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L-GLUTAMATE INDUCED CONTRACTIONS IN ISOLATED *SCHISTOSOMA MANSONI* FIBERS: EVIDENCE FOR A GLUTAMATE TRANSPORTER

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Cynthia Lynn Miller

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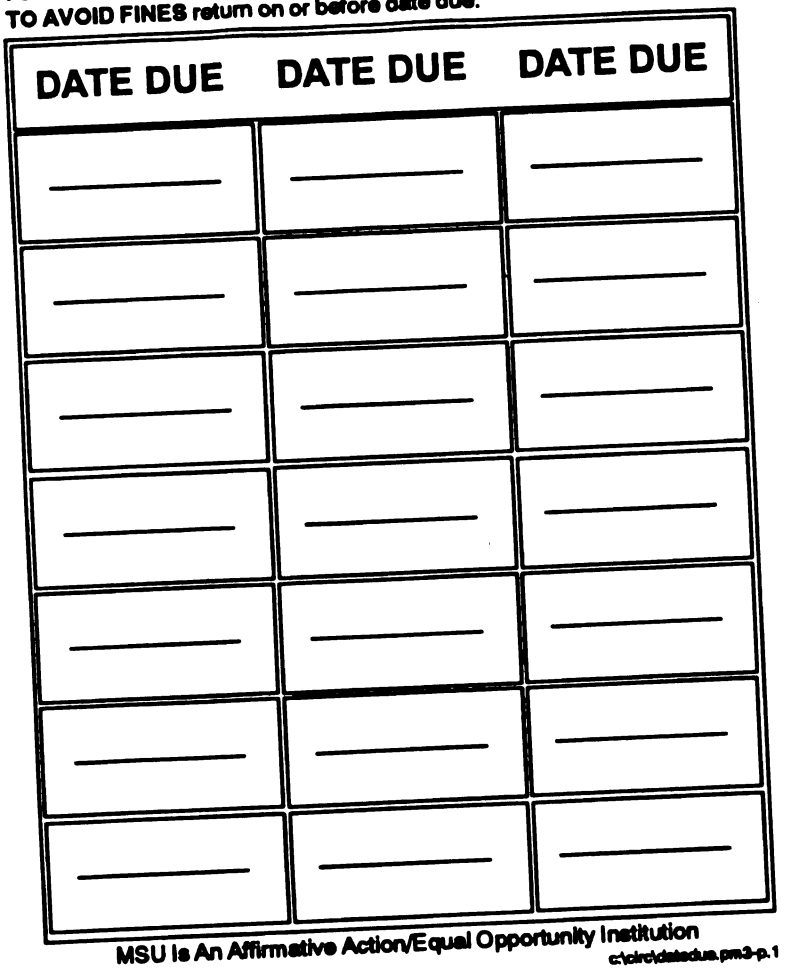

L-GLUTAMATE INDUCED CONTRACTIONS IN ISOLATED *SCHISTOSOMA MANSONI* MUSCLE FIBERS: EVIDENCE FOR A GLUTAMATE TRANSPORTER

by

Cynthia Lynn Miller

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ABSTRACT

L-GLUTAMATE INDUCED CONTRACTIONS IN ISOLATED *SCHISTOSOMA MANSONI* MUSCLE FIBERS: EVIDENCE FOR A GLUTAMATE TRANSPORTER

by

Cynthia Lynn Miller

Schistosoma mansoni muscle fibers contracted in response to L-glutamate in a dose-dependent manner (10^{-6}-10^{-3}M). D-Glutamate, L-aspartate and D-aspartate also caused contraction of the fibers. The glutamate receptor agonists NMDA, ibotenate, kainate, AMPA, quisqualate, ACPD, and L-AP4 produced little or no contraction at concentrations as high as 1 mM. The glutamate receptor antagonists, MK-801, CNQX, AP-5, and MCPG, did not block glutamate responses. However, other amino acids, L-aspartate, L-cysteate, and cysteine sulfinate, were found to elicit contraction of the muscle fibers. Contraction induced by L-glutamate is dependent on extracellular Ca^{++} and is blocked by the voltage-gated Ca^{++} channel blocker nicardipine (10 and 1 μM). [^{3}H]-L-Glutamate, incubated with the muscle fiber preparation, was taken up in a dose-dependent manner, which is also time- and temperature-dependent. Both the L-glutamate induced contractile response of the fibers and [^{3}H]-L-glutamate uptake are Na^+-

dependent, and can be blocked by specific inhibitors of the high-affinity transporter, DL-threo-β-hydroxyaspartate, and L-*trans*-pyrollidine-2,4-dicarboxylic acid (THA, PDC). This pharmacology suggests that there may be an electrogenic glutamate transporter on the muscle fibers. It is possible that the electrogenic nature of the transporter is causing the fiber membrane to depolarize, thereby opening voltage-gated Ca^{++} channels, and raising intracellular Ca^{++} concentrations leading to contraction. This experimental evidence supports the hypothesis that there is a Na^{+}-dependent high-affinity glutamate transporter on the schistosome muscle membrane.

To my David

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Best wishes for the future to my lab mates Kati Loeffler, Tim Day, Eunjoon Kim, and Ming Tien. I also would like to acknowledge contributions made by Mary Thomas, George Chen, Helen Cirrito and Mary Lou Pax.

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ABBREVIATIONS

AAD	DL-α-Aminoadipic acid
ACPD	*trans*-(±)-1-Amino-1,3-cyclopentanedicarboxylic acid
AMPA	(±)-α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrobromide
L-AP-4	L-amino-4-phosphonobutanoate
D-AP-5	D-amino-5-phosphonopentanoate
CA	Cysteaic acid
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
CSA	Cysteine sulfinic acid
DHK	Dihydrokainic acid
DMEM	Dulbecco's Modified Eagle's Medium
EAAC	Excititory amino acid carrier
EAAT	Excititory amino acid transporter
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether)N,N,N,N'- tetraacetic acid
GLAST	L-Glutamate/L-aspartate transporter
GLT	L-Glutamate transporter
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HCA	L-Homocysteic acid
I-DMEM	Inorganic Dulbecco's Modified Eagle's Medium
KA	Kainic acid
MCPG	α-methyl-4-carboxyphenylglycine
MK-801	(5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate
NMDA	N-methyl-D-aspartic acid
PDC	L-*trans*-pyrollidine-2,4-dicarboxylic acid
THA	DL-*threo*-β-hydroxyaspartic acid

INTRODUCTION

I. Glutamate is an Excitatory Chemical Messenger

L-Glutamate is the principle excitatory neurotransmitter in the mammalian central nervous system (Hediger *et al.*, 1995). In the past it was thought that an amino acid widely involved in protein synthesis and metabolism could not also function as a neurotransmitter. Now it is known that glutamate meets the criteria set for a neurotransmitter candidate. Glutamate is stored in synaptic vesicles, and it's release is Ca^{++}-dependent. Glutamate binds receptor subtypes with high-affinity, and termination of its action is thought to occur by high-affinity transport out of the synapse (Chamberlin & Bridges, 1993).

Glutamate neurotransmission is responsible for a broad spectrum of activities, ranging from neuronal plasticity to neurotoxicity. Long-term potentiation (LTP) is a process implicated in learning and memory acquisition (Seeburg, 1993). LTP is characterized by a sustained increase in synaptic efficacy (Riedel & Reymann, 1993), in which various glutamate receptors play a central role.

Glutamate has been implicated in several pathogenic processes. Over-stimulation of glutamate receptors, most notably the Ca^{++} permeable N-methyl-D-

aspartate (NMDA) receptor, has been shown to lead to neuronal degeneration during ischemia (Nakanishi, 1992). When rat astrocytes are placed in a hypoxic environment, glutamate uptake is reduced by 35-45% (Swanson *et al.*, 1995). It is speculated that glutamate is also involved in several neurodegenerative diseases. Amyotrophic lateral sclerosis is a neurodegenerative disorder characterized by death of motor neurons. It is thought to be associated with abnormal metabolism of glutamate involving the high-affinity glutamate transporter (Rothstein *et al.*, 1992). In the brains of humans with Alzheimer disease, it appears that different cortical regions have distinct glutamate transporter pharmacology when compared to normal controls at autopsy (Scott *et al.*, 1995). The study of glutamate neurotransmission and the involvement of glutamate in neurodegenerative disease are rapidly-growing fields.

