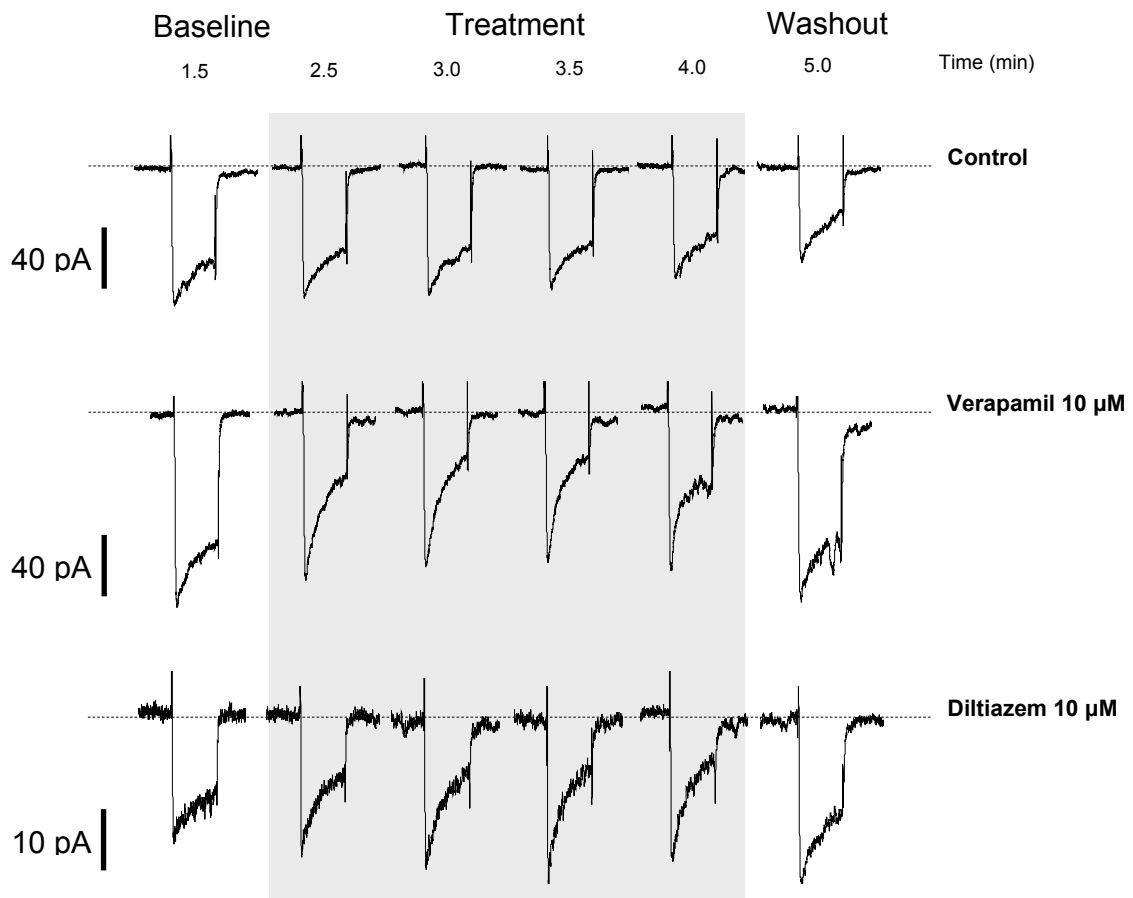
**Figure 23. Effects of phenylalkylamine  $\text{Ca}^{2+}$  channel blocker verapamil and benzothiazepine  $\text{Ca}^{2+}$  channel blocker diltiazem on whole cell  $I_{\text{Ca/Ba}}$  in *S. mediterranea* isolated muscle fibers.** For each cell tested at every time point, in control and test groups, peak and sustained currents were normalized to their current values at 1.5 min after the start of the trial.  $I_{\text{Ca/Ba}}$  peak current values used before normalizing corresponded to peak amplitudes within the first 50 ms of the 200 ms test pulse (determined by five points smoothing), and sustained current values corresponded to mean amplitudes within the last 20 ms of the 200 ms test pulse.

