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CHARACTERIZATION OF A VOLTAGE-GATED POTASSIUM CHANNEL GENE FROM *SCHISTOSOMA MANSONI*

by

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ABSTRACT

CHARACTERIZATION OF A VOLTAGE-GATED POTASSIUM CHANNEL GENE FROM *SCHISTOSOMA MANSONI*

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Voltage-gated K^+ channels modulate the excitability of many cells including neurons and muscle cells. In contrast to vertebrates and higher invertebrates, very little is known about the structure and diversity of voltage-gated K^+ channel genes in lower invertebrates. More information from these lower invertebrates would provide useful information on the evolution of K^+ channel genes and the nervous system.

Using *Schistosoma mansoni*, a parasitic flatworm that shows a centrally localized nervous system and many organs for the first time in the evolutionary pathway, we have isolated a cDNA (SKv1.1) encoding a *Shaker*-related voltage-gated K^+ channel. The deduced amino acid sequence (512 aa, 56.5 kDa) contains regions that are commonly found in other voltage-gated K^+ channels. SKv1.1 is grouped in the *Shaker* family, but forms a unique branch within this family, on the basis of dendrogram analysis. SKv1.1 shows significant sequence identity (64-74%) with most other *Shaker* channels in the core region (S1-S6), but not at the N- and C-terminal regions. Northern blot analysis detected a single primary transcript of 2.8 kb, indicating alternatively spliced transcripts of SKv1.1 gene may not exist. Southern blot analysis indicated that SKv1.1 is present as a single copy

in the genomic DNA of *S. mansoni*.

Expression of SKv1.1 in *Xenopus* oocytes produces a rapidly activating and inactivating outward K^+ current which is highly sensitive to 4-aminopyridine, but is insensitive to tetraethylammonium, mast cell degranulating peptide, dendrotoxin and charybdotoxin. Immunohistochemistry using SKv1.1-specific antibody (ASK1) demonstrated the expression of SKv1.1 gene in isolated muscle fibers. These results, in combination with the results of electrophysiological characterization, suggest that A-type currents that are measured in isolated muscle fibers are directed by the SKv1.1 gene. Immunohistochemistry on paraffin sections has also demonstrated that SKv1.1 proteins are expressed in the nervous system in a much higher density compared with the expression in muscle fibers and tubercles.

The presence of a *Shaker* homologue in *Schistosoma* suggests that the gene duplication event that generated four *Sh* subfamilies happened at least before the emergence of *Schistosoma*. The existence of a *Shaker* homolog in *Schistosoma* implies that *Sh* subfamilies may exist in other lower invertebrates as well as platyhelminths.

To Kyeonghee

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4-AP	4-aminopyridine
BSA	Bovine serum albumin
CTX	Charybdotoxin
DMEM	Dulbecco's modified eagle's medium
DTX	Dendrotoxin
EDTA	Ethylenedinitrilo tetraacetic acid
EGTA	Ethyleneglycol tetraacetic acid
KLH	Keyhole limpet hemocyanin
MCDP	Mast cell degranulating peptide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBT	Phosphate buffered saline with 0.3% Triton X-100
SDS	Sodium dodesyl sulfate
TEA	Tetraethylammonium
UTR	Untranslated region

INTRODUCTION

I. General background information on schistosome

A. Introduction

Schistosoma, a trematode flatworm (fluke), is the causative agent for schistosomiasis which is second only to malaria the world's most important tropical disease. Currently two hundred million people are infected resulting in 200,000 deaths every year, and the lives of 500-600 million people are currently at risk.

There are three important species of *Schistosoma* for human infection: *S. mansoni*, *S. haematobium* and *S. japonicum* differing in the regions of occurrence. *S. mansoni* occurs in Africa, Latin America and the Eastern Mediterranean while *S. haematobium* and *S. japonicum* occur in Africa and South-East Asia, respectively. The adult worms, female and male, of *S. mansoni* live primarily in the branches of the superior mesenteric vein and lay several hundred eggs every day. These eggs are trapped in the liver leading to the formation of granulomas in the liver and finally to liver failure which is the major cause of death in infected people. The eggs laid in the capillary vessels along the intestinal wall, by penetrating the wall of intestine, are excreted in the feces. Upon contact with water, eggs hatch into miracidia and infect snails which are the intermediate host. In the infected snail, the miracidium grows into a cercaria by asexual development, which takes about a month. Released

cercaria again find a human host by chemotaxis and active swimming, penetrate the skin and grow into adult worms.

In order to control the disease, research activities have been focused on the development of vaccines and chemotherapeutic drugs. Since adult worms easily escape the host immune system, current efforts on vaccine development are focused on minimizing the death caused by liver failure rather than completely blocking infections. Therefore, chemotherapy is the most important method to control schistosomiasis. To find new antischistosomal drugs, it is fundamental to identify novel targets by studying the basic biology of *Schistosoma* including the nervous system, neuromuscular system, digestive system, osmoregulation, reproduction, development and tegumental metabolism.

B. Nervous system

The nervous system of *S. mansoni* follows the general pattern of trematodes as described by Fripp (1967). Male and female worms have, anatomically, the same configuration (Figure 1). Two pairs of anterior ganglia, ventral and dorsal, interconnected by the central commissure are located in the anterior region of the worm. From the anterior ganglia, two pairs of longitudinal nerve cords, ventral and dorsal, extend anteriorly and posteriorly. Anterior nerve cords end near the oral sucker. Posterior nerve cords run down along the entire body and form a 'ladder' type

Figure 1. Diagram showing the central and peripheral nervous system of *S. mansoni*. (A) Longitudinal section of male worm. (B) Transverse section in the middle of the body of male worm. AG (anterior ganglia), CC (central commissure), OS (oral sucker), VS (ventral sucker), NC (nerve cord), T (tubercle), G (gut), DNC (dorsal nerve cord), VNC (ventral nerve cord), DNN (dorsal nerve net) and VNN (ventral nerve net).

structure horizontally interconnected by many commissures. Nerve plexuses of nerve cell bodies and nerve fibers emanating from the nerve cords comprise the peripheral nervous system and innervate many structures such as the oral and ventral suckers, gastrodermis and reproductive organs.

The ultrastructure of the nervous system including sensory structures has been well described by Silk and Spence (1969a). In the anterior ganglia or central ganglia, cell bodies with large nuclei and many organelles are found. Axons of these cell bodies are not myelinated. The closely packed axons in the central commissure vary considerably in shape, size and content. Adjacent axolemmas are separated by a uniform layer of cement substance which is approximately 12.5 nm wide. In the electron-lucent axoplasm, many inclusions are found including at least four types of synaptic vesicles and related structures such as granules resembling α-glycogen, mitochondria, microtubules and filaments. Types of synaptic vesicles found in nerve terminals include clear synaptic vesicles (type 1, 20-50 nm in diameter), dense osmiophilic granules (type 3, 32-90 nm), neurosecretory larger dense granules (type 4, 100-160 nm) and clear axoplasmic vesicles (type 5, 50-150 nm). Axons in the nerve cords contain fewer inclusions than axons in the anterior ganglia. Axo-axonal synapses are observed in the central commissure and in other regions. Nerve processes

packed with a variety of vesicles are also observed in the neuromuscular junctions.

Sensory nerve endings have been found around the tubercle in the tegument (Silk and Spence, 1969a). The structure of the nerve ending consists of a bulbous sac of nerve tissue and a single cilium extending into the apical side. The innervation of sensory receptors by peptidergic nerve fibers has been demonstrated by immunohistochemistry (Skuce *et al.*, 1990; Basch and Gupta, 1988; Gustaffson, 1987).

The nervous system of *S. mansoni* has many neurotransmitters and neuropeptides. The presence of acetylcholine has been reported by staining for acetylcholine esterase (Bueding *et al.*, 1967; Fripp, 1967). Biogenic amines such as 5-hydroxy tryptamine (5-HT), dopamine and norepinephrine have been shown to exist by biochemical, fluorescence immunohistochemistry and autoradiography (Bennett *et al.*, 1969; Bennett & Bueding, 1971; Chou *et al.*, 1972; Machado *et al.*, 1972; Gianutsos & Bennett, 1977; Dei-Cas *et al.*, 1979; Dei-Cas *et al.*, 1981; Gustafsson, 1987). Also, a variety of neuropeptides in *S. mansoni* include leu-enkephalin, FMRFamide, gastrin-17, luteinizing hormone releasing hormone, substance P, cholecystokinin, growth hormone releasing factor, pancreatic polypeptide, peptide YY and neuropeptide Y (Skuce *et al.*, 1990; Bash & Gupta, 1988; Gustafsson, 1987). Acetylcholine,

dopamine and norepinephrine are generally known as inhibitory neurotransmitters in *Schistosoma* while 5-HT is believed to be an excitatory neurotransmitter (Baker *et al.*, 1966; Hillman, 1983; Mellin *et al.*, 1983; Pax *et al.*, 1984; Semeyen *et al.*, 1982; Tomosky *et al.*, 1974).

C. Neuromuscular system

Three types of muscle fibers, circular, longitudinal and radial, have been described by Silk and Spence (1969b). Circular muscle fibers are located beneath the basement membrane of the tegument. Longitudinal muscle fibers are most abundant and located under the circular muscle fibers. Radial muscle fibers are disposed radially around the circular and longitudinal muscle fibers.

The arrangement of myofilaments is that of typical invertebrate smooth muscles in which thick filaments are surrounded by 8-14 thin filaments (Lowy & Hanson, 1962; Lumsden & Bryam, 1967). There is no evidence of striation in any region. There are no transverse invaginations of sarcolemma, and the sarcoplasmic reticulum is poorly developed. At the neuromuscular junction, axolemma and sarcolemma were closely apposed (10 nm). In the presynaptic nerve terminal, several synaptic vesicles are observed.

Muscle cells of *Schistosoma* have two peculiar features (Pax & Bennett, 1992; Silk & Spence, 1969b). First, the cell body of the muscle cell (myocyton), containing the

nucleus, is located more deeply and separately from the myofibril bundles, being connected together by one or more narrow cytoplasmic bridges. Electrical coupling between adjacent myocytons appears to be more prominent than that between myofibrils. Secondly, non-contractile cytoplasmic arms from the myofibril reach out to nerve fibers to make neuromuscular junction although this type of structure is best described in cestodes (Webb, 1987).

Motor activity of the whole worm is enhanced by exogenously applied 5-HT (Baker *et al.*, 1966, Fetterer *et al.*, 1977), phorbol ester (Blair *et al.*, 1994) and inhibited by dopamine (Tomosky *et al.*, 1974). Contractions of isolated muscle fibers have also been shown to be modulated by the invertebrate peptide, FMRFamide (Day *et al.*, 1994b), 5-HT (Day *et al.*, 1994a) and glutamate (C. Miller, unpublished observation). However, little is known about the mechanisms underlying *in vivo* spontaneous contractions or contractions induced by neurotransmitters in isolated muscle fibers (Blair *et al.*, 1994; Day *et al.*, 1994a; Day *et al.*, 1994b).

II. K^+ channels

A. Types and functions of K^+ channels

K^+ channels are roughly divided into four groups: voltage-gated K^+ channels, calcium-activated K^+ channels, receptor-coupled K^+ channels and other K^+ channels (Watson &

Abbott, 1992). Voltage-activated K^+ channels include delayed rectifiers, A-type channels, slow delayed rectifiers, inward rectifiers, sarcoplasmic reticulum channels and the muscarinic-inactivated K^+ channel (M channel). Calcium activated K^+ channels are further divided into three groups based on their conductance, either high, intermediate or small. Receptor-coupled K^+ channels include the atrial muscarinic-activated K^+ channel and the 5-HT inactivated channel (S channel). Other K^+ channels include an ATP-sensitive channel, a Na^+-activated channel, a cell-volume-sensitive channel, and a K^+ channel opener-sensitive channel.

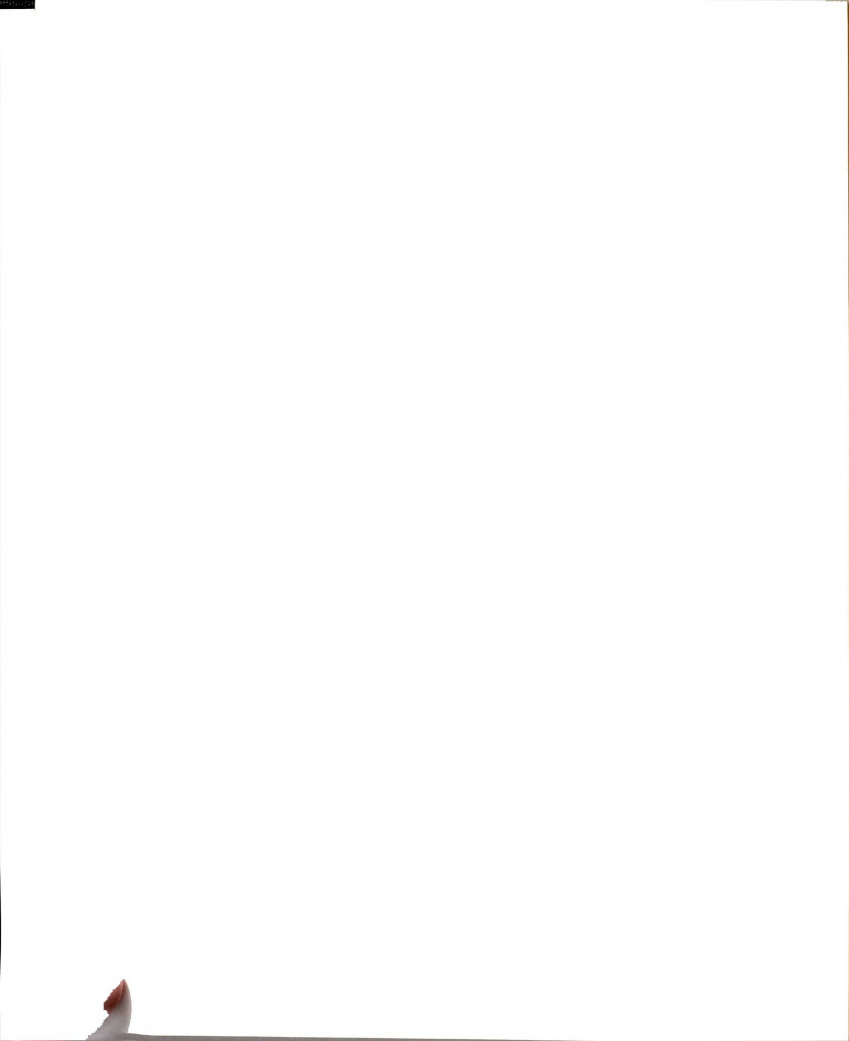
More accurate classification of K^+ channels is becoming possible due to isolation of various K^+ channel genes. One example is the finding of four types of genes, *Shaker*-related subfamilies, that are responsible for currents formerly classified only into two groups, the delayed rectifiers and A-type currents. Except for the relatively extensively studied *Shaker*-related voltage-gated K^+ channel genes, sequences of other genes are only beginning to emerge. Original isolation of genes in each category include *Shaker* (Kamb *et al.*, 1987; Papazian *et al.*, 1987; Schwarz *et al.*, 1988; Pongs *et al.*, 1988), slow delayed rectifier (Takumi *et al.*, 1988), Ca^{2+}-activated K^+ channel (Atkinson *et al.*, 1991), inward rectifier (Kubo *et al.*, 1993; Ho *et al.*, 1993), atrial muscarinic-activated K^+

channel (Kubo *et al.*, 1993; Dascal *et al.*, 1993) and ATP-sensitive K^+ channel (Ashford *et al.*, 1994).

K^+ channels are found in most excitable cells including neurons and muscles, as well as non-excitable cells such as lymphocytes (Douglass *et al.*, 1990). K^+ channels have very diverse functions, reflecting their diversity of structure (Hille, 1992; Rudy, 1988). In general, K^+ channels stabilize the membrane potential, thereby, regulating the excitability of the cell. In excitable cells, K^+ channels set the resting membrane potential, decrease the duration of the action potential, regulate the frequency of action potential, regulate the efficiency of excitatory input and are involved in learning and memory (Nelson and Alkon, 1992). In secretory cells, K^+ channels regulate the secretion of hormones by modulating the excitability of the cell (Philipson *et al.*, 1991). In glial cells, K^+ channels act as ion transporters to remove extracellular K^+ ions away from neighboring neurons.

In particular, the rapidly inactivating A-type K^+ channel is known to be involved in controlling the frequency (Segal et al., 1984) and duration (Sah and McLachlan, 1992) of the action potential, modulation of synaptic efficacy (Kaang et al., 1992) and postsynaptic excitability (Cassell et al., 1986).

B. K^+ channels in *S. mansoni*



Lower invertebrates show a remarkable diversity of ion channels that is comparable to that of vertebrates and higher invertebrates (Hille, 1992). A variety of voltage-gated K^+ currents have been detected in lower invertebrates including nematodes (Martin *et al.*, 1992), platyhelminths (Day *et al.*, 1993), coelenterates (Hagiwara *et al.*, 1981) and protozoa (Eckert & Brehm, 1979; Deitmer, 1989).