A. Mammalian Glutamate Receptor Subtypes

The mammalian glutamate receptors have been thoroughly characterized, both pharmacologically and by molecular biological techniques. The mammalian glutamate receptors are more recently referred to as the excitatory amino acid receptors, and can be divided into two broad categories, the ionotropic and metabotropic receptors (Table 1). The ionotropic receptors are multimeric and contain an intrinsic cation-specific ion channel (Seeburg, 1993), while

Table 1. The mammalian glutamate receptor subtypes according to their pharmacological characterization. Those agonists which are underlined were employed in the characterization of the glutamate contractile response of the isolated Schistosome muscle fiber. Although this categorization is oversimplified and incomplete, it provides a basic outline of the mammalian glutamate receptor subtypes. ACPD, *trans*-(±)-1-Amino-1,3-cyclopentanedicarboxylic acid; AMPA, (±)-α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrobromide; L-AP-4, L-amino-4-phosphonobutanoate; AP-5, D-amino-5-phosphonopentanoate; CNQX, 6-Cyano-7-nitroquinoxaline-2,3-dione; DCG-IV, (2s,1'R,2'R,3'R)--2-(2',3'-dicarboxycyclopropyl)glycine; DHPG, 3,5-dihydropheylglycine; DNQX, 6,7-dintroquinoxaline-2,3-dione; MAP4, α-methyl-L-AP4; MCCG 2s,1's,2's-2-methyl-2-(2'carboxycylopropyl)glycine; MCPG, α-methyl-4-carboxyphenylglycine; MK-801, (5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-mine hydrogen maleate; NBQX, 6-nitro-7-sulphamobenzo(*f*)quinoxaline-2,3-dione; NMDA, N-methyl-D-aspartic acid.

	IONOTROPIC			METABOTROPIC	
	NMDA	**AMPA**	**Kainate**	**L-AP4**	**ACPD**
Gene	nmda1 nmda2A-2D	glu1-glu4	glu5-glu7, ka1, ka2	mglu4, mglu6, mglu7	mglu1-mglu7
Agonists	NMDA Aspartate Ibotenate	AMPA Quisqualate	Kainate Domoate	L-AP4 ACPD	ACPD DCG-IV DHPG L-AP4
Antagonists	AP-5 MK-801 PCP	CNQX DNQX NBQX	CNQX DNQX	MAP4	MCPG MAP4 MCCG
Mechanism	ion channel Ca^{++},Na^{+},K^{+}	ion channel Na^{+},K^{+}	ion channel Na^{+},K^{+}	Gi, (-) AD	Gi, (-) AD Gs (+) AD Gq, (+) IP_3 G (+) AA G (+) PLD

metabotropic receptors are coupled to G proteins, and contain seven membrane spanning regions and modulate intracellular messengers (Schoepp & Conn, 1993).

The ionotropic receptor subtypes include the NMDA and (±)-∝-amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrobromide (AMPA)/kainate sensitive receptors. N-Methyl-D-aspartate (NMDA) selectively gates an intrinsic cationic channel that has a 10:1 preference for Ca^{++} to Na^{+} or K^{+}. This Ca^{++} permeability is implicated in both LTP and neurotoxicity (Monaghan *et al.*, 1989). The NMDA receptor has several unique properties, including voltage-dependent block by Mg^{++} (Sprengel & Seeburg, 1993). The NMDA receptor cation channel is open when glutamate is bound, however at negative potentials, Mg^{++} blocks current flow by binding inside the channel. The functional consequence of the Mg^{++} block is that the membrane in which the NMDA receptor resides must be depolarized for Mg^{++} to be dislodged and current to flow through the channel. This provides an important method of regulating the predominately Ca^{++} current. In addition, NMDA receptors are modulated by glycine, and without glycine the channel will not open even when glutamate is bound (Barnard, 1992). It has been found that the NMDA subunit composition and alternative splicing are responsible for altering ion specificity, size of the current, degree of susceptibility to Mg^{++} blockade, ability of glycine to stimulate, and affinity for agonists (Nakanishi, 1992).

The ionotropic receptor subtypes, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate, were first described pharmacologically (Monaghan *et al.*, 1989). It is now known that their sensitivity to agonists, and their electrical properties are derived from their molecular subunit composition (Seeburg, 1993). AMPA and kainate subunits may combine with themselves, or with each other, to form unique receptors regarding pharmacological profile, ion selectivity and kinetic parameters (Seeburg, 1993). In addition to subunit composition, alternate splicing and RNA editing both play a role to further diversify these receptors (Nakanishi, 1992).

Both AMPA and kainate receptor channels are predominately permeable to Na^+ and K^+ (Sprengel & Seeburg, 1993). AMPA receptor subtypes display fast kinetics, and are responsible for the majority of fast excitatory neurotransmission in the mammalian central nervous system (Seeburg, 1993). Usually AMPA type receptors are characterized by low Ca^{++} permeability, however, subunit assembly, RNA editing, and alternative splicing can alter the exact Ca^{++} permeability of these channels (Mishina *et al.*, 1991).

Metabotropic glutamate receptor subtypes produce their effects through the activation of G-proteins and subsequent modulation of intracellular messengers such as inositol 1,4,5-trisphosphate, cyclic adenosine monophosphate (cAMP), and phosphlipase D (PLD) (Schoepp & Conn, 1993; Boss & Conn, 1992). Recently

specific agonists and antagonists have become available to aid in characterizing the metabotropic receptors. At least seven metabotropic glutamate receptors have been cloned (Winder & Conn, 1995).

B. Mammalian Glutamate High-Affinity Na^+-Dependent Transport

The high-affinity glutamate transporter is thought to terminate the action of glutamate in the CNS by rapidly removing it from the synapse (Hediger *et al.*, 1995). This makes the transporter crucial for normal synaptic function. These high-affinity transporters have also been implicated in such processes as excitotoxicity, epilepsy, and neurodegenerative diseases. The first studies of glutamate transport employed synaptosomes and epithelial membrane vesicles (Kanai *et al.*, 1993). From this work, two types of glutamate transporters were characterized, the high-affinity (K_m=2 to 50 μM), and the low-affinity transporter (K_m>100 μM). Because high-affinity glutamate transporters are thought to be involved in termination of glutamate fast excitatory synaptic transmission, the majority of information collected to date, concerns the high-affinity uptake systems.

Historically, amino acid transporters have been characterized by substrate specificity and ionic dependence. Initial pharmacological studies in the vertebrate CNS indicated that there is probably more than one type of high-affinity

transporter for excitatory amino acids (Kanai *et al.*, 1993). Recently, several groups set out to clone the glutamate transporters (Bouvier *et al.*, 1994). However, traditional cloning strategies proved unsuccessful, because they relied on sequence similarity to other cloned transporters which belong to the superfamily which includes GABA, glycine, norepinephrine, dopamine, and serotonin transporters (Kanai *et al.*, 1993).

In 1992 it was independently discovered, by several groups, that the glutamate transporters do not belong in this superfamily of transporters, but are part of a new family, which includes *gltP* a Na^+-independent glutamate-proton transporter from *E. coli*, *gltT* the Na^+/proton glutamate transporter from *B. stearothermophilus*, *dctA* the C4-dicarboxylate transporter, and ASCT1 which is a human neutral amino acid transporter (Arriza *et al.*, 1993; Bouvier *et al.*, 1994). This new family of transporters is diverse in tissue distribution, kinetics, and pharmacological profile (Bouvier *et al.*, 1994).

The high-affinity glutamate transporters are electrogenic. It is currently hypothesized that two Na^+ ions are co-transported with each molecule of glutamate and that one K^+ and one OH^- are countertransported (Bouvier *et al.*, 1992; Kanai *et al.*, 1993). However unpublished results from the laboratory of M. Kavanaugh reveal that there may be three Na^+ ions co-transported with each molecule of glutamate (personal communication). Additional studies of the glutamate

transporters have shown that certain subtypes also mediate a chloride current (Wadiche *et al.*, 1995a). Regardless of the exact stoichiometry, inward transport of glutamate will produce a depolarizing current.

Presently the mammalian glutamate transporters can be grouped into three categories, the EAAC-like transporters, the GLAST-like transporters, and the GLT-like transporters. These categories have been named according to the name of the gene first cloned for each subtype. This categorization of the cloned high-affinity glutamate transporters is most likely an oversimplification of the actual number of transporter categories that exist (Arriza *et al.*, 1994). As more transporters are cloned, the relationships between the subtypes will become more clear.