For each cell tested current trace at each time point was the average of two runs. Data were plotted as mean  $\pm$  SEM. During the trial cells were constantly perfused with the bathing solution delivered through the perfusion system pipeline. Shaded box indicates time points where cells were exposed to the drug-containing bathing solution (for the test groups) or control solution itself (for the control group). (A) Verapamil and diltiazem did not significantly alter the amplitude of peak  $I_{\text{Ca/Ba}}$ . (B) Both blockers reversibly inhibited sustained  $I_{\text{Ca/Ba}}$ .

Figure 23



**Figure 24. Representative traces of  $I_{Ca/Ba}$  activated by depolarizing *S. mediterranea* muscle fibers from the holding potential  $V_h$  of -70 mV to +20 mV by 200 ms test pulse in the absence or presence of verapamil and diltiazem (10  $\mu$ M). Dotted line denotes zero current level and time scale of the experiment is shown above traces.**

**Figure 24**

## DISCUSSION

**I. The platyhelminth FMRFamide-like peptide (FLP) YIRFamide is more potent myoexcitatory on schistosome muscle than any of the putative FLPs encoded in the schistosome genome. FLP amino acid residue composition of the C-terminal as well as at positions 3 and 4 is crucial for their biological activity.**

FLPs or FMRFamide-like peptides are a large family of neuropeptides abundant amongst invertebrates. In invertebrates, FLPs constitute a large family of signaling molecules, responsible for muscle contraction (Day *et al.*, 1997; Money Penny *et al.*, 2001; Worden *et al.*, 1995; Mousley *et al.*, 2004), heart beat regulation (Price and Greenberg, 1977; Groome *et al.*, 1994; Skerrett *et al.*, 1995), ion channel function (Cottrell, 1990; Green *et al.*, 1994; Cottrell, 1997) and synaptic transmission (Skerrett *et al.*, 1995).

The FLP YIRFamide, isolated from the marine turbellarian *Bdelloura candida* (Johnston *et al.*, 1996), is very potent at inducing contraction of isolated *S. mansoni* muscle fibers. It contracts  $80 \pm 5$  % of muscle fibers when tested at 1  $\mu$ M. We also tested the myoexcitatory ability of a number of putative FLPs, identified from the genomes of *S. mansoni* and a model flatworm, *S. mediterranea* (Table 3). They were tested at 1  $\mu$ M concentration, where YIRFamide served as a positive control. The principal structural difference of the tested peptides was in the C-terminal amino acid composition. Based on this criterion they were divided into four different groups (Figure 1): peptides ending with RFamide (Arg-Phe-amide), RYamide (Arg-Tyr-amide), RLamide (Arg-Leu-amide) and RIamide (Arg-Ile-amide) at the C-terminal.

Contractions posed by FLPs discovered in flatworm genomes were qualitatively different from those observed with YIRFamide. They were very slower at the onset, seemed less complete, and resembled some twitching rather than smooth contraction. Peptides with RYamide end were the least potent in eliciting contraction. Smed-flp-1e peptide QSVFRYamide elicited  $9\pm 4\%$  of fiber cells contracting ( $n=4$ ) as compared to  $80\pm 5\%$  ( $n=5$ ) responded to YIRFamide. Peptides with Rlamide and Rlamide ending induced relatively low response of muscle fibers contracting: GFVRlamide ( $33\pm 12\%$ ,  $n=4$ ), AKYFRLamide ( $30\pm 5\%$ ,  $n=4$ ) and SFVRLamide ( $13\pm 1\%$ ,  $n=4$ ). Peptides with RF residues at the C-terminal induced contractions of a larger number of isolated fibers as compared to peptides from other groups, but not as much as YIRFamide itself. Sm-flp-1b peptide YTRFVPQRFamide elicited  $36\pm 9\%$  ( $n=4$ ) response and sm-flp-flp-1a peptide HFMPQRFamide gave  $34\pm 2\%$  ( $n=4$ ) of contracting fibers. It can be concluded that neuropeptides with polar amino acids at the C-terminal are very weak at stimulating myoexcitatory response in the muscles, while peptides with hydrophobic aromatic amino acids are able to trigger a relatively bigger response.