Several types of ion channels have been observed in *Schistosoma*. A calcium-activated K^+ channel with a large conductance (195 ps) has been measured in isolated muscle fibers (Blair *et al.*, 1991). A non-specific cation channel with high conductance has been detected in the outer tegument of the worm (Day *et al.*, 1992). Also, two types of voltage-gated K^+ channels have been observed in isolated muscle fibers (Day *et al.*, 1993). One is a delayed rectifier that shows very slow inactivation, while the other type is an A-type K^+ channel that shows very fast inactivation.

Electrophysiological studies of voltage-gated K^+ currents from lower invertebrates, including those from *Schistosoma*, have shown that these currents are essentially similar to those in higher phyla such as deuterostomes (*Chordata* and *Echinodermata*) and protostomes (*Annelid*, *Mollusc*, and *Arthropod*)(Hille, 1992). The existence of voltage-gated K^+ currents in lower invertebrates much like those of higher organisms could be due to the stable

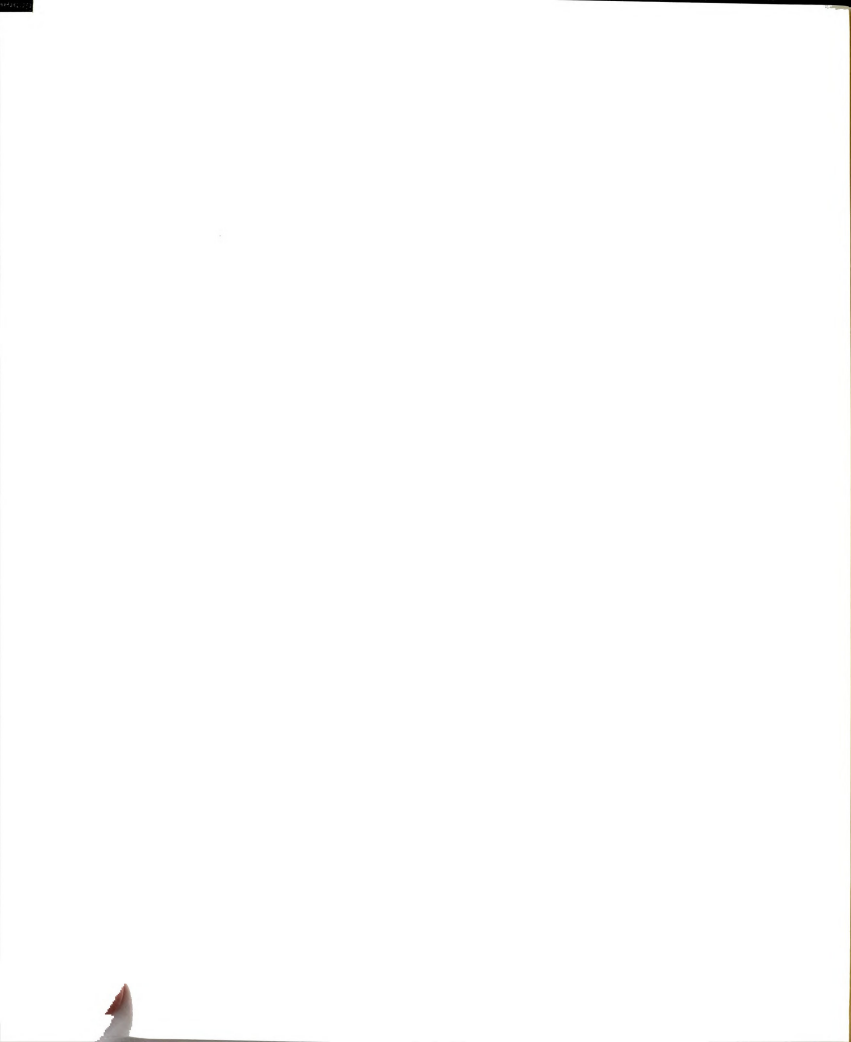
establishment of the voltage-gated *Sh* subfamilies in these organisms. However, molecular evidence for the existence of any member of *Sh* subfamilies in these more primitive phyla is limited. A *Shaw*-related K^+ channel gene has been mentioned to exist in *Caenorhabditis elegans* (Wei *et al.*, 1991), but the sequence has not been published. This lack of data is in contrast to the large number of K^+ channel genes that have been cloned and analyzed from vertebrates and higher invertebrates, such as *Drosophila*, *Aplysia* (Pfaffinger *et al.*, 1991) and leech (Johansen *et al.*, 1990).

C. Molecular biology of voltage-gated K^+ channel genes

Molecular analysis of voltage-gated K^+ channel genes started from the finding of a *Drosophila Sh* (*Shaker*) mutant that showed a violent shaking behavior when under ether anesthesia (Kaplan & Trout III, 1969). In the *Sh* mutant, nerve fibers showed an increased duration of action potentials (Tanouye *et al.*, 1986), and the neuromuscular junction exhibited prolonged neurotransmitter release (Jan *et al.*, 1977; Tanouye & Ferrus, 1985). Single channel and voltage-clamp analysis in larval muscle fibers of *Drosophila* indicated that the *Shaker* mutant has no, reduced or altered transient A-type K^+ channel current (Solc *et al.*, 1987; Wu & Haugland, 1989).

By extensive genetic analysis, several groups have independently isolated the *Shaker* channel gene (Kamb *et al.*,

1987; Papazian *et al.*, 1987; Schwarz *et al.*, 1988; Pongs *et al.*, 1988). The structure of the *Shaker* gene is roughly divided into three regions: the intracellular N- and C-terminal domains and the core region that contains six membrane spanning domains (S1-S6) and one pore forming domain (H5) (Figure 6) (Jan & Jan, 1994; Jan & Jan, 1992; Pongs, 1992). The S4 domain contains several positively charged and hydrophobic amino acid residues that are thought to be involved in voltage sensing and gating (Bezanilla *et al.*, 1991; Liman *et al.*, 1991; Logothetis *et al.*, 1992; MacCormack *et al.*, 1991; Papazian *et al.*, 1991; Stühmer *et al.*, 1991; Lopez *et al.*, 1991). The H5 domain, tucked into the membrane but not completely spanning the membrane, is involved in the formation of the pore of the channel, determining the selectivity, conductance and pharmacology (Hartmann *et al.*, 1991; Yellen *et al.*, 1991; Yool & Schwarz, 1991). The N-terminal of the *Shaker* gene has been proposed to form a ball structure for the rapid inactivation of the channel (Hoshi *et al.*, 1990; Zagotta *et al.*, 1990; Baldwin *et al.*, 1991). Also, a receptor region for the ball structure has been proposed to be located in the cytoplasmic mouth of the pore around the S4-S5 intracellular loop (Isacoff *et al.*, 1991). Post-translational modification of the extracellular loops of K^+ channels by the N-linked glycosylation has been demonstrated (Shen *et al.*, 1993; Rosenberg & East, 1992). Also, cytoplasmic loops have many



sites for phosphorylation by protein kinases such as protein kinase A, protein kinase C and tyrosine kinase.

Using the *Drosophila Shaker* channel gene as a probe, other *Shaker*-related subfamilies (*Shal*, *Shab* and *Shaw*) have also been isolated from *Drosophila* (Butler *et al.*, 1989, 1990; Wei *et al.*, 1990; Covarrubias *et al.*, 1991). These genes share about 40% amino acid sequence identity in the highly conserved core region and display distinct electrophysiological and pharmacological characteristics (Salkoff *et al.*, 1992). However, they share little sequence identity at the N- and C-terminal regions, implying functional importance of the core region in the K^+ channel function.

A functional K^+ channel is made by the assembly of four identical or different subunits. The tetrameric nature of the K^+ channel has been supported by sequence comparison to Na^+ or Ca^{2+} channels, analysis of heteromultimer made of subunits with different pharmacology (MacKinnon, 1991; Liman *et al.*, 1992) and analysis of a channel made of tandomly linked subunits (Liman *et al.*, 1992). Formation of heteromultimeric channels have been demonstrated by observing the hybrid behavior of channels when two electrophysiologically or pharmacologically distinct subunits are expressed together in *Xenopus* oocytes (Christie *et al.*, 1990; Isacoff *et al.*, 1990, Ruppersburg *et al.*, 1990; McCormack *et al.*, 1990). *In vivo* formation of

heteromultimers has been demonstrated recently by using antibodies that are specific to certain subunits (Wang *et al.*, 1993; Sheng *et al.*, 1993). However, the subunits from different subfamilies do not form heteromultimers (Covarrubias *et al.*, 1991).

After the discovery of the *Sh* subfamilies in *Drosophila*, numerous mammalian homologs have been isolated. Analysis of mammalian homologs revealed the presence of more than one member in a given subfamily, indicating the expansion of each subfamily by gene duplication during mammalian evolution. The plethora of mammalian K^+ channel genes has led to a suggestion for a standardized system of nomenclature (Chandy *et al.*, 1991; Gutman & Chandy, 1993). This nomenclature assigns names in the form of 'Kv3.1', in which 'K' means a K^+ channel, 'v' means voltage-gated and '3.1' means the first member of third group (Shaw-related).

Since K^+ channels are very diverse (Rudy, 1988), many mechanisms for the generation of diverse K^+ channels have been suggested. More than ten different K^+ channels can be generated by alternative splicing in the *Drosophila Shaker* channel (Iverson and Rudy, 1990; Jan and Jan, 1990; Kamb *et al.*, 1988; Schwarz *et al.*, 1988; Stocker *et al.*, 1990; Timpe *et al.*, 1988; Timpe *et al.*, 1988). Other mechanisms for the generation of diverse channels are the presence of subfamilies (Wei *et al.*, 1990), gene duplication within a subfamily in mammalian K^+ channels (Stühmer *et al.*, 1989),

the formation of heteromultimers (Isacoff *et al.*, 1990; MacKinnon *et al.*, 1991; McCormack *et al.*, 1990; Ruppersberg *et al.*, 1990), the density of channels in the membrane (Guillemare *et al.*, 1992; Honoré *et al.*, 1992; Moran *et al.*, 1992) and the presence of a K^+ channel ß subunit (Scott *et al.*, 1994; Rettig *et al.*, 1994).

The activity of voltage-gated K^+ channels are regulated by a variety of protein kinases. The fast inactivation of the *Shaker* channel is accelerated by protein kinase A and slowed by phosphatase (Drain *et al.*, 1994). Phorbol ester, an activator of protein kinase C, has been reported to suppress several K^+ currents (Attali *et al.*, 1992; Payet & Dupuis, 1992; Aiyar *et al.*, 1993). The phosphorylation site for protein kinase C has been found in almost all voltage-gated K^+ channels. Recently, a tyrosine kinase-dependent suppression of a delayed rectifier K^+ channel by the G protein-coupled m1 muscarinic acetylcholine receptor has been reported, indicating the presence of a novel mechanism by which neurotransmitters can modulate membrane excitability (Huang *et al.*, 1993).

Since there are multiple members of mammalian K^+ channels in each subfamily, temporal and tissue-specific regulation of expression of these channels has become an important topic in mammalian K^+ channel research (Drewe *et al.*, 1992). Modulators that have been reported to regulate the expression of K^+ channels include the H-*ras* oncogene

(Hemmick *et al.*, 1992; Perney *et al.*, 1992), depolarizing pulses (Perney & Kaczmarek, 1993), basic fibroblast growth factor (Perney & Kaczmarek, 1993), dexamethasone (Levitan *et al.*, 1991; Takimoto *et al.*, 1993), cAMP (Mori *et al.*, 1993), pentylenetetrazole (Tsaur *et al.*, 1992), nerve growth factor (Rudy *et al.*, 1992), heat shock or free radicals (Kuo *et al.*, 1992).

D. Significance of molecular analysis of a K^+ channel in *S. mansoni*

Molecular cloning of an ion channel allows sequence comparison among channels from *Schistosoma* and the host, which helps to assess the potential of the gene as a novel target for drug development for schistosomiasis. It also makes it possible to attempt drug screening by stably expressing the channel in a heterologous system such as an animal cell line.

Expression of the gene in a heterologous system provides a homogeneous population of the channel, which is difficult to obtain in conventional cell preparations due to contaminated currents. It also permits the study of K^+ channels that are not accessible by conventional methods due to the small size of cells and the difficulty of isolating intact cells, which is true for the neurons of *Schistosoma*.

Modulation of the channel can be studied. Putative modulation sites by protein kinases can be identified from

the amino acid sequence of the clone and expression and modulation analysis of the clone in a heterologous system can confirm the modulation. Furthermore, modification of the motifs in K^+ channel recognized by protein kinases by site-directed mutagenesis can provide convincing evidence.

Molecular analysis of the K^+ channel provides useful information for the correct classification of K^+ channels. Conventionally, voltage-gated outward K^+ currents have been classified into two groups, the rapidly inactivating (A-type) current and the non-inactivating (delayed rectifier) current. However, the molecular nature of the K^+ channel genes that underlie diverse potassium currents were not understood until the amino acid sequences of the *Shaker* and related genes became available.

Mechanisms for the generation of diverse K^+ channels *in vivo* can be studied by molecular analysis. The presence of more than eight different K^+ channels in cardiac cells (Carmeliet, 1989) is a simple example of the diversity of the K^+ channels. Little is known about the diversity of K^+ channels and the mechanisms underlying the diversity in *Schistosoma* and other lower invertebrates. However, once a K^+ channel gene becomes available, molecular analyses such as Southern and Northern hybridization and the cloning of related genes, will make it much easier to address questions on diversity.

Amino acid comparison among schistosome K^+ channels and

other K^+ channels also provides useful information on the evolution of ion channels. Most of the *Sh* K^+ channel genes have been isolated from higher organisms such as frog, mouse, rat, human and plant (*Arabidopsis*). In invertebrates, *Sh*-related K^+ channels have been cloned only from higher invertebrates such as *Drosophila* and *Aplysia* (Pfaffinger *et al.*, 1991; Quattrocki *et al.*, 1994). Therefore, there is no molecular evidence to answer questions, for instance, about when the gene duplication event that generated the four *Sh* subfamilies happened.

Immunological analysis of the K^+ channel has been used to study the *in vivo* distribution of the channels (Hwang *et al.*, 1993; Schwarz *et al.*, 1990). An antibody specific only to a certain subtype or to one of the alternatively spliced products can be raised by carefully designing a synthetic peptide based on the amino acid sequence of K^+ channels. Recently, immunohistochemical evidence for the segregation of two different K^+ channels at the subcellular level has been reported (Sheng *et al.*, 1992). Also, the formation of heteromultimers *in vivo* has recently been reported by using subunit specific antibodies (Sheng *et al.*, 1993; Wang *et al.*, 1993).

Expression of a cloned K^+ channel of *Schistosoma* in a heterologous system, frog oocytes or mammalian cell lines, would be a good model system for attempting the expression cloning of ion channels or receptor genes of *Schistosoma*.

The expression cloning technique has recently become a useful tool to isolate genes with very low homologies to the analogous genes of other species. *Schistosoma*, as one of the most primitive animals, is expected to have many genes with low homologies to the others, which makes it difficult to clone genes based on the PCR based cloning. A fundamental requirement for successful expression cloning is that the genes or mRNAs introduced into the heterologous system should be functionally expressed. To date, there have been few attempts to functionally express schistosomal mRNAs in heterologous systems such as *Xenopus* oocytes (Skelly *et al.*, 1993), monkey kidney cell (Hong *et al.*, 1993) and in an *in vitro* translation system (Crews and Yoshino, 1991; Eschete and Bennett *et al.*, 1990; Felleisen and Klinkert, 1990). Therefore, the expression of the K^+ channel in a heterologous system will provide useful information for future attempts to express schistosomal genes or mRNAs in heterologous system for expression cloning.

OBJECTIVES

Since voltage-gated K^+ channels have been observed in isolated muscle fibers of *S. mansoni*, the genes for the K^+ channels are expected to exist in the schistosome. The existence of these genes permit several questions: (1) How primitive are they? (2) How diverse are they? (3) How different are they compared to those of human host? (4) What are their functions?

There are three major goals of the present study: (1) understanding of the evolution of voltage-gated K^+ channel genes (2) assessment of a voltage-gated K^+ channel protein as a target protein for drug development for schistosomiasis (3) understanding of the functions of voltage-gated K^+ channel proteins in *Schistosoma*.

Firstly, determination of the structure of voltage-gated K^+ channels of *S. mansoni* will provide useful information about the evolution of voltage-gated K^+ channels and should help to answer the following questions. When did the gene duplication event that generated the four *Sh* subfamilies occur? How diverse are K^+ channel genes in lower invertebrates? Are the mechanisms that generate diverse K^+ channels in higher invertebrates or vertebrates the same as those in lower invertebrates? Since no structural information is available on any type of ion channel in lower invertebrates, results of the present study will provide the first molecular evidence on these

questions.

Secondly, the cloning of a K^+ channel gene is important for drug development for schistosomiasis. Historically, the nervous and neuromuscular system of helminths have been important targets for drug development (Geary *et al.*, 1992). K^+ channels are one of the most important components in the nervous and neuromuscular system. Cloning of a K^+ channel gene initially proves the presence of the channel. It also allows a direct amino acid sequence comparison between those from *Schistosoma* and host in addition to providing practical tools to try massive drug screening by stably expressing the gene in a heterologous system.