The first EAAC-like transporter to be cloned was from rabbit small intestine (EAAC1) using *Xenopus oocyte* expression methods (Table 2) (Kanai & Hediger, 1992). From this sequence, the analogous human and mouse genes have been cloned using PCR techniques (EAAT3 and MEAAC1). The EAAC-like transporters are 523-525 amino acids in length, are all found in the neuronal tissue in the brain, and have K_m values ranging from 12 to 28 μM for glutamate. It is difficult to compare the pharmacology of the transporters, because several different methods have been used to generate the pharmacological profiles. In general, the EAAC-like transporters appear to transport L-glutamate, and L-and

Table 2. The EAAC-like high-affinity glutamate transporter clones. EAAT3, the human transporter, has 92% identity with the rabbit transporter EAAC1, and MEAAC1, the mouse transporter, has 89.3% identity with EAAC1. AAD, aminoadipate; L-asp, L-aspartate; D-asp, D-aspartate; CA, cysteate; CSA, cysteine sulfinate; DHK, dihydrokainate; EAAC, excitatory amino acid carrier; EAAT, excitatory amino acid transporter; L-glut, L-glutamate; D-glut, D-glutamate; HCA, homocysteate, KA, kainate; PDC, *trans*-pyrollidine-2,4-decarboxylate, THA, DL-*threo*-β-hydroxyaspartate

	EAAC-like		
clone	**EAAC1 (rabbit)**	**EAAT3 (human)**	**MEAAC1 (mouse)**
immunohisto-localization	brain (neurons) intestine kidney liver heart	brain kidney placenta lung muscle	brain kidney lung muscle
size (amino acids)	524	525	523
L-glutamate	K_m = 12 μM oocyte (current)	K_m = 24±2 μM cos cell (transport) K_m = 28±6 μM oocyte (current)	na
other agonists	oocyte current K_m values THA=6.9μM DHK>1mM AAD=201μM	oocyte current K_m values THA=37±1 μM DHK (no current) KA (no current) PDC=27±5 μM L-asp=24±2 μM D-asp=47±8 μM D-glut =1.78mM	na
antagonists	IC_{50} for 20μM L-glutamate current THA 7.1μM DHK >1mM AAD 165μM	K_i for 1uM ^{3}H-glut transport PDC 61±14 μM THA 25±5 μM DHK > 3mM KA > 3mM CA 19±9 CSA 17±2	na
references	Kanai & Hediger, 1992	Arriza *et al.*, 1994; Shashidharan *et al.*, 1994; Wadiche *et al.*, 1995b	Freund *et al.*, 1995

D-aspartate, and both cysteate and cysteine sulfinate can block the uptake of L-[^{3}H]glutamate. The specific transport inhibitors *trans*-pyrollidine-2,4-decarboxylate (PDC), and DL-*threo*-β-hydroxyaspartate (THA) block [^{3}H]-L-glutamate uptake and inhibit the glutamate-induced depolarizing current in patch clamped oocytes injected with EAAC1 message (Kanai & Hediger, 1992; Wadiche *et al.*, 1995b). However, dihydrokainate (DHK), kainate, and aminoadipate (AAD) were not effective inhibitors.

The first GLAST-like transporter to be cloned was from a rat cDNA library which was screened with an oligonucleotide derived from the partial sequence of a purified protein responsible for transporter activity (Storck *et al.*, 1992). Based on this sequence, Inoue et al. (1995) cloned a transporter from the bovine retina, BNGLUAS; Tanaka (1993) cloned a transporter from mouse brain, mGLuT-1; and Arriza *et al.* (1994) cloned a human GLAST-like transporter, EAAT1. These transporter proteins are 542-543 amino acids in length and the K_m values range from 20 to 77 μM (Table 3). D-glutamate was not a good substrate or inhibitor of the GLAST-like transporters. In general, DHK and kainic acid were not able to block the GLAST-like transporters, with the exception of the transporter cloned from the mouse (MGlut-1) (Tanaka, 1993).

GLT-like transporters have been cloned from the rat, human and mouse. The rat transporter, GLT-1, was the first to be cloned by Kanner *et al.* (1992).

Table 3. The GLAST-like high-affinity glutamate transporter clones. The GLAST-like transporters have sequence similarity with the prokaryotic glutamate transporters GTLP (Kanai *et al.*, 1993). AAD, aminoadipate; L-asp, L-aspartate; D-asp, D-aspartate; CA, cysteate; CSA, cysteine sulfinate; DHK, dihydrokainate; GLAST, L-glutamate/L-aspartate transporter; L-glut, L-glutamate; D-glut, D-glutamate; HCA, homocysteate, KA, kainate; PDC, *trans*-pyrollidine-2,4-decarboxylate, THA, DL-*threo*-β-hydroxyaspartate

	GLAST-like			
clone	**GLAST (rat)**	**BNGLUAS (cow)**	**MGLuT-1 (mouse)**	**EAAT1 (human)**
immunohisto-localization	brain glial cells (bergmann)	retina na	brain lung muscle spleen testes	brain heart placenta muscle
size (amino acids)	543	542	543	542
L-glutamate	K_m = 77μM oocyte (current) K_m = 12 μM oocyte (current)	K_m = 38.1±14μM oocyte (current)	K_m = 72 μM oocyte (transport)	K_m = 20 μM oocyte (current) K_m = 48±10μM cos-7 (transport)
pharmacology	K_i for 50nM ^{3}H-glutamate oocyte transport L-glut 70μM L-asp 65μM CSA 80μM THA 65μM HCA 2.7mM KA 3mM DHK 3.1mM AAD 10mM	% Displacement of 1uM ^{3}H-glutamate transport using 100uM inhibitor THA 97% CA 87% L-glut 80% L-asp 70% PDC 41% D-asp 8% KA 5%	% Displacement of transport 50nM ^{3}H-Lglut by 0.1mM cold substrate L-glut 77±8% D-glut 2±0% L-asp 75±9% D-asp 66±5%	K_i for 1uM ^{3}H-glutamate oocyte transport CA 10±3μM CSA 14±7μM THA 32±8μM PDC 79±7μM DHK >3mM KA >3mM
	% Displacement of 100μM L-glut transport THA 9mM=90%		% Displacement of transport 50nM ^{3}H-Lglut by 100 μM cold substrate DHK 35±5% THA 57±4%	K_m oocyte (current) L-asp 16±1μM D-asp 23±2μM D-glut 595±50μM THA 33±3μM PDC 28±2μM KA no current DHK no current
references	Storck *et al.*, 1992; Klockner *et al.*, 1993, 1994; Tanaka, 1994	Inoue *et al.*, 1995	Tanaka, 1993	Arriza *et al.*, 1994; Wadiche *et al.*, 1995b

Table 4. The GLT-like transporters. AAD, aminoadipate; L-asp, L-aspartate; D-asp, D-aspartate; CA, cysteate; CSA, cysteine sulfinate; DHK, dihydrokainate; GLT, glutamate transporter; L-glut, L-glutamate; D-glut, D-glutamate; HCA, homocysteate, KA, kainate; PDC, *trans*-pyrollidine-2,4-decarboxylate, THA, DL-*threo*-β-hydroxyaspartate

	GLT-like		
clone	**GLT-1 (rat)**	**EAAT2 (human)**	**mGLT-1 (mouse)**
immunohisto-localization	brain astrocytes	brain placenta	brain
size (amino acids)	573	574	572
L-glutamate	K_m = 2 μM hela cells (transport)	K_m = 97±4 μM cos cell (transport) K_m = 18±3 oocyte (current)	
pharmacology	K_i [^{3}H]glutamate transport L-asp Ki=0.2uM D-asp Ki=0.6 uM CSA Ki=1.7uM PDC Ki=0.73uM THA Ki=1.0 uM	K_m (current) L-asp 7±1 μM D-asp 13±1μM D-glut 5.4±0.4 mM PDC 7±0 μM THA 10±1 μM	
	% inhibition using 100 uM inhibitor AAD = 81% DHK = 97%	K_i against 1μM L-[^{3}H]glutamate KA = 59±18 μM DHK = 23±6 μM PDC = 8±2 μM CA = 10±2 μM CSA = 6±1	
references	Pines *et al.*, 1992	Arriza *et al.*, 1994; Wadiche *et al.*, 1995b	Freund *et al.*, 1995

This group used an antibody to the purified glial transporter protein to screen a λ_{zap} library from rat brain. Again, using an oligonucleotide based on sequence similarity, the human EAAT2, and mouse mGLT-1 transporters were cloned (Arriza *et al.*, 1994; Freund *et al.*, 1995). The GLT-like transporters are 572-574 amino acids in length, and the K_m values for glutamate range from 2 to 97 μM (Table 4). The GLT-like transporters are pharmacologically different from the other transporters because DHK inhibits glutamate transport and is itself transported. Also, AAD inhibits glutamate transport in GLT-1. Otherwise, the pharmacology is quite similar to the previously-described glutamate transporter subtypes.