Amino acid composition of neuropeptides, specifically the C-terminal structure, seems to be crucial for their biological activity. Other isolated turbellarian RFamide FLPs such as GYIRFamide and RYIRFamide are also potent contractors of *S. mansoni* muscle (Day *et al.*, 1997). Previous studies of *S. mansoni* isolated muscles have shown that replacement of the phenylalanine residue at position 4 (Phe4) with tyrosine (Tyr4) yielded a peptide (YIRYamide) with greatly decreased potency (Day *et al.*, 1997). Truncated analogs (e.g. FRFamide, RFamide) and YIRF



free acid (no amide) were shown to be of very low potency or inactive (Day *et al.*, 1997). Amino acids residues at N-terminal were also shown to play important role in the ability of a certain neuropeptides to bind the FLP receptor and subsequently produce a response.

The structure-activity relationships for myoexcitatory neuropeptides were similar in the marine turbellarian *Procerodes littoralis* isolated muscle fibers (Money Penny *et al.*, 2001) and the whole worm preparations of monogenean *Diclidophora merlangi* (Money Penny *et al.*, 1997). There, YIRFamide and GYIRFamide were very potent myoexcitatory agents, while the GYIRFamide analog GYIRdFamide was not capable of inducing contraction of the muscle and blocked the ability of other peptides to induce them.

The similar potent effects of FLPs on different flatworm preparations suggest that the receptor FLPs acts on can be of similar structure across the phylum and requires specific sequence pattern to pose muscle activity.

In other species structure-activity relationship is of a great importance. In the arthropod *Limulus polyphemus* and crayfish *Procambarus clarkii* the N-terminal acid substitutions are less restrictive than the N-terminal extensions, where the extended FLPs (e.g. TNRNFLRFamide and SDRNFLRFamide) were shown to have cardioexcitatory activity, while tetrapeptide FLRFamide was inactive on this preparation (Groome *et al.*, 1994; Mercier *et al.*, 1991).

Since the peptidergic component of parasitic flatworms attracts special attention due to potential of new drug target discovery it is of interest how FLPs

execute their myoexcitatory action. Not solely the events triggered inside muscle cell, but the structural properties of peptides themselves, their distribution and their receptor localization are of interest. The diversity of neuropeptides that have been isolated and discovered in genomic data can shed the light on structural features responsible for a specific action. Sequencing and localization of biologically active peptides, as well as investigation of their physiological effects and behavioral roles in both vertebrate and invertebrate systems remains one of the important fields of research.

**II. FLP-induced contractions of isolated *S. mansoni* muscle require extracellular  $\text{Ca}^{2+}$  and use a DHP/PAA-sensitive channel as extracellular  $\text{Ca}^{2+}$  conduit.**

Flatworm FLPs have been shown to potently and specifically induce muscle contraction in flatworms (Day *et al.*, 1994). It is known that muscle contraction is dependent on a rise in cytosolic  $\text{Ca}^{2+}$ , and that the source of this  $\text{Ca}^{2+}$  can be either intracellular (Wray *et al.*, 2005) or extracellular (Greenberg, 2005). The extracellular  $\text{Ca}^{2+}$  recruitment by FLPs and the neuropeptide proctolin in inducing muscle contraction was shown in molluscan anterior bypass retractor muscle (Kizawa *et al.*, 1991) and in the hyperneural muscle of the cockroach *Periplaneta Americana* (Wegener and Nässel, 2000). When the extracellular  $\text{Ca}^{2+}$  was omitted from the bathing medium the contractions were diminished. In the presence of organic  $\text{Ca}^{2+}$  channel antagonists such as nifedipine, nifedipine and verapamil contractions were

reduced, but to a lesser extent, than were contractions induced by depolarization with elevated  $[K^+]$ .