Thirdly, results of the present study will provide useful information on the functions of voltage-gated K^+ channel proteins in *S. mansoni*. Voltage-gated K^+ channels modulate the excitability of many cells, being tightly regulated by a variety of neurotransmitters and neuropeptides. Therefore, in *Schistosoma*, K^+ channels are expected to be involved in many physiological processes such as the sensory and motor system, digestion, reproduction and development. The electrophysiology and immunolocalization of the channel will help to understand the diverse functions of K^+ channels in *Schistosoma*.

Specifically, the first goal will be an isolation of a *Shaker*-related voltage-gated K^+ channel gene from *Schistosoma mansoni*. The sequence of the gene will be

determined, analyzed and compared to other K^+ channel genes. The presence of alternatively transcribed transcripts of the gene will be tested by restriction mapping and sequencing of several clones as well as Northern blot analysis. Southern blot analysis will be employed to study the presence of multiple copies of the gene. For a better understanding of the function of the channel gene, functional expression in *Xenopus* oocyte and electrophysiological characterization of the K^+ channel gene will be attempted. Also, the distribution of the channel by the use of channel-specific antibody will be examined.

MATERIALS AND METHODS

I. Molecular cloning

A. Screening of a DNA fragment of a K^+ channel gene by PCR

Poly(A^+) RNA was purified from adult *S. mansoni* using the Fast Track mRNA isolation kit from Invitrogen, and used to make primary cDNA for PCR. Two oligonucleotide primers were designed and synthesized based on multiple sequence alignment of other *Shaker* K^+ channels: primer 1 (aa 387-393 in Figure 3), 5'-ACNGTNGGNTA(T/C)GGNGA(T/C)AT-3'; primer 2 (aa 425-431 in Figure 3), 5'-TC(G/A)TA(G/A)AA(G/A)TA(G/A)TT(G/A)AA(G/A)TT-3'. The first-strand cDNA was used for PCR in which 40 cycles of 40 sec at 94 °C, 2 min at 46 °C and 2 min at 72 °C were used. The condition for PCR was initially set by Oligo 4.0 program and optimized empirically. After visualization and elution on agarose gel, DNA fragments were subcloned into pCRII vector (Invitrogen) and sequenced.

B. Screening of *S. mansoni* cDNA library

Two non-degenerate inner oligonucleotides were synthesized using the sequence information from the amplified PCR fragment: primer 3 (nt 1526-1545 in Figure 3), 5'-ATGCGACCGGTTACTGTATG-3'; primer 4 (nt 1595-1614 in Figure 3), 5'-ATCACAGGAACAGGAAGTGC-3'. Chemiluminescent probes (Genius kit, Boehringer Mannheim) for screening of the cDNA library were prepared by random primed labeling or

by PCR (Lanzillo, 1990) using the two inner oligonucleotides. The cDNA library of adult *S. mansoni* (Davis *et al.*, 1988) was kindly provided by Dr. Davis at San Francisco State University. PCR with the two inner oligonucleotides was used as a first step to screen positive plates (Yu & Bloem, 1993). The positive plates were then used to isolate positive plaques by *in situ* hybridization. The plaque lift was incubated in 5x SSC/ 1% (w/v) blocking reagent (BM, Indianapolis)/ 0.1% N-lauroylsarcosine/ 0.02% SDS at 56 °C overnight and washed twice, 15 min per wash, with 2x SSC containing 0.1% SDS at room temperature followed by two final washing in 0.5x SSC containing 0.1% SDS at 58 °C for 15 min.

C. Subcloning and sequence determination

Lambda gt11 forward and reverse primers were used to amplify cloned genes from positive lambda phages. The amplified PCR products were ligated to pCRScript (Stratagene) or pCRII (Invitrogen) and used for restriction mapping and sequence analysis. Nested deletions of the m13 single strands containing the clones in both directions were generated for sequence determination (Shen & Waye, 1988). Sequence analyses were carried out using the Genetics Computer Group software.

D. Northern and Southern blot analyses

Three micrograms of poly(A^+) RNA from adult worms were resolved on a 1% formaldehyde gel, blotted to a positively charged nylon membrane. An *in vitro* transcribed message (1 ng) of SKv1.1 was used as a positive control. An SKv1.1-specific probe corresponding to the 3'-untranslated region of the cDNA (nt 2033-2703 in Figure 3) for both Southern and Northern blot was generated by PCR and labeled with [α-^{32}P] dCTP by the random primer DNA labeling method. We also made a bigger probe containing the whole region of SKv1.1 for Northern blot to investigate a possibility of alternative splicing. The membrane was hybridized at 42 °C in 5x SSC/ 2% blocking reagent/ 0.1% N-lauroylsarcosine/ 0.02% SDS/ 50% formamide and washed in 0.5x SSC/ 0.1% SDS at 50 °C. For Southern blot analysis, 10 μg of genomic DNA were restricted with *Eco*RI or *Hin*dIII and resolved on a 0.8% agarose gel and blotted on membrane. The membrane was hybridized at 65 °C in 5x SSC/ 1% blocking reagent/ 0.1% N-lauroylsarcosine/ 0.02% SDS and washed in 0.5x SSC/ 0.1% SDS at 50 °C.

II. Electrophysiology

A. *In vitro* transcription

In order to increase the efficiency of expression, the pBTG plasmid containing the 5'- and 3'-untranslated regions of the β-globin gene of *Xenopus* was used to make SKv1.1 mRNA (Guillemare *et al.*, 1992). Also, a sequence of six nucleotides (GCCACC) that is highly conserved in the region

around the translational start sites of eukaryotic mRNAs was added in the upstream region (nt -6 to -1) of the start codon of SKv1.1 (Kozak, 1991). A change of a nucleotide, T to G at nt +4 in the coding region resulting in Ser to Ala substitution of the second amino acid residue, was made in order to increase the efficiency of translation (Kozak, 1991). The coding sequence of SKv1.1 was amplified by PCR using two primers containing a *Bgl*II restriction site at the 5'-end: primer 5, 5'-GGAGATCTGCCACCATGTCCACCTTATCAGGAACAGC-3'; primer 6, 5'-CCAGATCTTTACTTTTTTATTGTAATGGAAT-3'. The PCR product was digested with *Bgl*II and inserted into the pBTG to make pBTGSK1. The pBTGSK1 was linearized with *Bam*HI and used for *in vitro* transcription by T3 RNA polymerase to make capped mRNA for injection.

B. Oocyte injection and recording

Mature female *Xenopus laevis* were anesthetized in 0.15% 3-aminobenzoic acid ethyl ester followed by surgical removal of ovarian lobes. After incubation of segments of lobes in Ca^{2+}-free ND-96 containing 2 mg/ml collagenase (Sigma, type II) for 30 min, individual oocytes were manually removed from the follicle cell sac. Mature oocytes at stage V and VI (Dumont, 1972) were used for injection and incubated in ND-96 for 48 hours before recording.

Each oocyte was injected with 50 nl of mRNA (0.1 μg/μl) and maintained in ND-96 supplemented with horse serum (Quick

et al., 1992) for two days before recording. Oocyte currents were recorded using the two-electrode voltage-clamping method (model OC-725B oocyte clamp, Warner Instrument Corp.). Both electrodes (1-2 MΩ) were filled with 3 M KCl and used to impale oocytes in ND-96. Stimulation, data acquisition and analyses were done using pClamp 5.5 software (Axon Instruments).

III. Immunohistochemistry

A. Antibody production

A synthetic oligopeptide (22 amino acid residues, LETDDDDITNKEQHINTKVRC) was made based on the sequence of the cytoplasmic side of the protein, aa 447-466 of SKv1.1 (512 aa and M.W.=56.5 kDa). The last amino acid (Cys), which is not in the original sequence, was added to facilitate the linking of the peptide to the carrier protein. The synthetic oligopeptide was coupled to keyhole limpet hemocyanin (KLH) using the NHS-Ester-Maleimide heterobifunctional crosslinker (Sulfo-MBS, Pierce) and injected into rabbits. Rabbits received 500 μg of the peptide-carrier conjugate followed by booster injections of 250 μg every three weeks. Test serum was collected 10 days after each booster injection and tested with a dot hybridization using the BSA-peptide conjugate as a target antigen.

An affinity column was used to isolate the SKv1.1-

specific antibody from the antiserum. The synthetic peptide was immobilized on a SulfoLink Gel affinity column (Pierce) and used to isolate peptide-specific antibody from the rabbit serum. The eluted antibody was named as ASK1 and dissolved in PBS at the concentration of 0.66 mg/ml.

For control reaction, the IgG fraction was isolated from preimmune serum using a protein A and protein G agarose column (Sigma). The concentration of preimmune IgG was 1.45 mg/ml.

B. *In vitro* translation and immunoprecipitation

A SKv1.1 protein, labeled with [^{35}S] methionine, was made using rabbit reticulocyte lysate (BRL) and *in vitro* transcribed mRNA from pBTGSK1. The SKv1.1 protein (5 μl) was diluted 25-fold in 1% Triton buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mg/ml bovine serum albumin) and incubated with ASK1 (3 μg/ml) for 1 hr at 0 °C. The antigen and antibody complex was precipitated by adding 10 μl of protein A sepharose (BRL) and incubating for 1 hr at room temperature. Immunoprecipitates were resolved on 10% SDS polyacrylamide gel. For control reaction, ASK1 was preincubated with an excess amount of free peptide for 1 hr before immunoprecipitation.

C. Isolation of muscle fibers

Muscle fibers were isolated from adult *S. mansoni* (Puerto Rican Strain) by the method described by Day *et al.* (1993). Adult female and male worms (20-30 pairs) were coarsely minced with a razor blade. The small pieces were incubated in the Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St. Louis) supplemented with 1 mM EGTA, 1 mM EDTA, 0.1% bovine serum albumin and 1 mg/ml papain (Sigma) at 37 °C for 45 min. The worm pieces were then incubated in the papain-free supplemented DMEM for 10 min, followed by pipetting up and down 75 to 100 times to break up the pieces into a cell suspension.

D. Immunohistochemistry

Isolated muscle fibers were attached to glass slides preincubated with 1 mg/ml poly-L-lysine, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min and permeabilized in 0.2% Triton X-100 in PBS for 10 min. The permeabilized muscle fibers were then incubated with ASK1 (3 μg/ml) in PBS containing 3% bovine serum albumin (BSA) for 20 min at room temperature followed by washing three times in PBS, 1% Triton X-100 for 5 min. For visualization of immunoreactivity, a goat anti-rabbit secondary antibody conjugated with, a red fluorescence dye, Cy3 (Jackson Immuno Research) was used at a dilution of 1:1000. After incubating with the secondary antibody in PBS containing 3% BSA for 30 min at room temperature, the muscle

fibers were washed three times in PBS, 1% Triton X-100 and observed on fluorescent microscope.

For immunohistochemistry of paraffin sections, a modified method described by Basch and Gupta *et al.* (1990) was used. Adult worm pairs were fixed in 10% neutral buffered formalin (NBF) for 3 hr at room temperature. Specimens were embedded in paraffin, and 2 μm sections were obtained on poly-L-lysine-coated slide glasses. The sections were de-paraffinized by incubating in xylene for 20 min and rehydrated by incubating in 100% and 95% ethanol. After equilibration in PBS containing 0.3% Triton X-100 (PBT) for 20 min, slides were blocked by incubating in PBT containing 5% normal goat serum and 0.1% bovine serum albumin for 20 min. Specimens were incubated in primary ASK1 antibody (3 μg/ml) in PBT containing 2% normal goat serum and 0.1% bovine serum albumin for 30 min at room temperature followed by washing in PBT for 10 min. The sections were again incubated with a secondary goat anti-rabbit antibody conjugated with Cy3 (1:1000 dilution) in PBT containing 2% normal goat serum and 0.1% bovine serum albumin for 30 min at room temperature followed by washing in PBT for 10 min.

RESULTS

I. Molecular cloning

A. Molecular cloning of a Shaker-related K^+ channel cDNA

A DNA fragment with high sequence identity to other *Shaker* channels was isolated by PCR using two oligonucleotides based on highly conserved regions (H5 and S6) of other *Shaker* channels and using primary cDNA from schistosomal poly(A^+) RNA as a template. Southern hybridization of the DNA fragment to genomic DNA of *S. mansoni* indicated the PCR fragment (134 bp, nt 1508-1641 in Figure 3) was from schistosomal cDNA. Also, the codon usage table of the PCR fragment fit well with the typical codon usage of schistosomal cDNA, which is highly biased (Meadows & Simpson, 1989). The G+C mol% of the PCR fragment was 41%, close to the typical G+C mol% (40%) of the schistosomal cDNAs. Two non-degenerate inner oligonucleotides, based on the sequence of the PCR fragment, were used to generate a K^+ channel-specific probe (89 bp, nt 1526-1614 in Figure 3) for cDNA library screening.

A total of six full length DNAs (clones 9, 30, 31, 56, 67 and 77) were isolated after screening of 8 x 10^5 plaques. The sizes of the inserts of the six clones were very similar, ranging from 2.7 - 2.8 kb. All six had identical restriction patterns for *Hin*dIII and *Vsp*I except for small differences in length at the 5'-ends. In order to differentiate the clones and to investigate the possibility

of alternative splicing, we subcloned and sequenced the three clones that showed different restriction patterns (clones 56, 67 and 77). The results indicated that clone 56 and 67 were identical, but that clone 77 contained an extra stretch of about 100 nt at the 5'-end, which could be due to multiple transcriptional initiation of the SKv1.1 gene. The longest cDNA (clone 77) was named SKv1.1 and used for further analyses (Figure 2).

Figure 2. Restriction map of SKv1.1 gene. Sites for restriction endonucleases are marked by vertical bars. A black box in the middle of the gene indicates the putative coding region of SKv1.1. 5' and 3' untranslated regions are shown as white boxes on both ends.

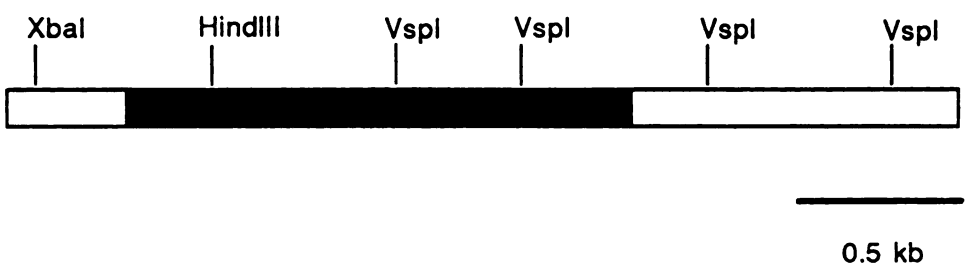
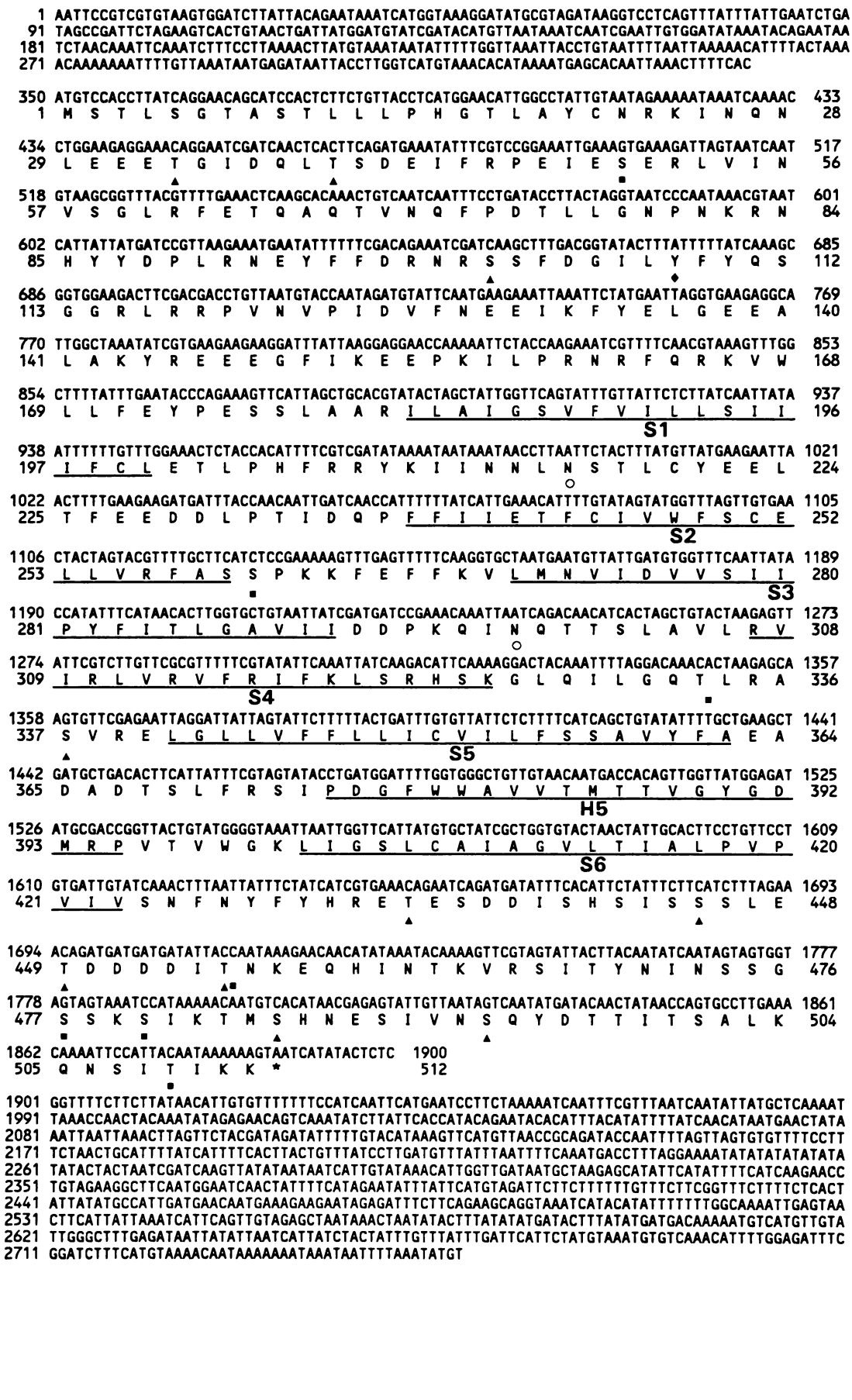

XbaI
HindIII
VspI
VspI
VspI
VspI
0.5 kb

Figure 3. Nucleotide sequence and predicted amino acid sequence of SKv1.1. The deduced amino acid sequence is shown below the nucleotide sequence. The numbers of the nucleotides and amino acids are given on both side of the sequence. The termination codon at the end of the open reading frame is marked by an asterisk. Six putative membrane spanning regions (S1 - S6) and the pore-forming region (H5) are underlined. Putative N-linked glycosylation sites are indicated under the corresponding amino acids by open circles (o). Putative sites for phosphorylation by protein kinases are also indicated under the corresponding amino acids: protein kinase C (■); casein kinase II (▲); tyrosine kinase (♦). The nucleotide sequence has been submitted to the GenBank™/EMBL Data Bank with the accession number L26968.