Although several of the transporters have been immunohistolocalized to tissues other than the brain (including intestine, kidney, heart, and muscle), little is known regarding their physiological function in these tissues. Hediger et. al. (1995), suggest that the transporters found in the intestine and kidney are involved in trans-epithelial glutamate transport. Glutamate transporters are also thought to be involved in cellular amino acid nitrogen metabolism (Arriza *et al.*, 1994). Presumably, glutamate transporters could also serve to transport glutamate in to cells to be incorporated into protein. While glutamate receptors and transporters have been widely studied in the mammalian central nervous system, less information is available about glutamate receptors or transporters in invertebrates.

C. Glutamate and Invertebrate Muscle

Glutamate is hypothesized to be a neurotransmitter in several invertebrates. It has been suggested that glutamate is a primordial neurotransmitter, which developed as a signal molecule before the evolution of specialized neurotransmitters (Schuster *et al.*, 1991). In the primitive Phylum Coelenterata, glutamate is the most abundant free amino acid in the sphincter muscle of the sea anemone *Actina equina*, and glutamate inhibits electrically-induced contraction of isolated sphincter muscle preparations (Carlyle, 1974; Walker & Holden-Dye, 1989). This work was done before mammalian glutamate receptor subtypes had been well-characterized, and the authors were hesitant to suggest that glutamate was functioning as a neurotransmitter.

Glutamate receptors are present on crayfish muscle at the neuromuscular junction, and glutamate serves as the excitatory neuromuscular transmitter in these animals (Takeuchi & Takeuchi, 1964). Solubilization and purification of a glutamate receptor from crustacean muscle yielded a protein which binds glutamate and quisqualate with high affinity (Gray *et al.*, 1991). Outside-out patches of crayfish muscle revealed glutamate-activated cation channels (Dudel *et al.*, 1990). In addition, Shinozaki *et al.* described an excitatory glutamate receptor that is found extra-junctionally on the crayfish muscle, and that is sensitive to kainate (Shinozaki & Ishida, 1992). This same group also characterized a

presynaptic metabotropic glutamate receptor which is sensitive to 2S,3S,4S-2-(carboxycyclopropyl)glycine (L-CCG-1).

Insect muscle membrane also contains glutamate receptors. Locust muscle membrane has glutamate-sensitive currents, and patch clamp studies by MacDonald *et al.*, revealed the presence of a quisqualate-sensitive receptor (Cull-Candy & Parker, 1982; Bates *et al.*, 1990; MacDonald *et al.*, 1992). A glutamate receptor subunit from *Drosophila* muscle has now been cloned, and it exhibits sequence similarity with ionotropic mammalian glutamate receptor subunits (Schuster *et al.*, 1991). Glutamate and aspartate elicit a current across the recombinant protein expressed in *Xenopus* oocytes. Based on sequence divergence, the authors concluded that this protein is an evolutionarilly-distant subtype of excitatory glutamate receptor.

In the smooth muscle of *Aplysia* anterior aorta, glutamate is a potential excitatory neurotransmitter (Sawada *et al.*, 1984). L-glutamate depolarizes the muscle and this response is blocked by 2-APB (a glutamate receptor antagonist) and mimicked by L-aspartate. This glutamate effect was not modulated by glycine.

It is difficult to relate information about possible invertebrate glutamate subtypes to the well-characterized mammalian glutamate receptor subtypes, because there is little evidence that invertebrate glutamate receptors have the same

pharmacological profiles or physiological roles as their mammalian counterparts. It is necessary to study the invertebrate glutamate-induced effects without the bias of mammalian subtypes.

D. Glutamate in Flatworms

Within the phylum platyhelminthes, glutamate has been hypothesized to be an excitatory neurotransmitter. Glutamate is the most abundant amino acid in *S. mansoni* proteins and is also an abundant free amino acid, second only to alanine (Chappell, & Walker, 1982; Webb, 1986). Several techniques have been used to visualize areas of concentration of the free amino acid glutamate.

Immunocytochemical techniques have been performed on *Trichibilharzia ocellata* and *S. mansoni* using freeze-drying-paraformaldehyde fixation. The results showed localization of immunoreactivity in both species in the main commissure and longitudinal nerve trunks of the cercaria (Solis-Soto & Brink, 1994). Glutamate-like immunoreactivity has also been shown in the longitudinal nerve cords and in sites of sacroneural intervention of muscle in the cestode *Hymenolepis diminuta* (Webb & Eklove, 1989). Sacroneural innervation is a term used to describe the cestode neural-muscular relationship, where the muscle contains a cytoplasmic extension which contacts the nerve cord (Webb, 1987). Webb and Eklove (1989) used a primary antibody directed toward a glutamate-

glutaraldehyde-protein conjugate, and fixed the flatworm tissue with glutaraldehyde. In addition, using histofluorecence methods, Keenan and Koopowitz (1982) have shown the presence of glutamate in the longitudinal nerve cords of *Gyrocotyle fimbriata*.

Because glutamate may play a metabolic role, as well as that of a neurotransmitter, there is commonly high background staining in each of these procedures. Tissues with a high level of metabolic activity may tend to stain more intensely for glutamate (Webb & Eklove, 1989). Therefore, distinguishing between transmitter pools and metabolic pools of glutamate is problematic. Nevertheless, each of these studies suggests that glutamate is relatively concentrated in the longitudinal nerve cord, which in turn supports the hypothesis that L-glutamate is a neurotransmitter in the Platyhelminths. However, this information does not necessarily suggest a physiological role for glutamate.

Studies employing *in vitro* flatworm preparations of selected neuronal tissues have shown that L-glutamate evokes excitatory responses. The primitive cestode *G. fimbriata* has longitudinal nerve cords that have increased spontaneous activity in response to applied glutamate and aspartate, and this activity can be blocked by 2-amino-4-phosphonobutyrate (APB), a non-specific glutamate receptor antagonist (Keenan & Koopowitz, 1982).

In support of the hypothesis that glutamate is a neurotransmitter in

flatworms, Webb *et al.* demonstrated glutamate high-affinity uptake into flatworm tissue. When [^{3}H]-L-glutamate is incubated with tissue slices of *H. diminuta,* it is transported into the tissue and can be released by K^+ depolarization (Webb, 1988). This release of glutamate is Ca^{++}-dependent and is enhanced by the presence of 5-HT. Webb *et al.* (1986) also measured the kinetics of glutamate uptake in *H. diminuta* tissue slices and described both a high-affinity glutamate transport system K_t=18 μM, and a low affinity glutamate transport system K_t=220 μM. In addition, glutamate is taken up by intact *S. mansoni* adults through the tegument (Asch & Read, 1975; Cornford & Oldendorf, 1979; Chappell & Walker, 1982; Cornford, 1985). Thompson & Mettrick (1989), demonstrated Ca^{++}-dependent stimulated release of glutamate and specific glutamate binding sites. The authors suggested that glutamate is released from nervous tissue and may serve as a neurotransmitter.

Early experiments tested putative neuromuscular transmitters on intact flatworms, and the effects on the musculature were measured by force transduction. These methods failed to reveal any effect for glutamate on the schistosome musculature. However, glutamate elicited powerful rhythmic contractions when applied to longitudinal muscle preparations of *H. diminuta* (Thompson & Mettrick, 1989; Webb, 1988). The contractile response of the *H. diminuta* muscle preparation is concentration-dependent and L-glutamate has a

greater effect than D-glutamate. The anatomy of the flatworms makes it difficult to isolate individual tissues. Therefore, these longitudinal muscle preparations most likely contain a variety of tissues, including neuronal tissue. It is therefore not possible to pinpoint the site of action of glutamate to a particular tissue on the basis of these studies.