We performed the series of *S. mansoni* isolated muscle contraction experiments to find out the importance of extracellular  $Ca^{2+}$  in mediating muscle contraction response triggered by FLPs. The results provided evidence for the crucial role of extracellular  $Ca^{2+}$ . In the culture medium with omitted  $Ca^{2+}$ , or with added  $Ca^{2+}$  chelator EGTA, both YIRFamide-induced contractions and depolarization-induced contractions were abolished, thus indicating extracellular  $Ca^{2+}$  requirement. At the same time the caffeine-induced contractions were almost full scale, thus indicating that the intracellular  $Ca^{2+}$  stores were enough to support contractile response if they were recruited by YIRFamide.

The mechanism of FLP-induced  $Ca^{2+}$  influx across the muscle in the parasite is of interest, as well as the signaling mechanisms that trigger it. The channels that control  $Ca^{2+}$  entry in other cell types studied so far were shown to be either voltage-operated  $Ca^{2+}$  channels (VOCCs) or transient receptor potential channels (TRPs) (Hofmann *et al.*, 2000). We applied a panel of putative VOCC blockers in contraction assays to see whether the contractile response to YIRFamide is altered in their presence. Dihydropyridine (DHP) nifedipine and phenylalkylamines (PAA) verapamil and methoxyverapamil inhibited the FLP-triggered myoexcitatory response, though at relatively high concentrations. The inhibitors failed to reduce the percentage of contracting cells when tested at 10  $\mu$ M, while at 100  $\mu$ M the response was significantly reduced. These results implicated VOCCs as the possible pathway

for extracellular  $\text{Ca}^{2+}$  entry in the FLP-induced contractions in *S. mansoni* muscle fibers.

### **III. Voltage-gated $\text{Ca}^{2+}$ currents of the isolated *S. mansoni* muscle are PAA-sensitive.**

Previously recordings of voltage-gated inward divalent currents in flatworms have been obtained from muscle fibers isolated from a free-living flatworm *Girardia tigrina* (Day and Cobbett, 2003) and a parasite *S. mansoni* (Mendonça-Silva *et al.*, 2006). These fibers were clamped at the holding potential of -70 mV and stepped to more positive voltages, which produced inward currents in the presence of extracellular  $\text{Ba}^{2+}$  used as a charge carrier. Recorded currents were relatively small in size (0.1-0.3 nA). Pharmacological characterization of flatworm VOCCs that carry these important currents has been difficult since the currents are relatively small in amplitude and rundown quickly. Day and Cobbett (2003) showed that the time course of inward current recorded in the presence of  $\text{Ba}^{2+}$  was somewhat variable, where after obtaining the whole cell configuration the current amplitude was rising to reach its peak amplitude and then gradually declined. The short life-span of the current has been a limiting factor in pharmacological experiments. The current rundown was not significantly decreased by the application of ATP, GTP or cAMP in the electrode solution.

In the current study we have been able to consistently record inward currents carried by VOCCs in isolated *S. mansoni* muscle fibers using whole cell configuration of the patch-clamp technique in voltage-clamp mode. Inward voltage-

activated current was recorded under conditions that suppressed outward  $K^+$  conductance by the replacement of internal  $K^+$  with  $Cs^+$ . We also enhanced inward currents with 15.0 mM  $Ba^{2+}$  as a charge carrier in addition to 2.0 mM  $Ca^{2+}$  typically present in the bathing medium ( $I_{Ca/Ba}$ ). Whole cell  $I_{Ca/Ba}$  was obtained from frayed muscle fibers by depolarizing them for 200 ms from a holding potential of -70 mV to more positive potentials. As in the case of *G. tigrina*, the *S. mansoni* inward current was relatively small in amplitude and was short-lived too. A simplified voltage-step protocol with a depolarization step from -70 mV to +20 mV, the voltage at which the largest current amplitude was recorded, allowed obtaining a time course of the current. On average, currents reached peak amplitude 1.5 minutes after the start of the trial and maintained a steady state within next 2.0-3.0 minutes followed by gradual decline in amplitude. The time frame when the current maintained the steady state was employed for extracellular application of common VOCC blockers and neuropeptides. High voltage-activated (HVA)  $I_{Ca/Ba}$  amenable to pharmacological manipulation was present in less than 30 % of patched cells.