S1
S2
S3
S4
S5
H5
S6

B. Sequence analysis of SKv1.1

The full nucleotide (nt) sequence of SKv1.1 is shown in Figure 3. The start and stop codons of the structural gene were assigned by primary sequence, amino acid sequence comparison, codon preference analysis and *in vitro* translation analysis using rabbit reticulocyte lysate (Figure 15A). The region a few nt upstream from the ATG start codon did not conform to the consensus sequence of other eukaryotic cDNAs (Kozak, 1991). Also, the last three amino acids of SKv1.1 (Ile-Lys-Lys) did not agree with the residues (Thr-Asp-Val) that are commonly found at the carboxyl terminal end of the voltage-gated K^+ channels from *Aplysia*, *Drosophila* and mammals. The open reading frame of SKv1.1 (nt 350-1888) encoded a protein of 512 aa with a calculated molecular weight of 56.5 kDa, and it was placed between the 5'-untranslated region (nt 1-349) and a long 3'-untranslated region (nt 1889-2757) containing two poly(A^+) signals (nt 2729-2734 and 2737-2742). The G+C mol% of the structural gene and of the flanking non-coding regions were 32% and 25%, respectively, which is lower than the average value of 40% and 30% in schistosomal cDNA genes (Meadows & Simpson, 1989). Therefore, the third bases of codons in the channel are thought to be occupied preferentially by nucleotide A or T.

SKv1.1 showed very high overall aa sequence identity with *Shaker* channels from invertebrates and vertebrates

(Table 1), for instance, showing 64% sequence identity to the *Shaker* K^+ channel (Ak01a) from *Aplysia* (Figure 4). The sequence identity was even higher in the core region including the six membrane spanning regions, ranging from 64-70% when compared to other *Shaker* channels from *Xenopus*, *Drosophila*, mouse, rat and human (Table 1). However, at the N-terminal and C-terminal ends, SKv1.1 showed little homology to other *Shaker* channels. Higher sequence similarity in the core region, not at the N- and C-terminal ends, is commonly observed amongst other *Shaker* channels, reflecting essential functions of various domains such as pore formation and voltage sensing. Interestingly, the highest sequence identity was observed in the region that is involved in the tetramerization of four subunits to form a functional K^+ channel (Li *et al.*, 1992; Shen *et al.*, 1993). This indicates that the domain for subunit assembly is as important as other domains involved in K^+ channel functions.

When SKv1.1 was compared to other *Sh* subfamilies such as *Shab*, *Shal* and *Shaw* from a variety of species, much lower sequence identities (40-50%) were observed in the core region (Table 2). This is consistent with the notion that there is about 70% sequence identity in the core region within a given subfamily (e.g. *Shaker*) while only about 40% sequence identities are observed among different subfamilies (*Shaker*, *Shab*, *Shal* and *Shaw*) (Salkoff *et al.*, 1992). In addition, the sequence comparison of the tetramerization

domain of SKv1.1 to other *Sh* K^+ channels exhibited much lower sequence identities (15-38%) supporting the fact that heteromultimers are not formed between subunits from different *Sh* subfamilies.

A dendrogram was constructed based on multiple sequence alignment of SKv1.1 and other K^+ channel genes (Figure 5). SKv1.1 was clearly grouped in the *Shaker* subfamily, not in any of the other *Sh* subfamilies. Also, within the *Shaker* subfamily, SKv1.1 formed a unique branch, which is in good accordance with the fact that schistosomes are among the most primitive organisms in the animal kingdom and are quite different from vertebrates and other highly developed invertebrates such as flies and snails.

Structural features that are commonly found in other voltage-gated K^+ channels were observed in the deduced amino acid sequence of SKv1.1. Six membrane-spanning domains (S1-S6) and one pore-forming domain (H5) were identified based on the hydropathy profile of SKv1.1 (Figure 6)(Kyte & Doolittle, 1982). Seven basic amino acids occurring at every third position were found in the S4 segment (Figure 6B and Table 3), which is thought to be involved in voltage sensing and gating current. A typical leucine-heptad, with the occurrence of a leucine residue at every seventh position, was found in the region spanning the S4 domain, S4-S5 loop and S5 domain (Table 4). An arginine residue, Arg394, is located around the outer mouth of the pore-

forming domain (Figure 7 and Table 5), predicting high resistance of SKv1.1 to external tetraethylammonium (TEA^+) (Kavanaugh *et al.*, 1991; MacKinnon & Yellen). Some positively charged amino acids and hydrophobic leucines were found at the N-terminal end implying the rapid inactivation of the SKv1.1 channel (Figure 8A). In the S4-S5 loop, the five important residues that have been suggested to form a putative receptor for the inactivation ball structure (Isacoff *et al.*, 1991) were well conserved compared with other *Sh* subfamilies (Figure 8B).

Two potential N-linked glycosylation sites (Asn216 and Asn298) were located in the extracellular loops (S1-S2 and S3-S4). A N-myristoylation site was detected at the N-terminal end (Gly6) of the channel. Several putative sites for phosphorylation by a variety of protein kinases were located on the intracellular side of the channel (Figure 3): seven sites for protein kinase C, ten sites for casein kinase II and one site for tyrosine kinase. However a site for phosphorylation by cAMP-dependent protein kinase A, which has been found in Ak01a of *Aplysia* (Pfaffinger *et al.*, 1991), is not seen in SKv1.1.

Table 1. Amino acid sequence identity between SKv1.1 and other *Shaker* K^+ channels.

	Overall (aa 1-512)	Core region (aa 182-423)	Tetramerization domain (aa 51-181)
Vertebrate			
Kv1.1	54	65	76
Kv1.2	54	65-66	75-76
Kv1.3	54	65-66	73
Kv1.4	52-55	63-66	72-73
Kv1.5	55-58	56	72-74
Kv1.6	57	56	69-71
Kv1.7	59	69	73
Invertebrate			
Shaker Al	59	69	78
Ak01a	64	74	84

Figure 4. Amino acid sequence comparison between SKv1.1 and Ak01a from *Aplysia* (Pfaffinger *et al.*, 1991). Putative membrane spanning domains (S1-S6) and the pore forming domain (H5) are underlined. Vertical lines indicate identical amino acids between the two sequences. Gaps introduced to maximize the alignment are indicated by periods.

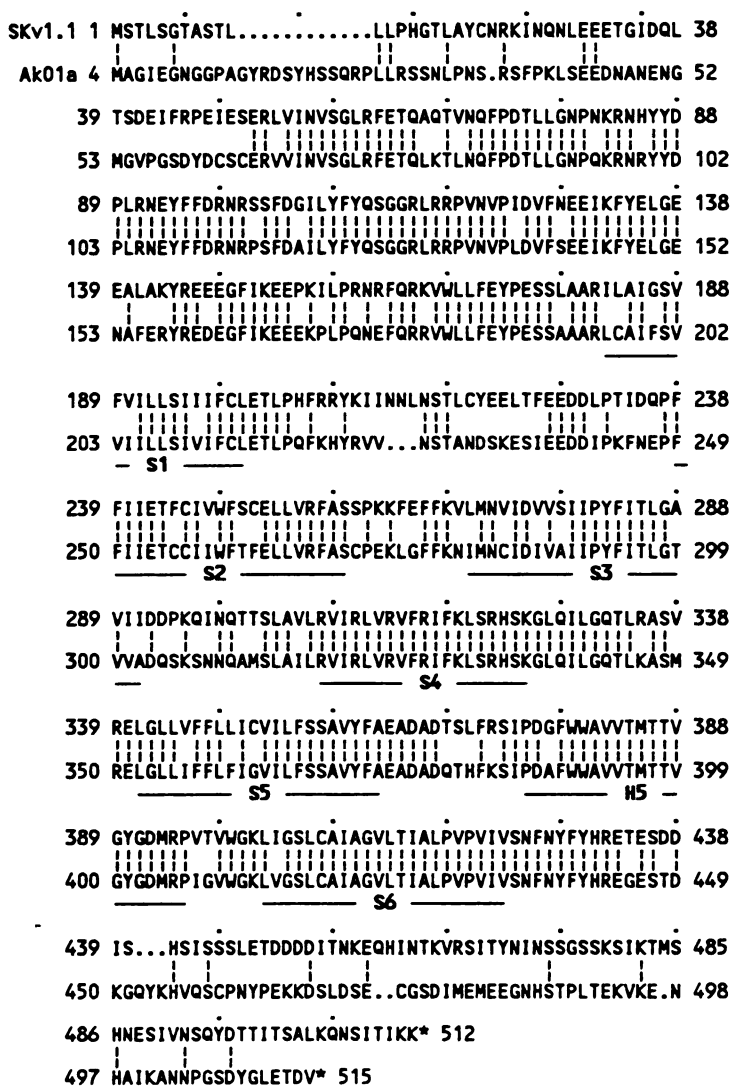

SKv1.1 1 MSTLSGTASTL.....LLPHGTLAYCNRKINQNLEEETGIDQL 38
Ak01a 4 MAGIEGNGGPAGYRDSYHSSQRPLLRSSNLPNS.RSFPKLSEEDNANENG 52
39 TSDEIFRPEIESERLVINVSGLRFETQAQTVNQFPDTLLGNPNKRNHYYD 88
53 MGVPGSDYDCSCERVVINVSGLRFETQLKTLNQFPDTLLGNPQKRNRYYD 102
89 PLRNEYFFDRNRSSFDGILYFYQSGGRLRRPVNVPIDVFNEEIKFYELGE 138
103 PLRNEYFFDRNRPSFDAILYFYQSGGRLRRPVNVPLDVFSEEIKFYELGE 152
139 EALAKYREEEGFIKEEPKILPRNRFQRKVWLLFEYPESSLAARILAIGSV 188
153 NAFERYREDEGFIKEEEKPLPQNEFQRRVWLLFEYPESSAAARLCAIFSV 202
189 FVILLSIIIFCLETLPHFRRYKIINNLNSTLCYEELTFEEDDLPTIDQPF 238
203 VIILLSIVIFCLETLPQFKHYRVV...NSTANDSKESIEEDDIPKFNEPF 249
S1
239 FIIETFCIVWFSCELLVRFASSPKKFEFFKVLMNVIDVVSIIPYFITLGA 288
250 FIIETCCIIWFTFELLVRFASCPEKLGFFKNIMNCIDIVAIIPYFITLGT 299
S2
S3
289 VIIDDPKQINQTTSLAVLRVIRLVRVFRIFKLSRHSKGLQILGQTLRASV 338
300 VVADQSKSNNQAMSLAILRVIRLVRVFRIFKLSRHSKGLQILGQTLKASM 349
S4
339 RELGLLVFFLLICVILFSSAVYFAEADADTSLFRSIPDGFWWAVVTMTTV 388
350 RELGLLIFFLFIGVILFSSAVYFAEADADQTHFKSIPDAFWWAVVTMTTV 399
S5
H5
389 GYGDMRPVTVWGKLIGSLCAIAGVLTIALPVPVIVSNFNYFYHRETESDD 438
400 GYGDMRPIGVWGKLVGSLCAIAGVLTIALPVPVIVSNFNYFYHREGESTD 449
S6
439 IS...HSISSSLETDDDDITNKEQHINTKVRSITYNINSSGSSKSIKTMS 485
450 KGQYKHVQSCPNYPEKKDSLDSE..CGSDIMEMEEGNHSTPLTEKVKE.N 498
486 HNESIVNSQYDTTITSALKQNSITIKK* 512
497 HAIKANNPGSDYGLETDV* 515

Table 2. Amino acid sequence identity of SKv1.1 to other *Sh* K^+ channels.

	Overall (aa 1-512)	Core region (aa 182-423)	Tetramerization domain (aa 51-181)
Vertebrate			
Shab			
Kv2.1	30	40	15
Shaw			
Kv3.1	24	43	27
Kv3.2	27	45	38
Kv3.3	25	45	25
Kv3.4	24-25	43	36
Shal			
Kv4.1	30	36	34
Kv4.2	29	37	31
Invertebrate			
dro*Shab*	35	46	33
dro*Shaw*	26	46	26
dro*Shal*	34	40	38

Figure 5. Dendrogram analysis of SKv1.1. The amino acid sequences of SKv1.1 and other *Sh* K^+ channels were clustered by the unweighted pair-group method using arithmetic average (UPGMA) described by Sneath & Sokal (1973). Each pairwise alignment was done by the method of Needleman & Wunsch (1970). The 'pileup' program of the Genetics Computer Groups software was used to align amino acid sequences and to generate the dendrogram. Standard names of K^+ channels (Chandy, 1991) followed by source organisms and gene names are given on the right side of the dendrogram. The standard name for *Drosophila Shaker* K^+ channels is not available. References: RCK1 (Baumann *et al.* 1988); RCK2 (Grupe *et al.* 1990); RCK3, RCK4 and RCK5 (Stühmer *et al.* 1989); KV1 (Swanson *et al.* 1990); DRK1 (Frech *et al.* 1989); Kv4 (Luneau *et al.* 1991); RK5 (Roberds & Tamkun, 1991).

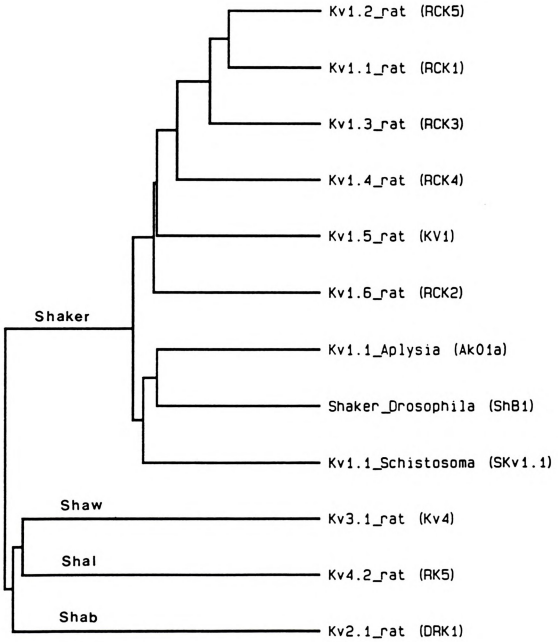
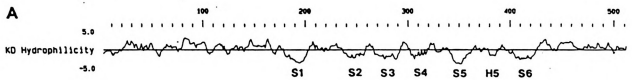

Kv1.2_rat (RCK5)
Kv1.1_rat (RCK1)
Kv1.3_rat (RCK3)
Kv1.4_rat (RCK4)
Kv1.5_rat (KV1)
Kv1.6_rat (RCK2)
Shaker
Kv1.1_Aplysia (Ak01a)
Shaker_Drosophila (ShB1)
Kv1.1_Schistosoma (SKv1.1)
Shaw
Kv3.1_rat (Kv4)
Shal
Kv4.2_rat (RK5)
Shab
Kv2.1_rat (DRK1)

Figure 6. (A) Hydrophilicity profile of SKv1.1 generated by the method of Kyte and Doolittle (1982). Six hydrophobic membrane spanning domains (S1-S6) and a pore-forming domain (H5) are indicated. (B) Putative membrane topology of the SKv1.1 K^+ channel based on the hydrophilicity profile of SKv1.1.