The microanatomy of the schistosome musculature was studied by Silk and Spence in 1969. They described unstriated longitudinal, circular, and radial muscle containing both thick and thin myofilaments (Silk & Spence, 1969). The schistosome muscle also contains glycogen granules and poorly-defined sarcoplasmic reticulum. The neuromuscular relationship of the schistosome has not been well-defined. However, in the cestodes, the musculature sends a cytoplasmic arm to contact the nervous system, which has been termed the sarconeural arm (Webb, 1987). This neuromuscular anatomy may also be present in the schistosomes.

The schistosomes are parasites that are able to infect humans and cause the disease complex schistosomiasis. The World Health Organization ranks schistosomiasis second only to malaria in terms of socioeconomic importance (World Health Organization, 1995). The adult *S. mansoni* parasites reside in the mesenteric veins of the host and the female produces approximately 300 eggs per day. The eggs normally pass through the mesenteric veins and into the intestine to

be released with the feces. However, 50% of the eggs become trapped in the liver. In the liver the host immune system forms granulomas around the eggs; fibrous tissue replaces these granulomas, and the resulting hepatic scarring leads to portal hypertension, esophageal varices and death.

Because several antiparasitic drugs produce marked effects on the schistosome musculature, our laboratory has strived to further understand schistosome neuromuscular physiology. Our laboratory has developed a procedure for isolating individual muscle fibers from the flatworm *S. mansoni* (Blair *et al.*, 1991). This preparation permits the direct application of neurotransmitters onto the individual muscle fibers, without other tissues present to confound results. Herein lies the first evidence of an effect produced by glutamate on the *S. mansoni* muscle fibers.

OBJECTIVES

The development of the procedure to isolate muscle fibers from the schistosome has changed the way our laboratory has studied the muscle physiology of this flatworm. It is now possible to apply neurotransmitters directly to the muscle fibers without experimental results being confounded by other tissue types. We have found that the excitatory amino acid, L-glutamate, produces contraction of the isolated muscle fibers. The main goal of this study was to characterize the glutamate-induced contraction of isolated *Schistosoma mansoni* muscle fibers to gain a greater understanding of the underlying mechanism. First, the pharmacology of the contractile response was characterized by using the microperfusion contraction assay. In addition, the ionic dependence of this glutamate-induced contractile response was characterized.

Based on the resulting data, it was determined that the glutamate-induced contractile response may be mediated by a high-affinity glutamate transporter. This putative transporter was characterized by measuring radiolabeled glutamate transport into the schistosome muscle fiber preparation. From these experiments the kinetics of the transport were determined. This response was further analyzed

in terms of ion dependence and transport pharmacology, employing the same tools used to describe the glutamate-induced contractile response in the isolated fibers. This now provides a way to compare the observations of glutamate-induced contraction and radiolabeled glutamate transport, and to understand if the electrogenic transport of glutamate could be responsible for the contraction observed in response to microperfusion of glutamate onto the isolated frayed muscle fibers.

MATERIALS AND METHODS

I. Muscle Fiber Isolation Procedure

S. mansoni muscle fibers were isolated using a modified version of the procedure previously published (Day *et al.*, 1994a). In short, Puerto Rican strain *S. mansoni* were surgically removed from mesenteric and portal veins of female ICR mice (Harlan Sprague-Dawley) 40-60 day post-infection. Adult parasites (35-45 pairs) were cut into approximately 2 mm pieces, and suspended in modified Dulbecco's Modified Eagle's Medium (DMEM) at 35-37°C. This medium has been described by Day *et al.*(1993), and consists of powdered DMEM stock dissolved in water to 67% of it's normal volume with the addition of 2.2 mM $CaCl_2$, 2.7 mM $MgSO_4$, 0.04 mM Na_2HPO_4, 61.1 mM glucose, 1.0 mM dithiothreitol (DTT), 10μM serotonin, and 0.1 mg/ml gentamicin replacing 10 mg/ml Pen-strep (pH 7.4) (Day *et al.*, 1994a). The resultant worm pieces were digested three times at 35-37°C for 10 minutes with gentle agitation in a solution of DMEM that contains 0.75 mg/ml papain, 1 mM EGTA and 1 mM EDTA. The pieces were then rinsed with enzyme-free DMEM containing 0.1% bovine serum albumin (BSA) for 10 minutes, and then washed three times with DMEM. The

individual fibers were released from the worm pieces by forcing the suspension through a Pasteur pipet approximately 30-60 times. This muscle fiber suspension was plated onto 35 mM petri dishes and left at room temperature for 30 minutes while fibers attached to the surface of the petri dishes. The media of the plated fibers was then replaced with an inorganic version of DMEM (I-DMEM) that contains 82.5 mM Na^+, 4.1 mM K^+, 3.6 mM Ca^+, 3.3 mM Mg^{2+}, 100.4 mM Cl^-, 79.9 mM glucose, 15.0 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 1.0 mM DTT, 10 μM serotonin, and 0.1 mg/ml gentamicin (pH 7.4). Fibers were stored at 18°C in I-DMEM until experimental procedure. This primary preparation was performed on the morning of each day in which data were collected, and the entire procedure takes approximately three and a half hours.

II. Microperfusion Procedure

Prior to microperfusion, the schistosome muscle fibers were incubated at 35°C for ten minutes and kept at 35-37°C throughout the microperfusion procedure by means of a heated microscope stage. Neurotransmitters and drugs were dissolved in I-DMEM, loaded into borosilicate glass micropipets (W-P Instruments, New Haven, Conn.) and perfused in a constant manner by applied positive pressure. The individual fibers were exposed to drug solutions by bringing the microperfusion pipet into the field of the fiber and applying the drug

solution directly onto the fiber. The data collected were visual observations of muscle fiber contraction. All observations were recorded on VHS tape, by means of a video camera and monitor. (Model CCD72, Dage MTI, Michigan City, IN, USA). A fiber was considered to have contracted if there was a detectable change in fiber length when viewed on the monitor. Each fiber which was microperfused was tallied as either contracting or not contracting in response to the applied drug solution. From these data, percentages of fibers responding to the drug solution were tabulated and averaged with at least three other trials. Only fibers described as the "frayed" type by Blair, *et al.* (1991), were tested in this study. Frayed fibers are easily distinguishable by their bifurcated endings and average length of 20μm length. Approximately 15-30 fibers in each petri dish were microperfused. Microperfusion of I-DMEM, which is the same media in which the fibers were bathed, served as a negative control. In some cases, microperfusion of 25 mM K^+ served as a positive control, to asses preparation viability. Often microperfusion of 1.0 and/or 0.1 mM L-glutamate was the positive control, depending on the concentration of other agonists or antagonists to be tested. Drugs and neurotransmitters were microperfused at designated concentrations and antagonists were normally added to the fiber's bath before the 37°C ten-minute incubation and remained in the dish through out the experiment. Where indicated, the inhibitors were also added to the microperfusion pipet at the same concentration. When

Table 5. Media employed in the microperfusion and glutamate uptake experiments (mM)

	modified DMEM	I-DMEM	25 mM K^+	Calcium free	Sodium Free
K^+	4.1	4.1	25.0	4.1	4.1
Na^+	82.6	82.5	82.5	82.6	-
$Ca2^+$	3.6	3.6	3.6	-	3.6
$Mg2^+$	3.3	3.3	3.3	3.3	3.3
Cl^-	93.7	100.4	16.4	93.2	17.9
SO_4^-	3.3	-	-	-	-
PO_4^-	0.04	-	-	-	-
Glucose	79.9	79.9	-	79.9	79.9
Phenol Red	11.3	-	-		-
HEPES	15.0	15.0	15.0	15.0	15.0
L-Arginine HCl	0.3	-	-	-	-
L-Cystine 2HCl	0.2	-	-	-	-
L-Glutamine	3.0	-	-	-	-
Glycine	0.5	-	-	-	-
L-Histidine HCl	0.2	-	-	-	-
L-Isoleucine	0.6	-	-	-	-
L-Leucine	0.6	-	-	-	-
L-Lysine	0.8	-	-	-	-
L-Methionine	0.2	-	-	-	-
L-Phenylalanine	0.3	-	-	-	-
L-Serine	0.7	-	-	-	-
L-Threonine	0.6	-	-	-	-
L-Tryptophan	0.1	-	-	-	-
L-Tyrosine	0.4	-	-	-	-
L-Valine	0.6	-	-	-	-
D-Ca-Pantothenate	3.0	-	-	-	-
Choline	-	-	-	-	-
Chloride	3.0	-	-	-	-
Folic Acid	3.0	-	-	-	-
Myo-Inositol	5.4	-	-	-	-
Niacinamide	3.0	-	-	-	-
Orotic Acid	37.5	-	-	-	-
Pyroxidine-HCl	3.0	-	-	-	-
Riboflavin	0.3	-	-	-	-
Thiamine Hcl	3.0	-	-	-	-
Sucrose	-	-	65.0	-	-
Gluconate	-	-	104.9	-	-
N-methyl-D-gluconate	-	-	-	-	-
EGTA	-	-	-	-	82.6
5-HT	-	-	-	0.5	-
	10^{-6}	10^{-6}	-	10^{-6}	10^{-6}

ionic concentrations of the I-DMEM was altered this modified I-DMEM was used both to bathe the fibers and to dissolve the drug to be tested (Table 5). Statistical comparisons were conducted using the two-tailed Mann-Whitney U-test ($P<0.05$).