The currents recorded from *S. mansoni* isolated muscle fibers were sensitive to PAA blocker verapamil, which reduced their amplitude in concentration-dependent manner. Due to the incompatibility with the vehicle, specifically, the organic blockers such as a common DHP nifedipine, precipitated in the high divalent cations, and therefore were not tested. Verapamil was effective against the sustained component of the current at both 10  $\mu$ M and 100  $\mu$ M concentrations tested, while only at 100  $\mu$ M it was able to reduce the amplitude of the peak component of  $I_{Ca/Ba}$ .

Electrophysiological data for most VOCC currents from invertebrates are not sufficient to allow sound conclusions about the specific type of channel mediating the current to be made. Most invertebrate currents studied so far were of L-type (Yeoman *et al.*, 1999). In mammals L-type  $\text{Ca}^{2+}$  channels have  $\alpha_1$  pore-forming subunit and are activated by high voltages and are sensitive to DHPs. Both L- and non-L-type *S. mansoni*  $\alpha_1$  subunits have been cloned (Kohn *et al.*, 2001) as well as HVA  $\beta$  subunit (Kohn *et al.*, 2001b). The recorded current might then be carried by one of these channels.

The necessity to apply relatively high concentration of the PAA and DHP blockers is in accordance with other invertebrate tissue preparations. Decreased or lack of sensitivity is observed in both lower and higher invertebrates compared to vertebrate L-type channels, which display sensitivity to DHPs and PAAs in the micromolar range. For example in the brain cells of a flatworm *Bdelloura candida* (Blair and Anderson, 1993)  $\text{Ca}^{2+}$  currents are relatively insensitive to standard panel of VOCCs blockers. There the current stimulated by test pulses is relatively unaffected by 10  $\mu\text{M}$  nifedipine ( $15 \pm 10$  % block) and 10  $\mu\text{M}$  verapamil ( $31 \pm 20$  % block). The VOCC  $\alpha_1$  subunit of jellyfish *Cyanea capillata* expressed in *Xenopus* oocytes produces current which is not greatly affected by DHP and PAA inhibitors as high as 100  $\mu\text{M}$  (Jeziorski *et al.*, 1998): nifedipine, isradipine and verapamil produce 50, 36 and 42 % of inhibition correspondingly. As compared to  $\alpha_1$  sequences of L- and non-L-type mammalian HVA channels, the jellyfish  $\alpha_1$  is more close to neuromuscular L-type VOCC. In the jellyfish *Polyorchis penicillatus* neurons, HVA channels are not sensitive to PAA verapamil, which is not able to block the current in

the 50-100  $\mu\text{M}$  concentration range, while DHP nifedipine at 100  $\mu\text{M}$  reversibly inhibits the sustained component of the current (Przysieznik and Spencer, 1992). In invertebrate neurons  $\text{Ca}^{2+}$  currents are sensitive to PAA verapamil and D-600 (Pearson *et al.*, 1993; Schäfer *et al.*, 1994), while they are not sensitive to DHPs (Wicher and Penzlin, 1997; Bickmeyer *et al.*, 1994; Pelzer *et al.*, 1989; Pearson *et al.*, 1993). The first increased DHPs sensitivity of  $\text{Ca}^{2+}$  currents in invertebrates has been reported for molluscan muscles (Jeziorski *et al.*, 1998).

The obtained VOCC pharmacology data allow us to make comparison between the *S. mansoni* electrophysiology and contraction assays as well as between species in search of similarities, which will allow application of similar more tractable model species in further research.