A
100
200
300
400
500
5.0
KD Hydrophilicity
-5.0
S1
S2
S3
S4
S5
H5
S6

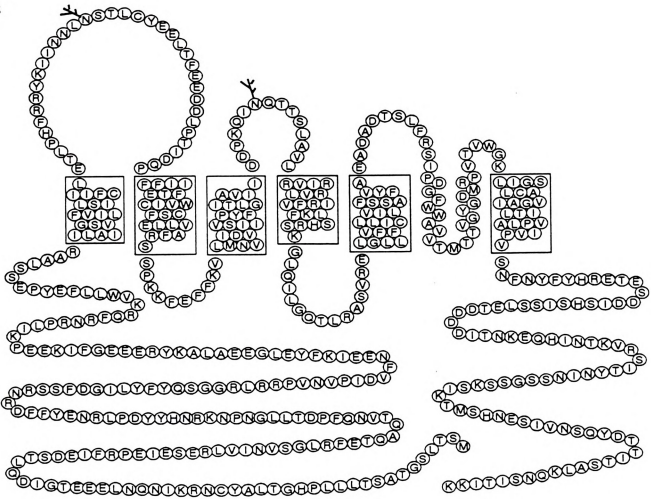

B

Table 3. Comparison of amino acid sequence of the S4 segment in SKv1.1 to other voltage-gated ion channels. References: *Drosophila* K^+ channels (Kamb *et al.*, 1988; Schwarz *et al.*, 1988, Wei *et al.*, 1990), sodium channel (Noda *et al.*, 1984), calcium channel (Tanabe *et al.*, 1987).

K channels																			
SKv1.1	R	VI	R	LL	R	VF	R	IF	K	LS	R	HS	K	GL
Shaker	R	VI	R	LV	R	VF	R	IF	K	LS	R	HS	K	GL
Shab	Q	VF	R	IM	R	IL	R	VL	K	LA	R	HS	T	GL
Shaw	E	FF	S	II	R	IM	R	LF	K	VT	R	HS	S	GL
Shal	F	VT	R	VF	R	VF	R	IF	K	FS	R	HS	Q	GL
Na channel	S	AL	R	TF	R	VL	R	AL	K	TI	S	VI	P	GL
Ca channel	S	VL	R	CI	R	LL	R	LF	K	IT	K	YW	T	SL

Table 4. Leucine-zipper motifs in SKv1.1 and other *Sh* K^+ channels.

	L1			L2			L3			L4			L5	
SKv1.1	K	L	SRHSKG	L	QILGRT	L	RASVRE	L	GLLVFF	L				
Shaker	K	L	SRHSKG	L	QILGRT	L	KASMRE	L	GLLIFF	L				
Shab	K	L	ARHSTG	L	QSLGFT	L	RNSYKE	L	GLLMLF	L				
Shaw	K	V	TRHSSG	L	KILIQT	F	RASAKE	L	TLLVFF	L				
Shal	K	F	SRHSQG	L	RILGYT	L	KSCASE	L	GFLNFS	L				
			S4							S5				

Figure 7. Amino acid sequence around the pore-forming region (H5) of SKv1.1. Putative binding sites for charybdotoxin (CTX) and tetraethylammonium (TEA) are indicated.

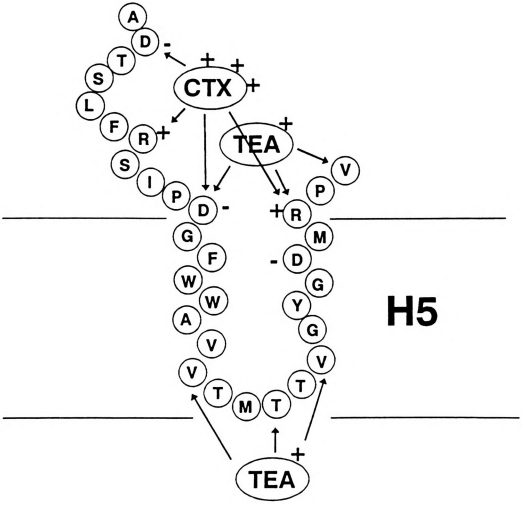

A
D
T
S
L
F
R
S
I
P
D
G
F
W
W
A
V
V
T
M
T
CTX
TEA
TEA
V
P
R
M
D
G
Y
G
V
T
H5

Table 5. Correlation between external TEA sensitivity and the presence of a particular amino acid in the H5 region of voltage-gated K^+ channels.

1	20	Channel	TEA[a](mM)	Ref.
PDAFWWAVVS	MTTVGYGDMYP	RCK1	0.6	Tempel *et al.* (1988)
.IG.....T	.....Y.	Raw3	0.3	Schroter *et al.* (1991)
.AS....TIT	.....IY.	m*Shab*	5	Pak *et al.* (1991)
.AS....TIT	.....IY.	DRK1	6	Frech *et al.* (1989)
.....T	.....Y.	RCK2	7	Grupe *et al.* (1990)
.....T	...L.....T.	*Shaker*	17	Hoshi *et al.* (1990)
.EA....GIT	.....IC.	*Shab*	10	Wei *et al.* (1990)
.....T	.....R.	RCK7	>40	Betsholtz *et al.* (1990)
.....T	.....H.	RCK3	50	Stuhmer *et al.* (1989)
.....T	.....K.	RCK4	>100	Stuhmer *et al.* (1989)
.....S	.....V.	RCK5	129	Christie *et al.*(1990)
.....T	.....R.	Ak01a	>100	Pfaffinger *et al.*(1991)
..G.....T	.....R.	SKv1.1	>100	

[a]Concentration of external TEA for 50% inhibition (IC_{50}) of the current.

Figure 8. (A) N-terminal amino acid sequence of SKv1.1 and other *Sh* K^+ channels. Basic residues are underlined. (B) Amino acid sequences in the S4-S5 loop region of SKv1.1 and other *Sh* K^+ channels. Five residues involved in fast inactivation are boxed. Dots indicate the same amino acid residues as SKv1.1 at the corresponding positions. References; *Shaker* (Hoshi et *al.*, 1990), *Shab*, *Shal* and *Shaw* (Butler et *al.*, 1989).

(A)

SKv1.1 MSTLSGTASTLLLPHGTLAYCNRKINQN

Shaker MAAVAGLYGLGEDRQHRKKQQQQQQHQKEQL

Shab MVGQLQGGQAAGQQQQQQQATQQQQHSKQQLQQQQQQQQQLQLKQHQQQ

Shal MASVAAWLPFARAAAIGWVPIATHPLPPPPMPKDRRKTDDEKLL

Shaw MNLINMDSENRVVLNVGGIRHETYKATLKKIPATRLSRLTEA

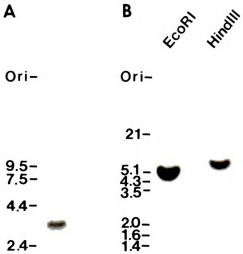
(B)

SKv1.1	GLQI	L	GQ	T	LRA	S	VR	EL	G
Shaker	....	.	.R	.	.K.	.	M.	..	.
Shab	...S	.	.F	.	..N	.	YK	..	.
Shal	..R.	.	.Y	.	.KS	C	AS	..	.
Shaw	..K.	.	I.	.	F..	.	AK	..	T

C. Northern and Southern blot analysis

Northern blot analysis using adult schistosome poly(A^+) RNA and a probe that is specific to the 3'-untranslated region of SKv1.1 detected a single transcript of 2.8 kb, which is comparable to the size of SKv1.1 gene (Figure 9A). In order to preclude the possibility that the probe targeting the 3'-untranslated region is detecting only one form of alternatively spliced transcripts showing variation in the 3'-untranslated region, a Northern blot analysis using a probe containing the whole region of SKv1.1 (2.8 kb), rather than the 3'-untranslated region, has been tried. Again, only single band has been observed in the blot indicating the SKv1.1 mRNA may occur as a single transcript in adult *S. mansoni*, not as multiple alternatively spliced transcripts. Southern blot analysis also suggested that the SKv1.1 gene exists as a single copy in the whole genomic DNA of *S. mansoni* (Figure 9B).

Figure 9. Hybridization analyses of SKv1.1. (A) Northern blot analysis of SKv1.1. Three micrograms of poly(A^+) RNA isolated from adult *S. mansoni* were fractionated on 1% formaldehyde gel, blotted and hybridized with a probe derived from the 3'-untranslated region of SKv1.1. Hybridization conditions are described in Materials and Methods. Size markers are indicated in kb. (B) Southern blot analysis of SKv1.1. Ten micrograms of genomic DNA were digested with *Eco*RI (E) and *Hin*dIII (H) and resolved on a 0.8 % agarose gel. The blotted membrane was hybridized with the same probe that was used in Northern blot. Size markers are indicated on left in kb.


A
B
EcoRI
HindIII
Ori–
Ori–
21–
9.5–
7.5–
5.1–
4.3–
3.5–
4.4–
20–
1.6–
2.4–
1.4–

II. Electrophysiology

A. Expression of SKv1.1 protein in *Xenopus* oocytes

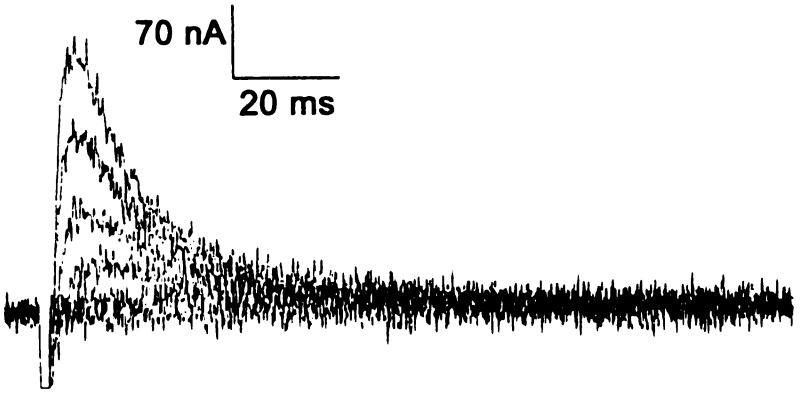
For the expression of SKv1.1 gene in *Xenopus* oocytes, we have initially made SKv1.1 mRNA without any modification, however, the injection of the mRNA did not result in a functional expression. Nor did the introduction of the Kozak's motif (Kozak, 1991) and a poly adenylated tail into SKv1.1 mRNA by the method described by Cestari *et al.* (1993). In order to maximize the functional expression of SKv1.1 gene in oocytes, two major modifications were made on SKv1.1 mRNA. Firstly, the coding region of SKv1.1 was placed between the 5' and the 3' untranslated regions (UTRs) of ß-globin gene of *Xenopus* (Melton *et al.*, 1984), in order to increase the stability or the translation efficiency of SKv1.1 mRNA. Secondly, in addition to introducing Kozak's motif, the fourth nucleotide (C) was changed to G in order to increase the translation efficiency of SKv1.1 mRNA. These two modifications increased the expression of SKv1.1 to a level which was high enough to be detected by oocyte voltage clamp.

Injection of SKv1.1 mRNA into *Xenopus* oocytes produced a rapidly inactivating outward potassium current (Figure 10). Expression of a rapidly inactivating A-type current is commonly observed in oocytes by injecting *Shaker* channel mRNAs from invertebrates such as *Drosophila*, *Aplysia* (Pfaffinger *et al.*, 1991). A-type currents have also

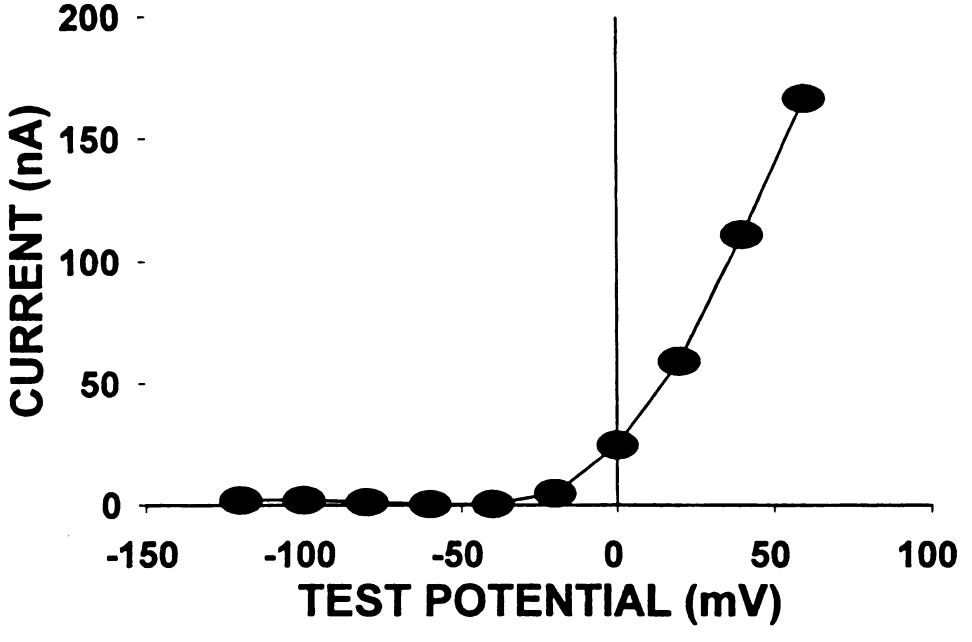
observed in some mammalian *Shaker* channels such as RCK4 (Stühmer *et al.*, 1989). However, most mammalian voltage-gated K^+ channel genes express the slowly inactivating delayed rectifiers. The putative structural motifs that are related with the fast inactivating current of SKv1.1 will are discussed in the discussion section.

Figure 10. Expression of SKv1.1 protein in *Xenopus* oocytes. (A) Currents evoked by depolarizing voltage steps from -80 mV to test potentials of -40 to +60 mV in 20 mV increments. (B) Current-voltage relationship averaged from 9 oocytes.

A


70 nA
20 ms

B


200
150
100
50
0
CURRENT (nA)
-150
-100
-50
0
50
100
TEST POTENTIAL (mV)

B. Voltage dependence of SKv1.1 current

The current mediated by SKv1.1 was voltage-dependent, with a threshold of activation near -20 mV (Figure 10). Although the conductance was not saturated at +60 mV, test pulses to more positive potentials produced other, non-ohmic currents that confounded the isolation of the SKv1.1 component (Figure 11). Therefore, for the purpose of analysis, the conductance at +60 mV was considered to be G_{max}.

The V_{50} for activation was +20 mV, with a gentle slope factor, a number mV required for an e-fold change of the relative conductance value, of 15 mV. When compared with other cloned voltage-gated K^+ channels, the V_{50} (+20 mV) of SKv1.1 current is located on the right edge of the normal range along the voltage axis. Most known *Shaker* channels have V_{50}s for activation ranging from -30 mV to +5 mV. The slope factor (15 mV) does not appear to be placed on the extreme edge, showing significant similarities to those measured from other *Shaker* currents (Papazian *et al.*, 1991).

The SKv1.1 current also displayed a voltage-dependent steady-state inactivation (Figure 11). The V_{50} for inactivation was -40 mV and the slope factor was 4 mV, which is steeper than that of voltage-dependent activation. Both the V_{50} and the slope factor for steady-state inactivation of SKv1.1 current fall into the normal range that are commonly observed in other *Shaker* channels. Voltage-

dependent activation and steady-state inactivation showed little overlap.

In the native A-type current measured from the isolated muscle fibers, a similar voltage dependence was observed (Day *et al.*, 1993; Table 6). The V_{50} for activation was +5 mV with a slope factor of 12 mV, the V_{50} for steady-state inactivation was -50 mV with a slope factor of 14 mV. This similarity strongly supports the idea that the expression of A-type current measured in muscle fibers is directed by SKv1.1 gene.

Figure 11. Voltage dependence of activation (●, n=9) and steady-state inactivation (○, n=5) of the SKv1.1 current. The conductance was calculated by dividing the amplitude of the SKv1.1 current by the difference between the test potential and the hypothetical reversal potential for K^+, -82 mV. The lines represent the fit of the data to the Boltzmann equation in the form $G/G_{max}=1/(1+e^{(V-V50)/k})$, where V = test potential, V_{50}= voltage of half activation or inactivation, k = slope factor. For steady-state inactivation, the oocytes were held at different voltages for 5 sec before applying a depolarizing voltage step to +60 mV. Peak currents obtained by during the test pulse after a prepulse to the indicated voltage were divided by the peak currents obtained during a test pulse obtained during a test pulse after a prepulse to -80 mV.