III. Glutamate Uptake Experiments

L-[2,3,4-^{3}H]glutamate was incubated with the fiber preparation to assess the possibility that glutamate was being taken up by the muscle fibers. The preparation used in these studies was as described by the muscle fiber isolation procedure in this methods section, with the following modifications. At least 45-60 worm pairs were used for each preparation. The digested pieces were separated into two parts and the fibers in each were released by pipetting into approximately 1.7 ml of I-DMEM to produce a concentrated preparation. This concentrated fiber suspension was then allowed to rest for two minutes by which time the large unbroken worm pieces settled to the bottom and the fiber suspension could be drawn off and placed in microcentrifuge tubes (200 μl/ tube). One 200 μl aliquot was frozen to be assayed for protein content by the Albro method (Albro, 1975). Each sample was preheated to 37°C for ten minutes prior to the addition of [^{3}H]-L-glutamate. During this time, heat-shocked samples were submerged in boiling water for two minutes, indicated samples were sonicated, and specified samples received inhibitors. Unless otherwise indicated, each sample was incubated for 30

minutes at 37°C with 1 μCi of L-[2,3,4-^{3}H]glutamate, to yield a final concentration of 84 nM glutamate. After incubation, the samples were microcentrifuged (Reliable Scientific, Quick Spin-18, 16,000g) for 45-60 seconds. The supernatant was discarded and the pellet resuspended in I-DMEM. This suspension was again microcentrifuged for 45-60 seconds, and the rinse procedure repeated. Each sample was resuspended with its own Pasteur pipet, because the fibers tended to adhere to the side of the pipets. After the last rinse, the pellets were resuspended in 0.5 ml of I-DMEM and transferred to scintillation vials, and the microcentrifuge tube and pipet were rinsed with scintillation fluid. The samples were dissolved in 5 ml scintillation fluid, and deteriorations per minute were measured (BetaTrac 6895, TmAnalytic). Variations of this experiment include a time-dependant experiment in which samples are incubated for increasing time periods in the presence of [^{3}H]-L-glutamate (1 μCi). During these experiments, the incubation and pre-incubation was preformed at 37°C. For the temperature-dependence experiment, the samples were held at the designated temperature from pre-incubation to the end of the 30-minute incubation, at which time the samples were pelleted and rinsed with I-DMEM at room temperature. In experiments where normal I-DMEM was replaced with low Na^+-I-DMEM, or I-DMEM containing no Na^+, the preparation samples were treated with an additional 60-second microcentrifugation step. The resultant pellet was then resuspended in

the appropriate buffer. Control samples were resuspended in normal DMEM-I. For complete media contents listing see Table 5.

The effects of glutamate uptake inhibitors were tested by adding them to the fiber suspension prior to the 10-minute pre-incubation. This experimental paradigm is similar to the microperfusion experiments, where the inhibitor was added prior to the 10-minute incubation preceding microperfusion. The inhibitors remained present until the end of the 30-minute incubation with [^{3}H]-L-glutamate.

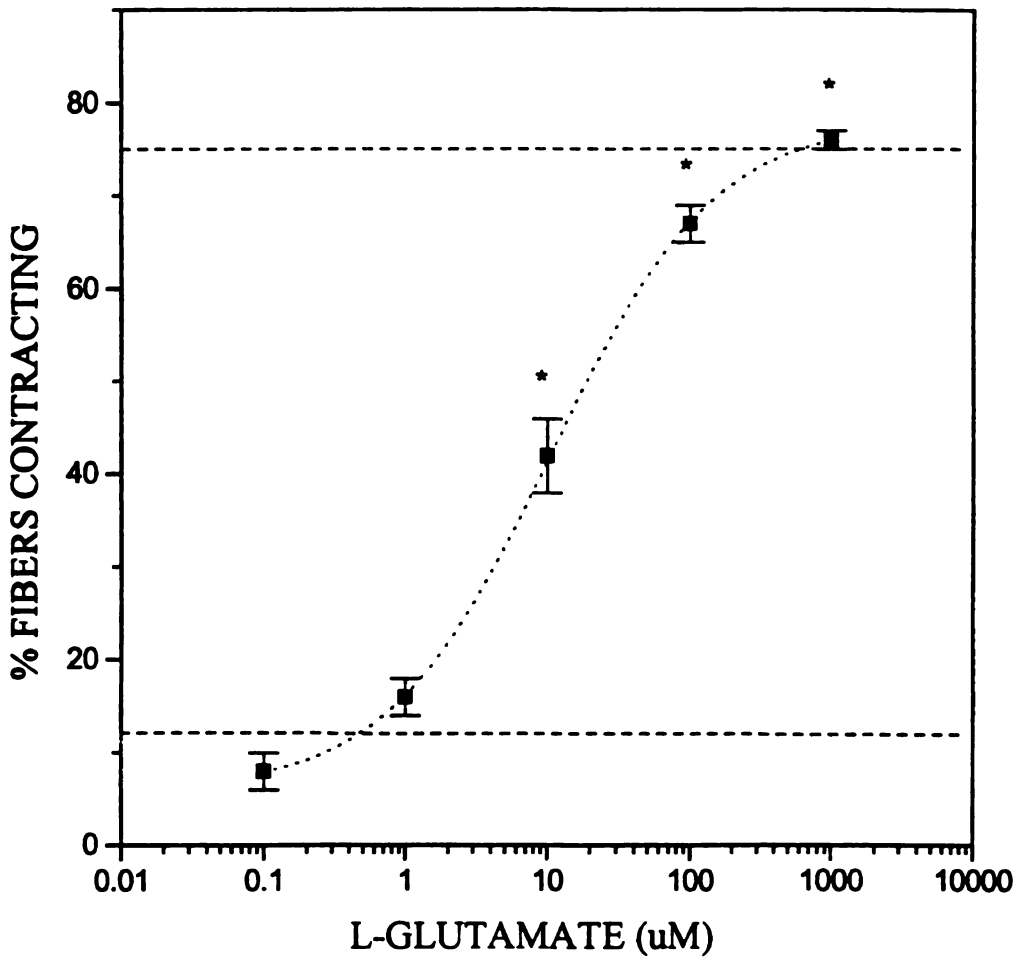
RESULTS

I. Microperfusion Experiments

A. L-Glutamate dose response curve

L-Glutamate microperfused onto *S. mansoni* frayed muscle fibers elicited contractions in a dose-dependent manner (Figure 1). The EC_{50} for this effect was approximately 11±3 μM, as calculated by the sigmoidal curve fit to these data. The negative control in this series of experiments was microperfusion of I-DMEM. I-DMEM is the same medium in which the fibers were bathed. An average of 12±1% of the fibers contracted in response to control medium. This contraction represents non-specific effects of microperfusion and may be accounted for by the frayed fibers' known mechanosensitivity and the ability of the fibers to spontaneously contract (Day *et al.*, 1994a). Often glutamate contractions were compared on corresponding days to the contraction elicited by 25 mM K^+. This elevated K^+ solution is a positive control, which has served as an indicator of muscle preparation viability (Day *et al.*, 1994a). Muscle fibers contracted 75±2% in response to microperfusion of 25 mM K^+ solution. This has been considered to represent maximal contraction according to the dose-response relationship of increasing amounts of K^+ (Day *et al.*, 1994a). The percent contraction produced by microperfusion of 1 mM L-glutamate (76±1%) was not significantly different

Figure 1. ***Schistosoma mansoni*** **muscle fibers contract in response to microperfused L-glutamate in a dose-dependent manner.** Frayed fibers were microperfused with DMEM without or with various concentrations of L-glutamate. The EC_{50} value calculated from this curve is 11±3 μM. *Significantly different from I-DMEM negative control medium ($P<0.01$). The upper dashed line represents the % fibers contracting in response to 25 mM K^+, and the lower dashed line represents the % fibers contracting in response to control medium (I-DMEM). In each petri dish, 15-30 fibers were microperfused, and each data point represents the average percentage (± 1 S.E.M.) of fibers contracting from at least 8 dishes. Mann Whitney-U Test.