#### **IV. The FLP YIRFamide enhances the inward current carried by PAA-sensitive $\text{Ca}^{2+}$ channels and this $\text{Ca}^{2+}$ current enhancement is reduced in the simultaneous presence of PAA VOCC blocker verapamil.**

The effect of FLP YIRFamide on voltage-dependent  $I_{\text{Ca/Ba}}$  was studied in isolated *S. mansoni* muscle fibers. YIRFamide at 1  $\mu\text{M}$  produced significant increase in the amplitude of both peak and sustained components of the current. The current amplitude increase caused by the YIRFamide occurred almost immediately, and reached its maximal level within a minute of constant application. This effect was reversed by washout. The enhancing effect of YIRFamide on  $I_{\text{Ca/Ba}}$  was reduced in the simultaneous presence of a putative PAA L-type channel antagonist verapamil at 100  $\mu\text{M}$ . The obtained result indicates possible involvement of VOCCs in carrying

Ca<sup>2+</sup> influx triggered by the FLP. Additional studies will be required to study macroscopic whole cell currents in the presence of PLC, PKC and PKA inhibitors. Also experimental work at the single channel level would be of interest to see how single channel events influence the current.

**V. The signal transduction pathway between GPCR and final contraction response of isolated *S. mansoni* muscle triggered by FLPs is equivocal.**

Of great interest is the intracellular mechanism that links the FLP receptor activation and final contractile response. Modulation of Ca<sup>2+</sup> channels by neuropeptides requires activation of G-protein-dependent signaling cascades (Dolphin, 1995; Kits and Mansvelder, 1996) or direct gating of channels (Cottrell, 1997). In mollusks their modulation by transmitters is mediated by phosphorylation via protein kinase A (PKA) or protein kinase C (PKC) pathways, which enhance Ca<sup>2+</sup> channels. Negative channel regulation occurs directly through G proteins or by activation of phosphodiesterases and phosphatases by cGMP (Kits and Mansvelder, 1996). Previous studies done on a free-living flatworm *Procerodes littoralis* (Totten, unpublished) and liver fluke *Fasciola hepatica* (Graham *et al.*, 2000) provided evidence for the involvement of G-protein coupled receptor, cyclic adenosine monophosphate (cAMP) and activation of PLC, protein kinase C (PKC) second messenger pathways in mediating the myoexcitatory cellular response to stimulation with the FLP GYIRFamide.

We conducted a series of contraction experiments in order to get more knowledge on signaling mechanisms involved between activation of FLP receptors



and the final *S. mansoni* isolated muscle contraction response. The PKC and PKA signaling cascades were of particular interest, since they are the major regulators of  $\text{Ca}^{2+}$  channels activity.

Several observations in this study confirm the involvement of PKC in mediating FLP-triggered response. This conclusion is based on the observation that the contraction response was reduced in the presence of PKC inhibitors with  $\text{IC}_{50}$  specific for the reported PKC inhibition range for some of them. Calphostin C, one of the most selective PKC inhibitors, was very potent at inhibiting the response. The extremely high specificity of Calphostin C is determined by its ability to bind to the phorbol ester/diacylglycerol binding site that is unique to PKC (Bruns *et al.*, 1991).

Two inhibitors tested, the staurosporine analog Ro 31-8220 and chelerythrine chloride were effective at blocking the contractions too, but to a lesser extent. However, because these inhibitors are less specific, these data are not as conclusive as the Calphostin C results. Ro 31-8220 failed to provide inhibition of YIRFamide-induced response in the suggested range of concentrations reported as specific for PKC. Ro 31-8220, which acts by competitive inhibition of ATP binding to the kinase, was reported to have rather low specificity of action on PKC since this ATP binding site is conserved among different members of protein kinases family (Hartzell and Rinderknecht, 1996). Chelerythrine chloride that affects translocation of PKC from cytosol to plasma membrane belongs to a group of drugs that are not widely used as specific PKC inhibitors (Bruns *et al.*, 1991).