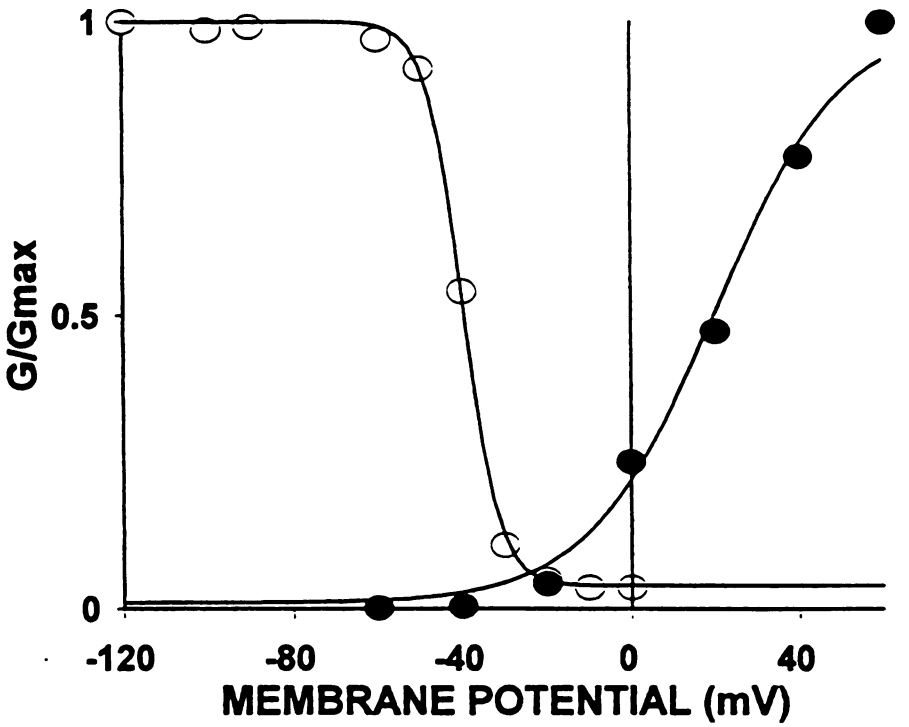

1
0.5
0
G/Gmax
-120
-80
-40
0
40
MEMBRANE POTENTIAL (mV)

Table 6. Functional properties of the current mediated by SKv1.1 compared to those of a native current in muscle fibers of *S. mansoni* (Day *et al.*, 1993; Day *et al.*, 1994).

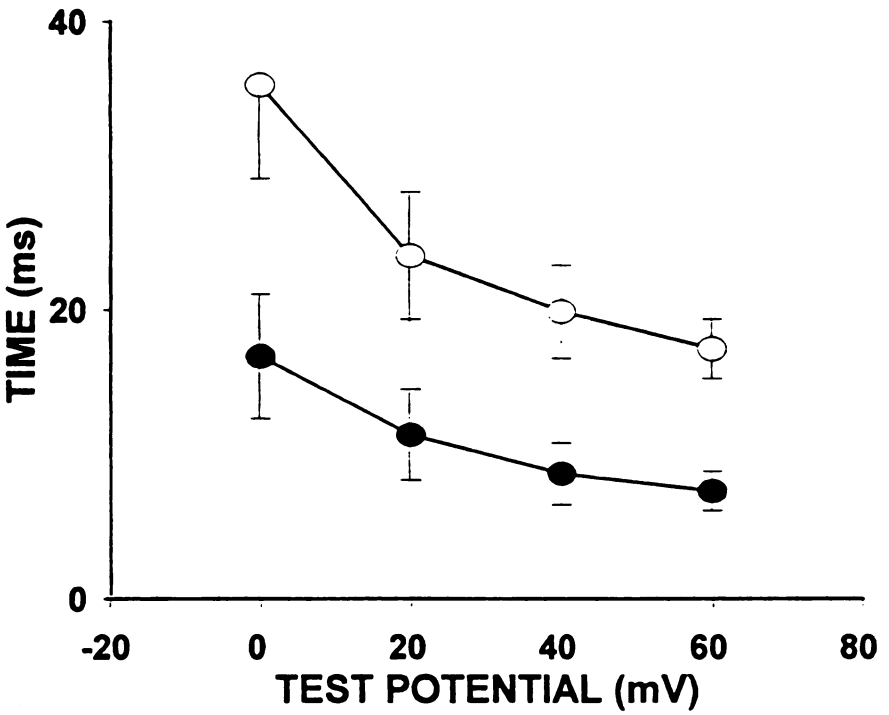
	SKv1.1 current	muscle current
Activation		
Threshold	-20 mV	-30 mV
Time to peak	8 ms	8 ms
V_{50}	+20 mV	+5 mV
Slope factor	-15 mV	-12 mV
Inactivation		
V_{50}	-40 mV	-50 mV
Slope factor	4 mV	14 mV
Time constant	17 ms	13 ms
Recovery from inactivation		
Time constant	7s	
Pharmacology (IC_{50})[a]		
4-AP	0.2 mM	1 mM
TEA^+	>100 mM	30 mM
DTX	>300 nM	>300 nM
MCDP	>300 nM	
CTX	>100 nM	

[a]Concentration of blockers for 50% inhibition of the current.

C. Kinetics of SKv1.1 current

The currents mediated by SKv1.1 were rapidly activated and inactivated with a time to peak of 8 ms and a time constant of inactivation of 17 ms in response to a test pulse to +60 mV from a holding potential of -80 mV (Figure 12). Inactivation of the current fit well with a single exponential. Both activation and inactivation were voltage-dependent, becoming faster at more positive potentials. The time to peak and the time constant of inactivation of SKv1.1 were similar to those from other A-type currents as well as the native A-type currents of isolated muscle fibers (Table 6).

Figure 12. Time to peak (●, n=4) and the time constant of inactivation (○, n=4) of the SKv1.1 current. The holding potential was -80 mV and the time constants of inactivation were derived by fitting the data to the equation $I=A(1-e^{-t/\tau})^n$, where t = time, τ = time constant and with the single exponential n=2. Error bars indicate standard deviations.


40
20
0
TIME (ms)
-20
0
20
40
60
80
TEST POTENTIAL (mV)

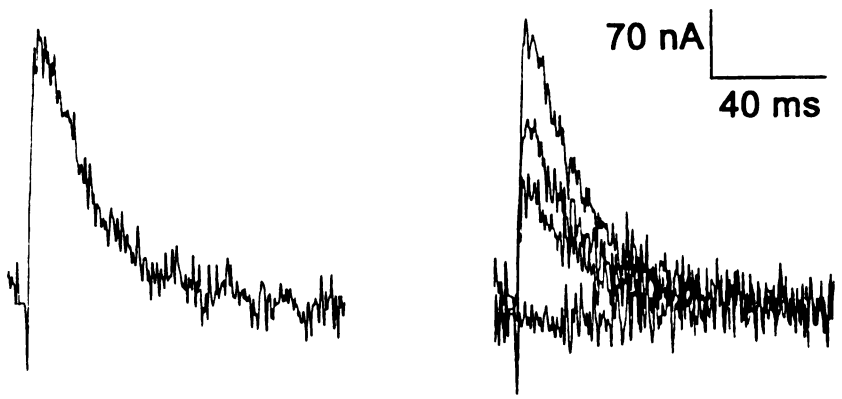
D. Recovery of SKv1.1 current from inactivation

Recovery from inactivation was measured using two consecutive pulses with different intervals. The ratio of the amplitude of the second peak current to that of the first peak current was plotted against the duration of intervals. (Figure 13). The time course of recovery fit well with a single exponential with a time constant of 5 sec.

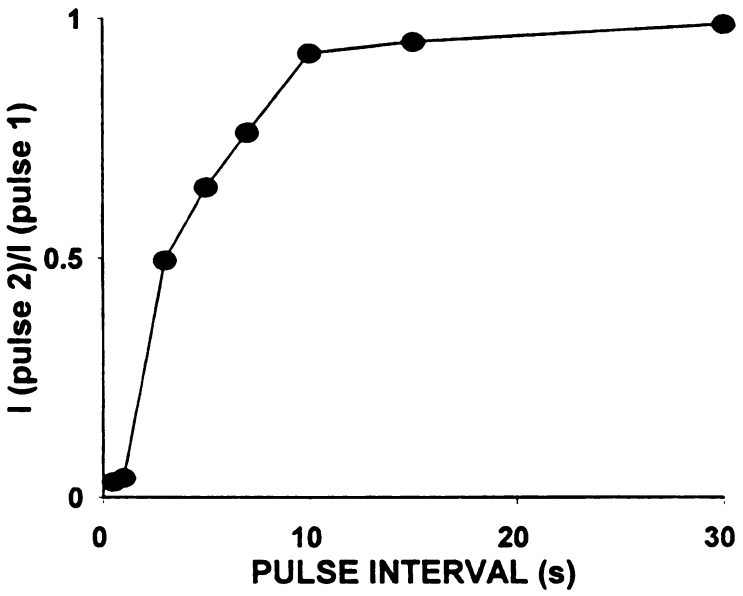
SKv1.1 showed a very slow recovery from inactivation. The slow recovery from inactivation has also been observed form the *Aplysia Shaker* channel (time constant of 2.3 sec) and the mammalian RCK4 channel with a time constant in the range of seconds (Pardo *et al.*, 1992).

Figure 13. Recovery of SKv1.1 current from inactivation (n=10). (A) Current traces show a control current at a +60 mV test potential, followed by currents elicited by the same pulse following variable intervals. Shown are intervals of 1s, 3s, 5s and 15s. (B) The amplitude of SKv1.1 current induced by a step to a test potential of +60 mV against the interval between the two pulses.

A



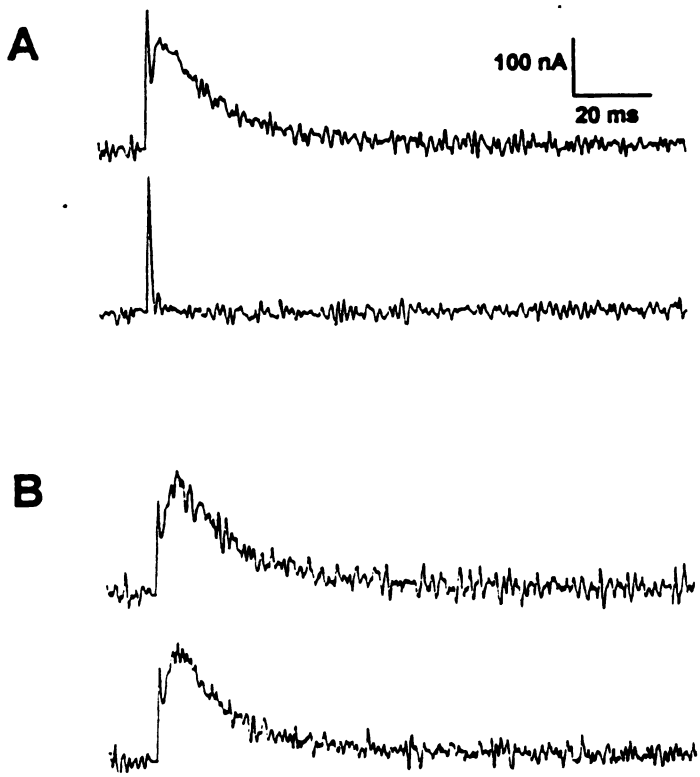
B



E. Pharmacology of SKv1.1 current

The SKv1.1 current was extremely sensitive to block by external 4-aminopyridine (4-AP) (Figure 14A). The current was reduced 93±5% (n=5) by 1 mM 4-AP. The current was strikingly resistant to external TEA^+, with 100 mM TEA^+ producing no discernable blockade (n=4)(Figure 14B). Insensitivity to external TEA^+ was predicted by the amino acid sequence analysis of the SKv1.1 channel, which showed the presence of Arg394 in the outer pore region. The current was not sensitive to 100 nM Charybdotoxin (CTX), which would also be predicted by the presence of the positively charged Arg394 in the pore region. The current was insensitive to mast cell degranulating peptide (MCDP) and dendrotoxin (DTX) at concentrations as high as 300 nM.

Figure 14. Pharmacological properties of the currents mediated by SKv1.1. The blockers were applied by perfusing the chamber with ND-96 containing the blockers (1-2 ml/min). A voltage step to +60mV was given to oocytes from the holding potential of -80 mV. (A) SKv1.1 current before (top) and after (bottom) the application of 1 mM external 4-aminopyridine. (B) SKv1.1 current before (top) and after (bottom) the application of 100 mM external tetraethylammonium.


A
100 nA
20 ms
B

III. Immunohistochemistry

A. *In vitro* translation and immunoprecipitation

A synthetic oligopeptide based on the amino acid sequence of the cytoplasmic domain (aa 447-466) near the C-terminal end of SKv1.1 was made. Since different *Sh* subfamilies have unique amino acid sequences in the region around the N- and C-terminals, an antibody raised against the unique region of the *Shaker* protein would be expected to recognize only the *Shaker* channel protein and not other *Sh* family proteins in *Schistosoma*. After immunization, a SKv1.1 protein-specific antibody (ASK1), was purified using an affinity column in which the synthetic oligopeptide was immobilzed. The purified ASK1 was highly specific to the oligopeptide motif, not to the carrier proteins such as BSA and KLH.

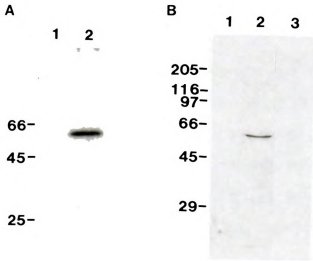
In vitro translation of the SKv1.1 gene was used to test the specificity of the ASK1 antibody to its target protein by immunoprecipitation analysis. In addition, the *in vitro* translation was employed to test the expression of SKv1.1 gene in a heterologous system and to measure the molecular weight of the protein. The SKv1.1 mRNA for *in vitro* translation was prepared by *in vitro* transcription using linearlized pBTGSK1 as a template. The *in vitro* translation product of SKv1.1 mRNA showed a molecular weight of 55 kDa, which is close to the predicted molecular weight of 56.5 kDa based on the amino acid sequence of SKv1.1

(Figure 15A).

Immunoprecipitation analysis showed that ASK1 can precipitate the *in vitro* translation product of SKv1.1 (Figure 15B, lane 2). On the other hand, the preimmune serum and the ASK1 preincubated with an excess amount of oligopeptide were unable to precipitate the SKv1.1 protein (Figure 15B, lane 1 and 3).

Western blot analysis has also been tried using both the *in vitro* translation product of SKv1.1 and membrane proteins of *S. mansoni* as target proteins. No specific band was found in the Western blot membrane, indicating that the peptide motifs in the SKv1.1 protein that are recognized by ASK1 without any problem in a non-denaturating condition may be completely denatured by the presence of SDS, resulting in the loss of their native configuration. This has been observed in some peptide-specific antibodies (Sheng *et al.*, 1992). However, since most conditions that are used in immunohistochemistry, except for Western blotting, do not eliminate the native configuration of target proteins, ASK1 has been shown to successfully recognize the target protein in immunohistochemistry on isolated muscle fibers and tissue sections.

Figure 15. *In vitro* translation and immunoprecipitation of SKv1.1 protein. (A) Autoradiograph of *in vitro* translation products of SKv1.1 protein. The mRNAs from SKv1.1 gene were added to the rabbit reticulocyte lysate *in vitro* translation system containing [^{35}S]-methionine. Lane 1, water was used instead of mRNA as a negative control; lane 2, SKv1.1 mRNA. Translation products were resolved on 10 % SDS PAGE gel. (B) Immunoprecipitation of *in vitro* translation product by ASK1 antibody. Lane 1, preimmune antibody as a negative control; lane 2, immunoprecipitation with ASK1; lane 3, ASK1 was preincubated with an excess amount of free peptide before the immunoprecipitation.

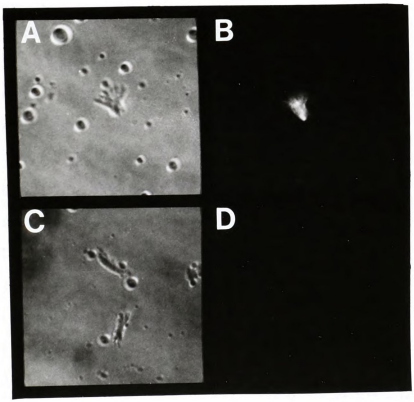

A
1
2
66-
45-
25-
B
1
2
3
205-
116-
97-
66-
45-
29-

B. Immunohistochemistry on isolated muscle fibers

Since native A-type currents were initially measured from isolated muscle fibers of *S. mansoni* (Day *et al.*, 1993), we, by immunohistochemistry, tested whether SKv1.1 protein is expressed in the isolated muscle fibers of *S. mansoni*. During this analysis, we have focused on finding the expression of SKv1.1 protein only in muscle fibers, not in any other cells, because the morphology of muscle fibers is relatively well characterized compared with other cell types. The expression of SKv1.1 gene in other cell types was studied using immunohistochemistry on paraffin sections.

Among three types, frayed, crescent and spindle, of isolated muscle fibers of schistosome, strong immunoreactivities were observed in the most abundant frayed fibers and in the crescent fibers, where A-type currents were originally measured. ASK1 specifically recognized its target protein (Figure 16A and B) while the ASK1 preincubated with free peptide did not identify the target (Figure 16C and D). Also, the preimmune serum did not show any specific binding to the muscle preparation. This result, together with the results from the electrophysiology of SKv1.1, provides strong evidence that the expression of A-type currents measured in the frayed and the crescent muscle fibers are directed by the SKv1.1 gene.

Figure 16. Immunolocalization of SKv1.1 protein in the isolated muscle fibers. Light (A and C) and fluorescent (B and D) microscopy of the isolated frayed muscle fiber stained with ASK1 (A and B) and with the ASK1 preincubated with an excess amount of free peptide (C and D).