% FIBERS CONTRACTING
0
20
40
60
80
0.01
0.1
1
10
100
1000
10000
L-GLUTAMATE (uM)

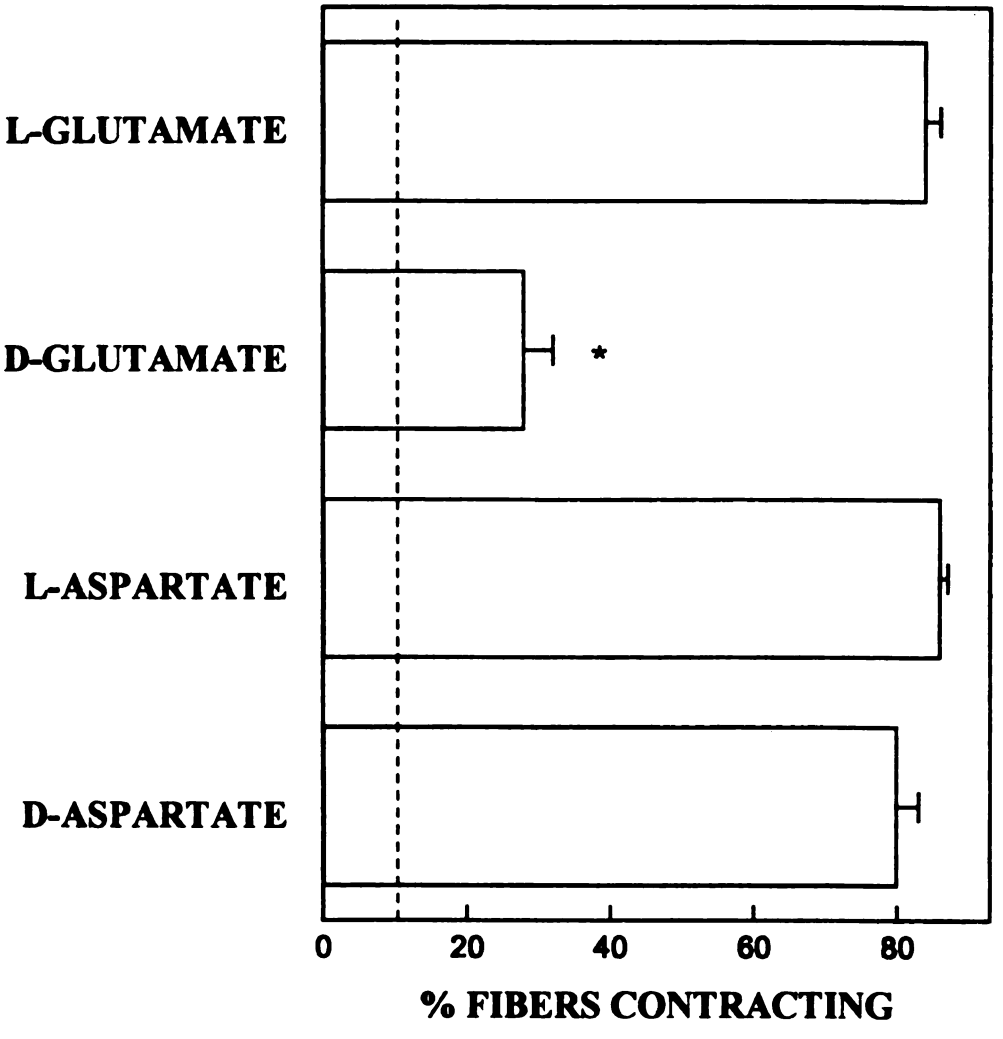
from that of 25 mM K^+.

The contraction in response to microperfused glutamate was qualitatively different from the contractions previously described for both FMRFamide and 25 mM K^+. The contraction of frayed fibers induced by FMRFamide is a slow, smooth contraction, and the contraction elicited by 25 mM K^+ is a rapid, twitching contraction (Day *et al.*, 1994a, b). In contrast, L-glutamate caused the fibers to contract rapidly and smoothly, without twitching, or shortening beyond approximately half of their original length.

B. Stereospecificity

To further characterize the contraction produced by glutamate, both enantiomers of glutamate and aspartate were microperfused onto frayed fibers (Figure 2). Each enantiomer elicited contraction in a significantly greater percentage of fibers than microperfusion of negative I-DMEM control. The percentage of fibers contracting in response to D-glutamate was significantly lower than that for L-glutamate, demonstrating that the contractile effect of glutamate is stereospecific.

Figure 2. The enantiomers of glutamate and aspartate elicit different percentages of contraction in *Schistosoma mansoni* muscle fibers. Each enantiomer was tested at the concentration of 1mM, and produced levels of contraction which were significantly different from I-DMEM control values, which are represented by the dashed line (9±1%). *Significantly different from L-glutamate ($P<0.05$). Data are represented as ±1 S.E.M. Each bar represents $N \geq 8$.

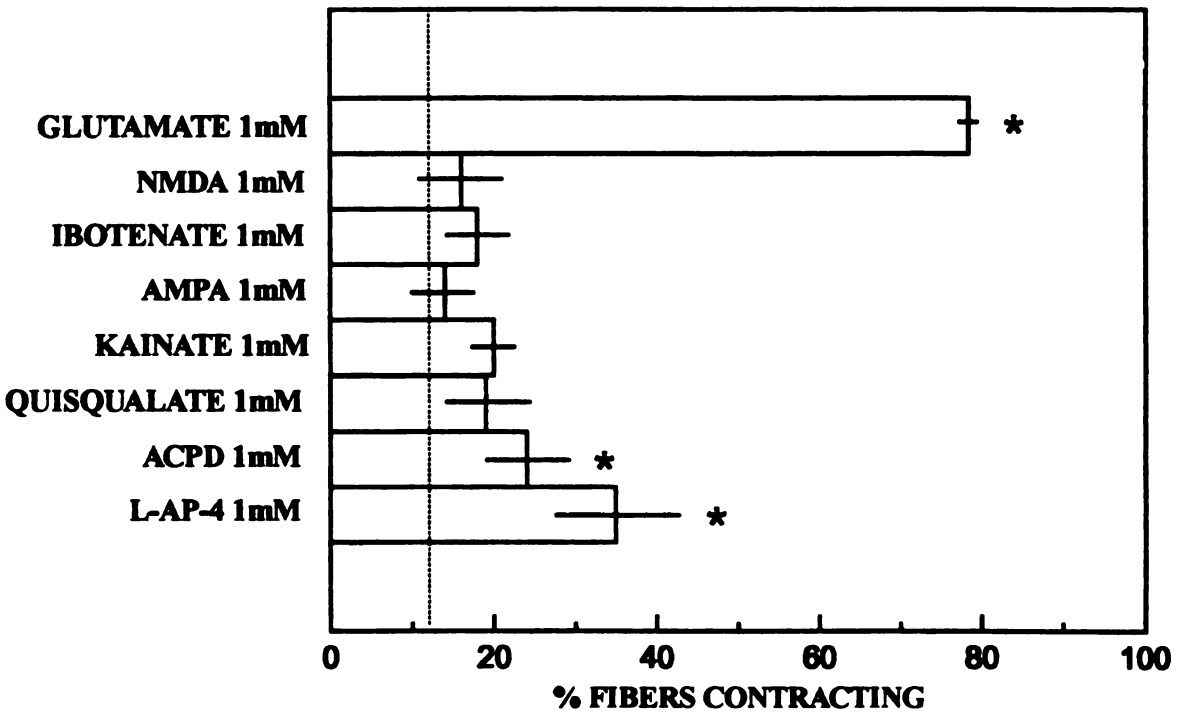

L-GLUTAMATE
D-GLUTAMATE
*
L-ASPARTATE
D-ASPARTATE
0
20
40
60
80
% FIBERS CONTRACTING

C. Glutamate Receptor Pharmacology

1. Agonists

Agonists of the well characterized mammalian glutamate receptor subtypes were microperfused onto *S. mansoni* muscle fibers to determine if the contractile response was mediated by a glutamate receptor that fits the described subtypes (Figure 3). All agonists were tested at 1 mM, a concentration of L-glutamate which elicited the maximal percentage of contraction of the muscle fibers. NMDA microperfused onto *S. mansoni* muscle fibers produced no significant amount of contraction above I-DMEM control values. Because the NMDA receptor in the mammalian system requires glycine in order to function, 100 μM glycine was included in the bath and the microperfusion pipet. This concentration of glycine did not increase the percentage of fibers contracting in response to 1 mM NMDA. In addition, Mg^{++} is known to block the NMDA receptor at negative membrane potentials. It was necessary to address the possibility that Mg^{++} could be blocking a NMDA type channel in the isolated fibers. The resting membrane potential of the muscle tissue of the schistosome has been estimated to be approximately -39 mV (Fetterer *et al.*, 1981). If this is true, then the NMDA receptor may be partially blocked by Mg^{++}. The NMDA current is augmented by reducing the concentration of extracellular Mg^{++} (Ascher *et al.*, 1988; Shannon & Sawyer, 1989). Consequently, the fibers were microperfused with NMDA without Mg^{++} in