We also had a set of experiments aimed to investigate possible PKA-dependent pathway. However, no solid grounds for that were established. We used a panel of adenylate cyclase (AC) and PKA inhibitors, which included some very specific inhibitors and some that are less specific. A common AC inhibitor MDL 12.330A was found to be very potent at inhibiting neuropeptide-stimulated contractions. The results obtained for MDL 12.330A could not be definitely ascribed to inhibitory action on AC, since MDL 12.330A has been reported to exert some other action besides AC inhibition. It was reported to reversibly inhibit slow  $\text{Ca}^{2+}$  current in a voltage-dependent manner in rat anterior pituitary cells at 1  $\mu\text{M}$  (Rampe *et al.*, 1987). Also MDL 12.330A was shown to have negative inotropic effect on the isolated guinea pig heart ( $\text{IC}_{50}$ , 1.1  $\mu\text{M}$ ), which was reversible on addition of  $\text{Ca}^{2+}$  ions (Grupp *et al.*, 1980). Inhibitory action on store-operated  $\text{Ca}^{2+}$  entry has also been reported for it (van Rossum *et al.*, 2000). Besides the above mentioned actions, MDL 12.330A has been reported to have biphasic effects on UDP-glucuronosyltransferase and glucose-6-phosphatase enzymes' activity in liver microsomal preparations (Guellaen *et al.*, 1978). Due to the broad reported spectrum of the inhibitor it might be hard to draw conclusions, since the action might not be connected with AC inhibition, but with  $\text{Ca}^{2+}$  channels or with transient receptor potential channels (TRPs). Similar confusing results were obtained when testing MDL 12.330A on *F. hepatica* muscle strips (Graham *et al.*, 2000). There it was considered to be an unsuitable pharmacological tool since GYIRFamide-induced response was inhibited, while excitatory response to 50  $\mu\text{M}$  forskolin was not altered. In addition MDL 12.330A itself stimulated mechanical activity of muscle

strips. A more specific AC inhibitor SQ 22.536 did not show dose-dependent inhibition pattern of the contractions.

In the present study it was also hard to judge effects posed by a common PKA inhibitor H-89 and cell-permeable myristoylated PKI (14-22) amide PKA inhibitor on muscle contractions evoked by 1  $\mu$ M YIRFamide. H-89 was quite potent, suggesting that PKA might be involved, but many other studies have shown that it can inhibit a number of other enzymes. In a study on specificity of different protein kinase inhibitors H-89 has been shown to inhibit eight protein kinases at 10 $\mu$ M, three of which (MSK1, S6K1 and ROCK-II) were inhibited with a similar or a greater potency than that for PKA (Davies *et al.*, 2000). H-89 has unspecific effects on sarcoplasmic reticulum Ca<sup>2+</sup> ATPase (Hussain *et al.*, 1999; Lahouratate, 1997) and blocks shaker-type K<sup>+</sup> channels (Choi *et al.*, 2001). H-89 has already been shown to inhibit depolarization-induced contractions of *S. mansoni* muscle fibers, while the cAMP analogue 8-Br-cAMP did not significantly increase percentage of fibers contracting in response to increased [K<sup>+</sup>] (Day *et al.*, 1994). H-89 failed to block excitatory response to cell permeable 8-Br-cAMP in muscle strips from *F. hepatica* too (Graham *et al.*, 2000).

The use of highly specific inhibitors of AC and PKA could have provided evidence for the involvement of the enzymes in mediating the contraction response to YIRFamide. As with SQ 22.536, the inhibitory effect posed by the PKI (12-22) amide, was doubtful as being related to inhibition of FLP-activated PKA, since the concentration at which it was very effective fell far beyond the range specific for PKA

inhibition. As an explanation of the observed effects, the suggestion that elevated levels of cAMP are just needed to stimulate energy production, necessary to support muscle contraction to any of stimulatory agents, can be used.