A
B
C
D

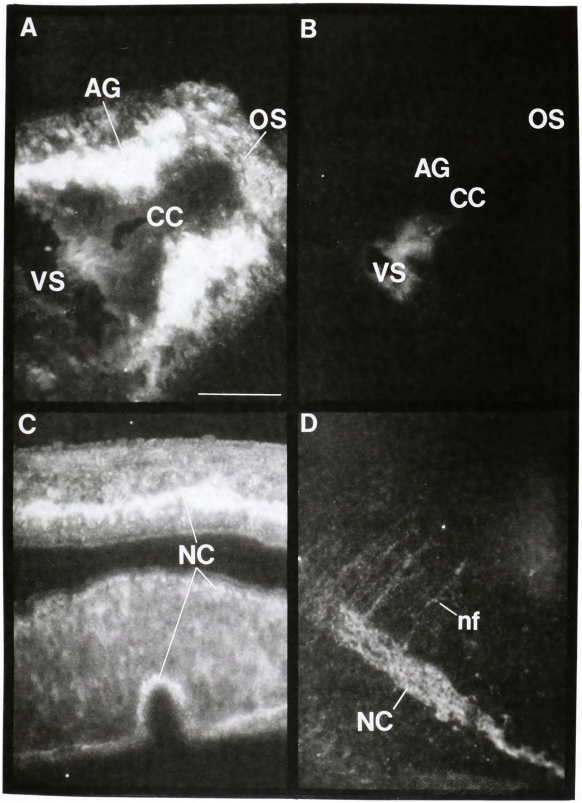
C. Immunohistochemistry on paraffin sections

In order to study the expression of SKv1.1 protein in tissues other than muscle fibers, paraffin sections (2 μm) of adult female and male worms were immunostained with ASK1 antibody.

Strong immunoreactivities were observed in the central and peripheral nervous system of both female and male worm (Figure 17). In the head region of the male worm, ASK1 strongly stained the anterior ganglia and the central commissures from which longitudinal nerve cords extend out both anteriorly and posteriorly (Figure 17A). On the other hand, preabsorbed ASK1 did not recognize these structures (Figure 17B). Also, strong immunoreactivities were localized in the longitudinal nerve cords in the body region of both female and male worms (Figure 17C). Fine nerve fibers that emanate from main nerve cords were observed (Figure 17D). Relatively strong immunoreactivities were also observed in the nervous system innervating the oral sucker (Figure 17E), the ventral sucker (Figure 17F) and the tail region (Figure 17G).

In addition to the nervous system, tubercles as well as muscle fibers including longitudinal, circular and ventral muscle fibers were mildly stained (Figure 17H).

Figure 17. Immunolocalization of SKv1.1 protein in paraffin sections of *S. mansoni* (x400). *Scale bar*: 50 μm. (A) Head region of male showing strong immunoreactivity in the anterior ganglia (AG) and the central commissure (CC). Oral sucker (OS), ventral sucker (VS). (B) Negative control. Head region of male in an adjacent section in which ASK1 antibody was inactivated by incubating with an excess amount of free peptide. The fluorescence around the ventral sucker is an autofluorescence. Oral sucker (OS), ventral sucker (VS), anterior ganglia (AG) and central commissure (CC). (C) Longitudinal nerve cords (NC) of female (bottom) and male (top) worm located in the middle of the body. (D) Fine nerve fibers (nf) emanating from the main nerve cords (NC). (E) Oral sucker (OS) in the head region of male. Anterior ganglia (AG). (F) Ventral sucker (VS) in the head region of male. (G) Tail region of the male showing immunoreactivity in the main nerve cords (NC). (H) Tubercles (TB), longitudinal muscle fibers (LM) and circular muscle fibers (CM) in the dorsal surface of male.


A
AG
OS
CC
VS
B
OS
AG
CC
VS
C
NC
D
nf
NC

DISCUSSION

I. Molecular cloning

A. What does the existence of a *Shaker* channel gene in *Schistosoma* mean in an evolutionary sense?

The first organisms that show the presence of a nervous system are coelenterates such as hydra and jelly fish. The Phylum Platyhelminthes, including *Schistosoma* emerged soon after the coelenterates in the evolutionary pathway about 600 million years ago, and the platyhelminths are the first that show both central and peripheral nervous system and multiple organs. Since ion channels are one of the most important constituents of the nervous system, ion channels have evolved in parallel with the evolution of the nervous system.

Based upon the comparative analysis of amino acid sequences of various ion channels, it appears ancient duplications gave rise to three groups of ion channels: sodium and calcium channels, potassium channels and nucleotide-binding channels (Strong *et al.*, 1993). K^+ channels have further evolved into several groups including voltage-gated and calcium-activated K^+ channels, generating much more diverse channels compared with other types of ion channels. Again, voltage-gated K^+ channels have evolved into several families such as the *Sh* family (six membrane-spanning domains), inward rectifiers (two domains), and slow

K^+ channels (one domain).

In the *Sh* family, a common ancestor has given rise to four subfamilies (*Shaker, Shal, Shab and Shaw*) by a gene duplication event, and each subfamily again has undergone a considerable expansion by another gene duplication during the evolution of mammalian species. For example, there are seven *Shaker* K^+ channel genes in mammals. When did the first gene duplication event that generated the four *Sh* subfamilies happen? Before the cloning of the SKv1.1 gene, the most primitive *Shaker* channels that have been cloned were those from *Drosophila* and *Aplysia*, which are highly developed invertebrates. Also it was not clear whether the duplication happened before or after the divergence of deuterostomes (*Chordata* and *Echinodermata*) and protostomes (*Annelid*, *Mollusc*, and *Arthropod*) which occurred approximately 570 million years ago. Therefore, the presence of a *Shaker* homologue in *Schistosoma* indicates the gene duplication happened at least before the emergence of *Schistosoma* about 600 million years ago.

Another interesting question arises when we consider the fact that the gene duplication event that generated the four *Sh* subfamilies happened before the emergence of *Schistosoma*. If the gene duplication event happened before the emergence of schistosome, do other *Sh* subfamilies such as *Shal*, *Shab* and *Shaw* exist in *Schistosoma* and contribute to the diversity of schistosomal K^+ currents? They might

exist in modern *Schistosoma* unless these genes were lost in the evolutionary pathway. One possible way to prove it is to try to screen other *Sh* subfamilies in the schistosomal cDNA library using the SKv1.1 as a probe using a low-stringency hybridization condition.

Do *Sh* subfamilies exist in other phyla of lower invertebrates? Theoretically, *Sh* subfamilies should be found in organisms of any higher phyla such as nematodes. Indeed, a *Shaw* homolog was found in *C. elegans* (Wei *et al.*, 1991), but the full sequence of the gene has not been published. Do *Sh* subfamilies exist in more primitive phyla such as protozoa, sponges, coelenterates? A variety of voltage-gated K^+ currents that have properties similar to mammalian channels have been detected in these phyla including coelenterates (Hagiwara, Yoshida & Yoshii, 1981) and protozoa (Eckert & Brehm, 1979; Deitmer, 1989), supporting the idea that these currents might be directed by *Sh* subfamilies.

B. What does the analysis of SKv1.1 gene say about the diversity of K^+ channel currents in *Schistosoma*?

With limited molecular and physiological data, it is not easy to discuss the diversity of K^+ channel currents in *Schistosoma*. There are several mechanisms for the generation of diverse K^+ channels in the *Sh* family: the presence of subfamilies (Wei *et al.*, 1990), the formation of

heteromultimers (Isacoff *et al.*, 1990; MacKinnon *et al.*, 1991; McCormack *et al.*, 1990; Ruppersberg *et al.*, 1990), alternative splicing (Iverson and Rudy, 1990; Jan and Jan, 1990; Kamb *et al.*, 1988; Schwarz *et al.*, 1988; Stocker *et al.*, 1990; Timpe *et al.*, 1988; Timpe *et al.*, 1988), gene duplication within a subfamily (Stühmer *et al.*, 1989) and the level of expression (Guillemare *et al.*, 1992; Honoré *et al.*, 1992; Moran *et al.*, 1992).

Are there multiple copies of SKv1.1 gene in *Schistosoma*? Southern hybridization analysis has been tried, and the result shows that there is only a single copy of SKv1.1-related gene in the schistosomal chromosome. This suggests that, in contrast to mammalian K^+ channel genes, multiple copies of identical or closely related genes for a given channel do not exist in invertebrates as also reported in *Drosophila* and *Aplysia*.

In general, invertebrates employ alternative splicing as a primary mechanism to generate diverse K^+ channels while mammals use extensive gene duplication in a given *Sh* subfamily even though alternative splicing has also been reported in some mammalian *Shaker* (Attali *et al.*, 1993) and *Shaw* channels (Rettig *et al.*, 1992; Luneau *et al.*, 1991a; Luneau *et al.*, 1991b). This has led us to investigate the presence of alternatively spliced transcripts of the SKv1.1 gene. However, evidence for the existence of alternatively spliced transcripts of the SKv1.1 gene could not be found.

The six positive clones showed the same restriction patterns. Three of the six clones contained the same nucleotide sequence in the coding region. Also, Northern blot analysis detected only a single primary transcript with a small size (2.8 kb), which may not be long enough to allow the generation of diverse alternatively spliced transcripts.

What does the lack of alternative splicing and multiple copies of SKv1.1 mean? Does this mean *Schistosoma* might have a relatively simple profile of voltage-gated K^+ channel genes? Although no conclusion can be drawn at this point due to the lack of data, *Schistosoma* appears to have diverse types of excitable tissues that may need diverse K^+ channels. *Schistosoma,* as a member of the Phylum Platyhelminthes, is the first organism that shows multiple organs, tissue layers and primitive central and peripheral nervous system. In addition to several ion channels that have been measured in schistosomes (Blair *et al.*, 1991; Day *et al.*, 1992; Day *et al.*, 1993), *Schistosoma* respond to a variety of neurotransmitters and neuropeptides that may be directly or indirectly related to K^+ channels (Baker *et al.*, 1966; Hillman 1983; Mellin *et al.*, 1983; Pax *et al.*, 1984; Bennett *et al.*, 1969; Bennett and Bueding, 1971; Chou *et al.*, 1972; Dei-Cas *et al.*, 1971; Dei-Cas *et al.*, 1981; Gianutsos and Bennett, 1977; Gustafsson, 1987; Machado *et al.*, 1972; Tomosky *et al.*, 1974; Basch and Gupta, 1988; Gupta and Bash, 1989).

Therefore, if any further diversity of voltage-gated K^+ channels exists in the schistosome, it could results from the presence of other *Sh* subfamilies, alternative splicing in other *Sh* subfamilies, the formation of heteromultimers or the density of channels in the membrane.

C. Does the SKv1.1 have a potential to be a novel target for drug development for schistosomiasis?

The availability of SKv1.1 gene provides several advantages for drug development. It permits to compare the amino acid sequence of SKv1.1 to that of the host. The expression of SKv1.1 channel protein in a heterogenous system also permits a high volume screening for drug development.

The nervous system and the neuromuscular system of helminths are attractive targets for drug development (Geary *et al.*, 1992). Many anthelminthic drugs act on receptors and ion channels in the nervous system. In order for SKv1.1 to be considered as a target for drug development, the following questions need to be answered. Does SKv1.1 protein exist in the nervous or neuromuscular system of *S. mansoni*? Is the function of SKv1.1 protein important enough to make the chemotherapeutic intervention of SKv1.1 protein valuable? Is the amino acid sequence of the SKv1.1 protein significantly different from that of the host?

Several lines of evidence in the present study show

that SKv1.1 proteins exist in the nervous system and the neuromuscular system of *S. mansoni*. The expression of SKv1.1 protein in the nervous system has been demonstrated by immunohistochemistry on paraffin sections of *Schistosoma*. Also, immunohistochemistry on isolated muscle fibers using the antibody raised against SKv1.1 shows SKv1.1 proteins are indeed expressed in these muscle fibers.

The functions of SKv1.1 in *Schistosoma* are not obvious from current data. The fact that SKv1.1 shows a A-type current implies that, if this channel is present in neurons, it may regulate the frequency and shape of action potentials. On the other hand, the function of SKv1.1 protein in muscle fibers is difficult to investigate due to technical problems such as the lack of a specific blocker for the channel and the small size of the muscle fibers. However, the presence of sites for phosphorylation by protein kinase C or tyrosine kinase in SKv1.1 gene suggests that SKv1.1 channel might be modulated by many neurotransmitters and neuropeptides participating in the regulation of cellular excitability.

The comparison of the amino acid sequence of SKv1.1 to other mammalian *Shaker* channels shows that, despite the evolutionary distance between the *Schistosoma* and mammals, they share high amino acid sequence identity in the middle of the channel including the tetramerization domain, the six membrane spanning domains and the pore-forming domain.

However, the N-terminal (aa 1-50) and C-terminal (aa 430-512) ends show almost no sequence identity to other *Shaker* channels, making this region a possible novel target.

Nonetheless, the presence of highly variable regions at both ends, not in the middle, of the gene holds several disadvantages for drug development. Most K^+ channel blockers target the pore-forming domain (H5) in the middle of the channel, where almost all *Shaker* channels share high amino acid sequence identities (Pongs, 1992). Also, the diversity of the K^+ channel blockers is far less than that of K^+ channels. Another problem arises from the fact that both N- and C-terminal regions of the channels are located intracellularly, so any drugs targeted against these region would have to penetrate the cell membrane. However, the N- or C-terminal ends are targeted in drug development, there are some possibilities that novel drugs might be found, for instance, a drug interfering with the inactivation of the channel because the N-terminal is involved in the rapid inactivation of channel.

II. Electrophysiology

A. Why does SKv1.1 current show a fast inactivation?

The injection of SKv1.1 mRNA into *Xenopus* oocytes results in the expression of a fast inactivating current. What are the structural elements that are responsible for the fast inactivation of SKv1.1 current?

Fast inactivating currents have been observed in various *Shaker* channels from *Drosophila*, *Aplysia* and some mammals. Mechanisms underlying the fast inactivation of some *Shaker* channels have been very well characterized by deletion and mutation analysis. The fast inactivation of the channel appears to be the result of an interaction between the ball structure at the N-terminal end and the receptor structure in the intracellular S4-S5 loop. Application of an oligopeptide mimicking the N-terminal sequence of the channel gene accelerated the inactivation of the current (Ruppersberg *et al.*, 1991). Deletion of the N-terminal sequence also slowed down inactivation of mouse *Shal* current (Pak *et al.*, 1991). Deletion of three basic amino acid residues (aa 35 - 37) in the ball structure also slowed down inactivation of rat *Shal* current (Baldwin *et al.*, 1991). At the N-terminal end, in addition to the positively charged amino acids, the importance of a hydrophobic residue has been demonstrated by replacing the seventh leucine residue with a hydrophilic residue. This substitution inhibits the fast inactivation almost completely. At the receptor region in the S4-S5 loop, five important amino acids including one glutamate interact with the inactivation ball (Isacoff *et al.*, 1991).

In SKv1.1, the N-terminal region contains two positively charged amino acid residues (Arg-Lys, aa 23-24) and four hydrophobic leucines (aa 4, 11-13). The receptor

region at the S4-S5 loop contains all five amino acids that are highly conserved in other rapidly inactivating *Shaker* channels. Therefore, these structural features of SKv1.1 provide possible explanations for the rapid inaction of SKv1.1 current although deletion or site-directed mutagenesis analysis of the N-terminal and the receptor structure of SKv1.1 will give direct evidence for this possibility.

In addition to the fast inactivation currents (N-type) described above, a slow inactivation (C-type) has been observed in some A-type currents. No slowly inactivating currents could be observed in SKv1.1 after leak-subtraction. In other *Shaker* channels, some channels show only the N-type inactivation, while others show both the N- and C- type inactivations. Alanine in the S6 domain is an important determinant of slow inactivation as shown by site-directed mutagenesis study (Wittka *et al.*, 1991). Based on the sequence comparison between SKv1.1 and *Shaker* channels, SKv1.1 current is expected to show both the N-type and the C-type inactivation. The reason for the absence of C-type inactivation in SKv1.1 current is not clear. One possibility is that the amplitude of the slowly inactivating current is very small compared with that of the rapidly inactivating (N-type) current. Another possibility is that there are determinants other than the Ala residue in the S6 domain, for the slowly inactivating component of the

currents.

Another interesting question that needs to be answered is whether there is a ß-subunit in SKv1.1. Most of the mammalian *Shaker* channels show very slow inactivations when expressed in frog oocytes. However, when coexpressed with the ß-subunit, the α and ß complex show an altered inactivation. For instance, the RCK1, which normally shows a slow inactivation when expressed alone, exhibits a fast inactivation when expressed together with the ß-subunit (Rettig *et al.*, 1994). A more surprising result is that RCK4, which normally shows a fast inactivation even when expressed alone, exhibited a faster rate of inactivation by the coexpression. Is there a ß-subunit of SKv1.1 in *Schistosoma*? Unfortunately, the fact that SKv1.1 alone can make a fast inactivating channel does not necessarily give any clue for the presence of a ß-subunit because the inactivation of a fast inactivating channel can also be accelerated by the presence of a ß-subunit as shown in mammalian *Shaker* channels.

B. Why is the voltage dependence of activation of SKv1.1 current and native muscle current shifted to the right edge of the normal range along the voltage axis when compared with other *Shaker* channels?

In SKv1.1 current, the V_{50} of the relative conductance vs. voltage relationship is +20 mV, being placed in the

right extreme edge of the normal range. Native A-type current from isolated muscle fibers also shows a very high V_{50} of +5 mV. What are the structural features that underlie this unique voltage-dependence of schistosomal A-type current?