In mammalian system muscle phosphofructokinase (PFK), a crucial enzyme in glycolysis, has been shown to be phosphorylated as a result of elevated cAMP and activated PKA (Foe and Kemp, 1982; Valaitis *et al.*, 1989; Alves and Sola-Penna, 2003). PFK kinetics of extracts obtained from different mollusks was shown to be modulated by phosphorylation, which increases enzyme activity, and dephosphorylation (Michaelidis *et al.*, 1993). Serotonin or cAMP administration increases the activity of PFK in the homogenates obtained from *F. hepatica* (Mansour and Mansour, 1962). Serotonin stimulates PFK phosphorylation in *F. hepatica* and *Ascaris suum* (Kamemoto *et al.*, 1989; Harris *et al.*, 1982). In the foot muscle of gastropod *Patella caerulea* incubation of PFK preparation with cAMP and cGMP increased enzyme activity (Lazou, 1991). These considerations in addition to the observed inhibitory effect posed by AC inhibitor MDL 12.330A on caffeine-induced contractions, which do not recruit intracellular signaling cascades, suggest the energy supply hypothesis as a possible explanation.

#### **VI. *Schmidtea mediterranea* muscle possesses a complement of similar K<sup>+</sup> and PAA-sensitive Ca<sup>2+</sup> channels to *S. mansoni* muscle.**

We examined the nature of the ensemble of currents activated by depolarization to characterize isolated *S. mediterranea* muscle. The predominant voltage-gated current is a large outward K<sup>+</sup> current. The identity of the ions

responsible for carrying this outward current was determined by analyzing the so called 'tail' currents of the outward current - these are the transient currents that persist after repolarization of the membrane back toward the resting potential. Analyzing tail currents gives an opportunity to study the direction of current flow at the potentials where channels are normally closed and thus to see the point where current changes in direction, referred to as the reversal potential of the current.

As mentioned previously, the presence of voltage-gated inward currents carried by divalent cations has previously been demonstrated in studies on muscle fibers isolated from flatworms *B. candida* (Blair and Anderson, 1993), *G. tigrina* (Cobbett and Day, 2003) and *S. mansoni* (Mendonça-Silva *et al.*, 2006). As well as in these flatworms, experiments proposed to characterize the pharmacology of whole isolated flatworm muscle fiber membrane inward currents posed a challenge since these inward currents rapidly run down. As with VOCCs in all cells, there is often a rapid decrement in the amplitude of inward currents soon after the whole cell configuration is obtained. In these small muscle fibers of flatworms that rapid rundown is dramatic and presents a challenge to measuring the effects of putative blockers.

Using the same approach, as when recording inward voltage-gated currents in *S. mansoni* muscle fibers, we have been able to consistently record inward currents carried by VOCCs in muscle fibers isolated from the free-living flatworm *S. mediterranea* in the presence of  $\text{Ca}^{2+}/\text{Ba}^{2+}$  cationic mixture. They are very labile and run down within first minutes after obtaining the whole cell configuration as well. A

special voltage clamp protocol with reduced number of depolarizing sweeps from the holding potential of -70 mV to +20 mV, voltage at which the largest current was recorded, has been developed to not only record the currents but also to determine the time frame within which application of pharmacological agents is made possible. On average currents reach maximum amplitude on the 1.5 min after the start of the trial, remain relatively stable in amplitude within next 90 – 150 sec and are almost gone by end of 8.0 min time span. Using the SF-77B perfusion Fast-Step system from Warner Instrument Corporation we were able to compare inward currents in muscles in which the putative blockers are applied to muscles in which the control vehicle is applied. The currents were sensitive to classical blockers of vertebrate L-type  $\text{Ca}^{2+}$  channels, phenylalkylamine verapamil and benzothiazepine diltiazem. The blockers require relatively high concentrations ( $>1 \mu\text{M}$ ) and are more effective against sustained component of the current in contrast to the peak component.

The comparison of obtained outward and inward currents profiles in *S. mansoni* and *S. mediterranea* is of importance, since the similar general nature of the complement of voltage-gated ion channels present in the somatic musculature of both species can validate further use of this much more tractable species for experiments elucidating flatworm muscle physiology.

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