Several regions that are involved in the voltage-dependence have been suggested by extensive site-directed mutagenesis studies combined with electrophysiological studies. The first region is the S4 membrane spanning domain or the S4 segment. This domain is well known as a voltage-sensor which detects voltage changes in the membrane and initiates the conformational change of the protein for the opening of the channel. In most *Shaker* channels, the S4 segment contains seven positively charged amino acid residues which occur at every third position. Their movement toward the extracellular side is thought to be responsible for the gating current that is observed during the opening of the channel (McCormack *et al.*, 1994).

The importance of positively charged amino acid residues of the S4 segment in determining the voltage-dependence of the channel has been studied by substituting all the basic residues in the S4 segment with non-charged glutamines or with other conserved basic residues (Papazian *et al.*, 1991). These mutant channels exhibit a wide range of changes in their voltage-dependences, indicating the importance of positively charged residues. This study also

showed that every positively charged amino acid does not contribute equally to the voltage-dependence of the channel. Detailed mechanisms for these changes have also been studied using single channel analysis of mutant channels (Shao & Papazian, 1993).

The importance of hydrophobic amino acids, in addition to positively charged amino acids, in the S4 segment has also been investigated by site-directed mutagenesis analysis (Lopez *et al.*, 1991). In the S4 segment, conservative mutations of hydrophobic residues, for instance, Leu to Ala, Leu to Val or Ala to Leu, that cause slight changes in the hydrophobicity of each residue resulted in large shifts of voltage-dependence of the current, indicating the importance of hydrophobic amino acids in determining the voltage-dependence.

In addition to the S4 segment, the S4-S5 loop structure has been shown to play an important role in determining the voltage-dependence (McCormack *et al.*, 1991). A leucine zipper motif, with the occurrence of a Leu at every seventh position, is present in this loop structure. The leucine zipper is a motif commonly found in transcription factors and participates in protein-protein interaction, however, this motif in K^+ channels has not been proven to be participating in the assembly of the K^+ channel subunits. When each Leu is substituted by Val, these mutations cause significant changes in the voltage-dependence such as

voltage-dependent activation and steady-state inactivation.

In order to determine if the sequence of SKv1.1 has any unique features, amino acid sequences of the S4 segment and the S4-S5 loop structure containing the leucine zipper motif were compared to the corresponding regions of other *Shaker* channels. Surprisingly, the sequence of the S4 segment of SKv1.1 was identical to the corresponding regions of *Shaker* channels from *Drosophila, Aplysia* and most mammals which obviously show a wide range of V_{50}'s of voltage-dependence of activation. This suggests that, although the S4 segment is an important determinant for the voltage-dependence, the S4 segment may not be the element that is responsible for the various voltage-dependences observed in many K^+ channels. On the other hand, when the S4-S5 loop structure of SKv1.1 containing the leucine zipper was compared to other sequences, differences were observed in three amino acid residues (Arg335, Val338 and Val345), which are non-Leu residues located within the leucine zipper motif.

Unfortunately, no data are available for the possible effects of mutations of non-Leu residues in the leucine zipper motif on the voltage-dependence. An interesting experiment will be to make mutations of these three residues to highly conserved residues and observe if there is a leftward shift of the voltage-dependence of SKv1.1 along the voltage axis.

Another possible explanation for the extreme voltage-

dependence of activation of SKv1.1 is that the voltage-dependence may be affected by regions other than the S4 segment and leucine zipper motif. Supporting evidence comes from the sequence comparison between SKv1.1 and the *Aplysia* K^+ channel (Ak01a). Ak01a has an identical amino acid sequence both in the S4 segment and leucine zipper motif, but, it displays a different voltage-dependence of activation in a more negative position (V_{50} of +5 mV) compared with that of SKv1.1 (V_{50} of +30 mV).

C. Why is SKv1.1 current resistant to external TEA and CTX?

The current mediated by SKv1.1 is highly sensitive to 4-AP, but highly resistant to external TEA, CTX, DTX and MCDP. In general, the amino acid residues that are responsible for the sensitivity of voltage-gated K^+ currents to external TEA and CTX, but not for 4-AP, DTX and MCDP, are relatively well characterized. In order to understand the structural bases for the pharmacology of SKv1.1 current, the amino acid sequence of the pore region of SKv1.1 gene was compared with those from other *Shaker* channel genes.

For external TEA, the residue at position 19 in Table 5 appears to be the most important residue in determining the sensitivity of the channel to external TEA. The channel shows a high sensitivity to external TEA when the residue is Tyr or Phe. The presence of Thr or His at this position gives the channel a medium sensitivity to external TEA.

However, when the residue is a positively charged residue (Arg or Lys), the channel shows a high resistance to external TEA.

The importance of residue 19 has also been studied by extensive site directed mutagenesis. A Thr to Tyr mutation makes the *Shaker* channel current highly sensitive to TEA while mutations from Thr to positively charged residues (Arg or Lys) make the channel highly resistant to TEA (MacKinnon & Yellen, 1990). In rat brain *Shaker* channels, a mutation (Val to Tyr) increases the TEA sensitivity of the channel while the reverse mutation (Tyr to Val) decreases the sensitivity (Kavanaugh *et al.*, 1991).

Further evidence for the importance of residue 19 comes from an experiment using heteromultimers that are made of subunits with different pharmacology. When the channel pore is blocked by external TEA, all four subunits participate in binding one molecule of TEA (Kavanaugh *et al.*, 1992; Heginbotham & MacKinnon, 1992). This cooperative interaction of four subunits to bind one TEA has been proven by demonstration a linear relationship between the number of Tyr-containing subunits and the sensitivity of the channel to external TEA.

SKv1.1 contains an Arg (amino acid 394) at the corresponding position, which makes it possible to predict a high resistance of the SKv1.1 channel to external TEA. Indeed, the SKv1.1 current was highly resistant to external

TEA when expressed in *Xenopus* oocytes, proving the importance of the Arg residue at position 19 in determining the sensitivity of the channel to external TEA.

SKv1.1 current was highly resistant to CTX, being insensitive to as much as 100 nM of CTX. CTX is a scorpion venom toxin, from *Leiurus quinquestriatus,* that block Ca^{2+}-activated K^+ channels (Miller *et al.*, 1985) as well as voltage-gated K^+ channels (Garcia-Calvo *et al.*, 1992). The amino acids that mediate molecular recognition between CTX and *Shaker* K^+ channel have been well characterized by site directed mutagenesis analysis of all the amino acid residues of both CTX and the receptor region in the channel (Goldstein *et al.*, 1994).

Mutations of amino acid residues in the receptor region, especially on the two extracellular loop regions of S5-H5 and H5-S6 (Figure 8B), revealed five important residues for the determination of the sensitivity of the channel to CTX. When these amino acids are compared with the corresponding residues of SKv1.1, two differences in the SKv1.1 sequence were detected: L (370) rather than G in the S5-H5 loop and R (394) rather than T in the H5-S6 loop. Both differences provide reasonable explanations why SKv1.1 channel is resistant to CTX.

Firstly, the presence of a bigger side chain, L rather than G, at the first position (370) gives a clue for the resistance of SKv1.1 to CTX. In the *Shaker* channel, when

the Gly residue was mutated to other residues with increasing sizes of the side chain, for instance from Gly to Ala, Val, Leu and Phe, the affinity of the toxin to the receptor was inversely related with size of the side chain (Goldstein *et al.*, 1994). This implies that bigger side chains are sterically hindering CTX from fitting into the receptor site of the channel. For example, the Gly to Leu mutation decreased the affinity of CTX to the receptor by 2000-fold.

Secondly, in position 394, the presence of a positively charged residue (Arg) probably results in the resistance of SKv1.1 current to CTX. Other *Shaker* channels, rat Kv1.5 and *Aplysia* Ak01a, that have Arg at this position are highly resistant to CTX (Pak *et al.*, 1991; Pfaffinger *et al.*, 1991). When the Thr residue at this position in the *Drosophila Shaker* channel is mutated to a positively charged Lys, the channel becomes extremely resistant to CTX, implying an electrostatic repulsion between the toxin and the receptor although an electrostatic repulsion has not been demonstrated experimentally. Taken altogether, the resistance of SKv1.1 channel to CTX appears to be due to the presence of two amino acid residues (Leu370 and Arg394) around the pore region.

It is interesting to note that the residue 394, Arg in SKv1.1, is known to be an important determinant for both TEA and CTX sensitivity. These two blockers bind to the pore in

a mutually exclusive manner (Miller, 1988; Goldstein and Miller, 1993). This residue is also important in determining the requirement of external K^+ for channel opening (Pardo *et al.*, 1992). The rKv1.4 channel, which has a positively charged Lys at this position, shows an extreme requirement of the presence of external K^+ for channel opening, but the dependence disappears when the Lys is replaced by Tyr. Since SKv1.1 has an Arg at this position, one would expect it to require the presence of external K^+ for channel opening. However, this hypothesis has not been tested in this study.

Only limited information is available for the molecular mechanisms for the blocking actions of 4-AP (Kirsch et al., 1993; Yao & Tseng, 1993), DTX (Hurst et al., 1991) and MCDP. Amino acid sequence comparison among SKv1.1 and other *Shaker* channels has not been attempted for these blockers.

D. Is the expression of A-type current measured in isolated muscle fibers directed by SKv1.1 gene?

The easiest way of addressing this question is to compare the electrophysiological and pharmacological properties of SKv1.1 current to those of native A-current. The electrophysiological properties of SKv1.1 current are much like those of the rapidly inactivating outward current that has been measured from the isolated muscle fibers of *S. mansoni* (Table 6)(Day *et al.*, 1993; Day *et al.*, 1994). In

terms of voltage-dependence, the SKv1.1 current and the native current share very similar V_{50}'s for both activation and inactivation and a similar slope factor for activation. In terms of kinetics, SKv1.1 current and the native current display very similar time constants of activation and inactivation. The most apparent difference between the SKv1.1 current and the native muscle current is their pharmacological properties. The current mediated by SKv1.1 is almost completely blocked by 1 mM 4-AP, while the native current requires 30 mM 4-AP for complete blockade. Whereas the SKv1.1 current was not blocked by 100 mM TEA^+, 30 mM TEA^+ produced a 50% inhibition of the native current.

There are several factors that make the direct comparison between oocyte current and native current difficult. Firstly, one-electrode patch clamping has been employed to measure native current in isolated muscle fibers while two-electrode recording has been used to measure SKv1.1 current in oocytes. Secondly, the native currents are contaminated by other currents, making it difficult to isolate a pure current. Thirdly, different cells provide different environments for the expression as well as the modulation of the channel.

There are some examples in which the same channel gene, when expressed in different cells, shows different properties including conductance, kinetics and pharmacology. When rat Kv1.1 channel gene is expressed in frog oocytes and

in a fibroblast cell line, the channel shows different conductances (Stühmer *et al.*, 1988; Koren *et al.*, 1990). The time course of inactivation is much slower in oocytes compared with the expression in a mammalian cell (Grissmer *et al.*, 1992). Also, when rat Kv1.3 channel is expressed in frog oocytes and the pharmacology of the current is compared to the native lymphocyte current, different sensitivities to external TEA, quinine and verapamil have been observed (Douglas *et al.*, 1990).

Some possible mechanisms for these differences have been suggested. Different cells have different abilities to express the channel protein resulting in a different amount of the channel protein in the cell membrane. Although the exact mechanism has not been revealed, the level of expression determines the electrophysiology and pharmacology of the channel (Guilemare *et al.*, 1992; Honore *et al.*, 1992). The sodium channel, when expressed in frog oocytes and in *Electophorus electroplax*, exhibits different sensitivity to CTX, which proves to be due to a different posttranslational modification, the glycosylation of the channel (Thornhill & Levitan, 1987). There are also many modulatory factors that affect the properties of the channel in different cells including the presence of different cytoplasmic factors (Marom *et al.*, 1993), ß-subunit of K^+ channel (Rettig *et al.*, 1994) or functionally associated protein kinases (Esguerra *et al.*, 1994).

The SKv1.1 current measured from frog oocytes displayed characteristics similar to native muscle current except for the pharmacology. Further evidence comes from the immunohistochemistry on the isolated muscle fibers in which SKv1.1-specific antibody (ASK1) specifically labels two types of muscle fibers from which A-type currents were originally measured. Therefore, based on results from both electrophysiological and immunohistochemical studies, the expression of A-type currents in isolated muscle fibers appears to be directed by SKv1.1 gene.

III. Immunohistochemistry

A. What is the function of SKv1.1 current in the nervous system and the neuromuscular system?

Strong immunoreactivity to SKv1.1 has been observed in many excitable cells including neurons and muscle fibers.

A-type currents modulate the excitability of neurons. In axons, A-type currents modulate the onset, duration and frequency of action potentials (Connor and Stevens, 1971; Segal *et al.*, 1984; Storm, 1987; Kaang *et al.*, 1992). In nerve terminals, A-type currents also regulate neurotransmitter release and synaptic efficacy (Shimahara, 1981; Jan *et al.*, 1977; Kang *et al.*, 1992). A-type currents also modulate postsynaptic excitability (Cassell and McLachlan, 1986).

Consistent with their diverse functions, A-type

currents are localized in various regions of neurons including dendrites, axon fibers and nerve terminals. Recently, subcellular segregation of various types of mammalian *Shaker* channels have been demonstrated *in vivo* indicating differential roles of K^+ channels depending on different subcellular locations (Sheng *et al.*,1992; Sheng *et al.*, 1994). Obviously, determination of subcellular localization of SKv1.1 protein in the nervous system of *S. mansoni* could be useful for understanding the detailed functions of SKv1.1 protein in neurons.

The function of SKv1.1 protein in muscle fibers is not clear. Neither oscillation of membrane potentials nor the firing of action potential have been observed in the isolated muscle fibers of *S. mansoni*.

In addition to the distribution of SKv1.1 protein, the voltage-dependence of SKv1.1 may help to understand the function of SKv1.1 in the regulation of action potentials. A-type channels modulate the excitability of excitable cells in three ways. First, they delay the initiation of the action potential. Secondly, they decrease the duration of action potentials by facilitating the repolarization of the membrane potential. Thirdly, they, activated by an afterhyperpolarization following action potential repolarization, delay the onset of the next action potential, decreasing the frequency of action potentials.

The V_{50} of the steady-state inactivation of SKv1.1

is -40 mV. The resting membrane potential of isolated or *in situ* (Bricker *et al.*, 1982) muscle fibers is around -30 mV. At this membrane potential, only about 10% of SKv1.1 channels are available, not providing enough channels for the first and second functions described above. Therefore, it is more likely that SKv1.1 protein, activated by afterhyperpolarization, may modulate the frequency of action potentials rather than the onset or duration, unless there is some mechanism that cycles the membrane potential and recruits more SKv1.1 channels without hyperpolarization.

Based on the sequence comparison, the SKv1.1 channel is expected to require extracellular K^+ for channel opening like mammalian RCK4 channel (Pardo *et al.*, 1992). Higher extracellular K^+ concentration also increases the amplitude of the current mediated by RCK4. Therefore, it is possible that SKv1.1 may regulate the excitability of many cells in an activity-dependent manner, being more active when the surrounding concentration of K^+ increases as a result the activity of neighboring cells.

SKv1.1 also contains many putative sites for phosphorylation by protein kinases such as protein kinase C, tyrosine kinase and casein kinase II. Isolated muscle fibers react to many neurotransmitters and neuropeptides that may be linked to the modulation of the SKv1.1 channel. This indicates that SKv1.1 may modulate the excitability of cells in combination with other receptors.

SUMMARY

1) A cDNA (SKv1.1) encoding a *Shaker*-related K^+ channel has been isolated from an adult cDNA library of the human parasitic trematode *Schistosoma mansoni*.

2) The deduced amino acid sequence (512 aa, 56.5 kDa) contains structural features that are common to other voltage-gated K^+ channels.

3) Dendrogram analysis shows that SKv1.1 is classified in the *Shaker* family. The presence of a *Shaker* channel in *Schistosoma* supports the conclusion that the gene duplication event that generated four *Sh* subfamilies happened before the emergence of *Schistosoma*.

4) SKv1.1 shows significant sequence identity with most other *Shaker* channels, with 64-74% identity in the core region (S1-S6), but not at the N- and C-terminal ends.

5) Evidence for the presence of alternative transcripts of SKv1.1 was not found. Northern blot analysis detected a single primary transcript of 2.8 kb. Southern blot analysis indicated that SKv1.1 is present as a single copy in the genomic DNA of *S. mansoni*.

6) Expression of SKv1.1 in *Xenopus* oocytes produced a rapidly activating and inactivating outward K^+ current which is highly sensitive to 4-AP, but is insensitive to external TEA, MCDP, DTX and CTX.

7) Immunohistochemistry on isolated muscle fibers using

antibody specific to SKv1.1 demonstrated the expression of SKv1.1 gene in frayed fibers and crescent fibers where A-type currents have been measured. In combination with the results of the electrophysiological characterization, A-type currents in the isolated muscle fibers appear to be directed by SKv1.1 gene.

8) Immunohistochemistry on paraffin sections of *S. mansoni* has demonstrated the presence of SKv1.1 proteins in neurons as well as muscle cells. Much stronger immunoreactivity was found in the central and peripheral nervous system of female and male worms including the anterior ganglia, the central commissure, the longitudinal nerve cords, the nerve plexus and many cell bodies in the head region and the body. Mild immunoreactivities have been observed in the longitudinal, circular and ventral muscle fibers as well as tubercles of male worm.

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