Figure 3. Relative ineffectiveness of mammalian glutamate-receptor agonists in eliciting contractions in *Schistosoma mansoni* muscle fibers. Fibers were microperfused without (dashed line) or with various glutamate receptor agonists at the concentration of 1 mM. Both metabotropic agonists tested, ACPD and L-AP-4 elicited contraction of the frayed muscle fibers at values significantly above control. *Significantly different from I-DMEM control values ($P<0.05$). Each value represents the mean ±1 S.E.M. for at least 7 determinations.


GLUTAMATE 1mM
NMDA 1mM
IBOTENATE 1mM
AMPA 1mM
KAINATE 1mM
QUISQUALATE 1mM
ACPD 1mM
L-AP-4 1mM
*
*
*
0
20
40
60
80
100
% FIBERS CONTRACTING

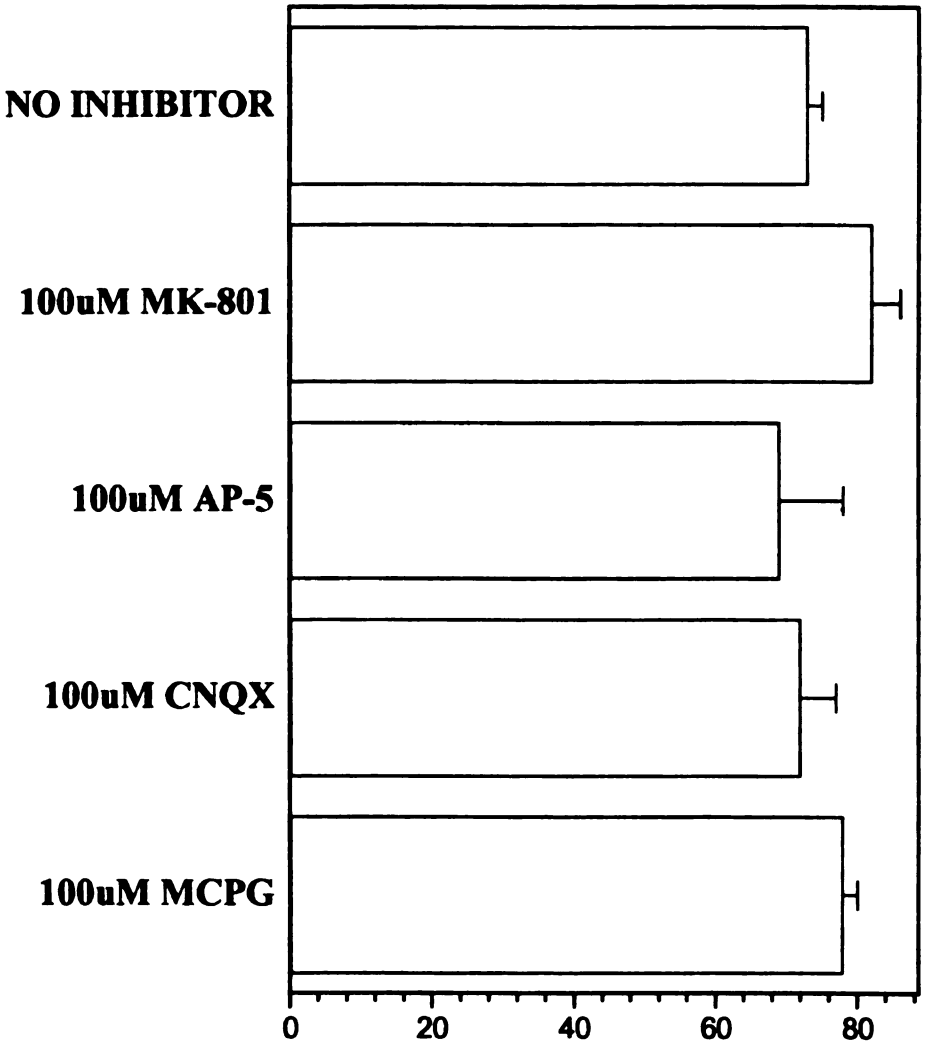
the microperfusate or bath solution. Alteration of the Mg^{++} concentration did not significantly affect the percentage of fibers contracting in response to 1 mM NMDA. In addition, ibotenate, a less specific agonist at the mammalian NMDA receptor, also did not elicit contraction of the muscle fibers.

Non-NMDA ionotropic glutamate agonists, AMPA and kainate, also were ineffective at producing contraction in the frayed fibers (Figure 3). Quisqualate, a fairly non-specific glutamate receptor agonist, did not produce contraction of the *S. mansoni* frayed muscle fibers. ACPD is a non-specific agonist for all cloned mammalian metabotropic receptor subtypes, and L-AP-4 is an agonist at a subset of these receptors which negatively modulate cAMP levels. ACPD and L-AP-4 microperfused at the concentration of 1 mM both produced levels of contraction that were statistically significantly different from control values, 24±4% and 35±6% respectively. However, none of the tested glutamate receptor agonists were as effective as L-glutamate in eliciting contraction of the isolated frayed muscle fibers.

2. Antagonists

Antagonists for the mammalian glutamate receptor subtypes were also employed in the characterization of the glutamate contractile response (Figure 4). For these experiments each antagonist was present in the bath during the 10 minute

Figure 4. The glutamate receptor antagonists tested do not inhibit the contraction produced by microperfusion of 100 μM L-glutamate. The response of the fibers to 100 μM L-glutamate (a concentration which produces sub-maximal percentage of contractions) in the presence of the antagonists tested was not significantly less than the response to 100 μM L-glutamate alone. Data are represented as ± 1 S.E.M. Each bar represents N≥7.


NO INHIBITOR
100uM MK-801
100uM AP-5
100uM CNQX
100uM MCPG
0
20
40
60
80
% FIBERS CONTRACTING
IN RESPONSE TO 100uM L-GLUTAMATE

pre-incubation, included in the 100 μM L-glutamate microperfusion medium, and remained present throughout the experiment. None of the antagonists tested were able to block the contraction induced by 100 μM L-glutamate. MK-801, an antagonist of the NMDA receptor subtype which blocks the intrinsic cation channel of the receptor, did not significantly inhibit the response of the frayed muscle fibers to 100 μM L-glutamate. The competitive NMDA antagonist AP-5 also did not produce a significant decrease in the percentage of fibers responding to 100 μM L-glutamate. CNQX, a non-specific antagonist at the AMPA/kainate mammalian subtype glutamate receptor, did not produce a decrease in the percentage of fibers responding to glutamate, nor did the general metabotropic receptor antagonist α-methyl-4-carboxyphenylglycine (MCPG).

Because no specific receptor agonists or antagonists were particularly effective, a receptor subtype could not be assigned to the *S. mansoni* contractile response, and little could be extrapolated from these data regarding the mechanism that results in contraction. Therefore, it was necessary to explore mechanisms other than a normal receptor-mediated response by which L-glutamate might be causing contraction in the schistosome muscle fibers. High-affinity glutamate transporters are known to be electrogenic, producing a depolarizing current when the transporter is actively taking up glutamate. If such a transporter exists on the schistosome muscle membrane, then activation of this transporter might cause

sufficient depolarization to result in contraction of the muscle fiber, much like depolarization of the fibers by microperfusion of elevated K^+ causes contraction.

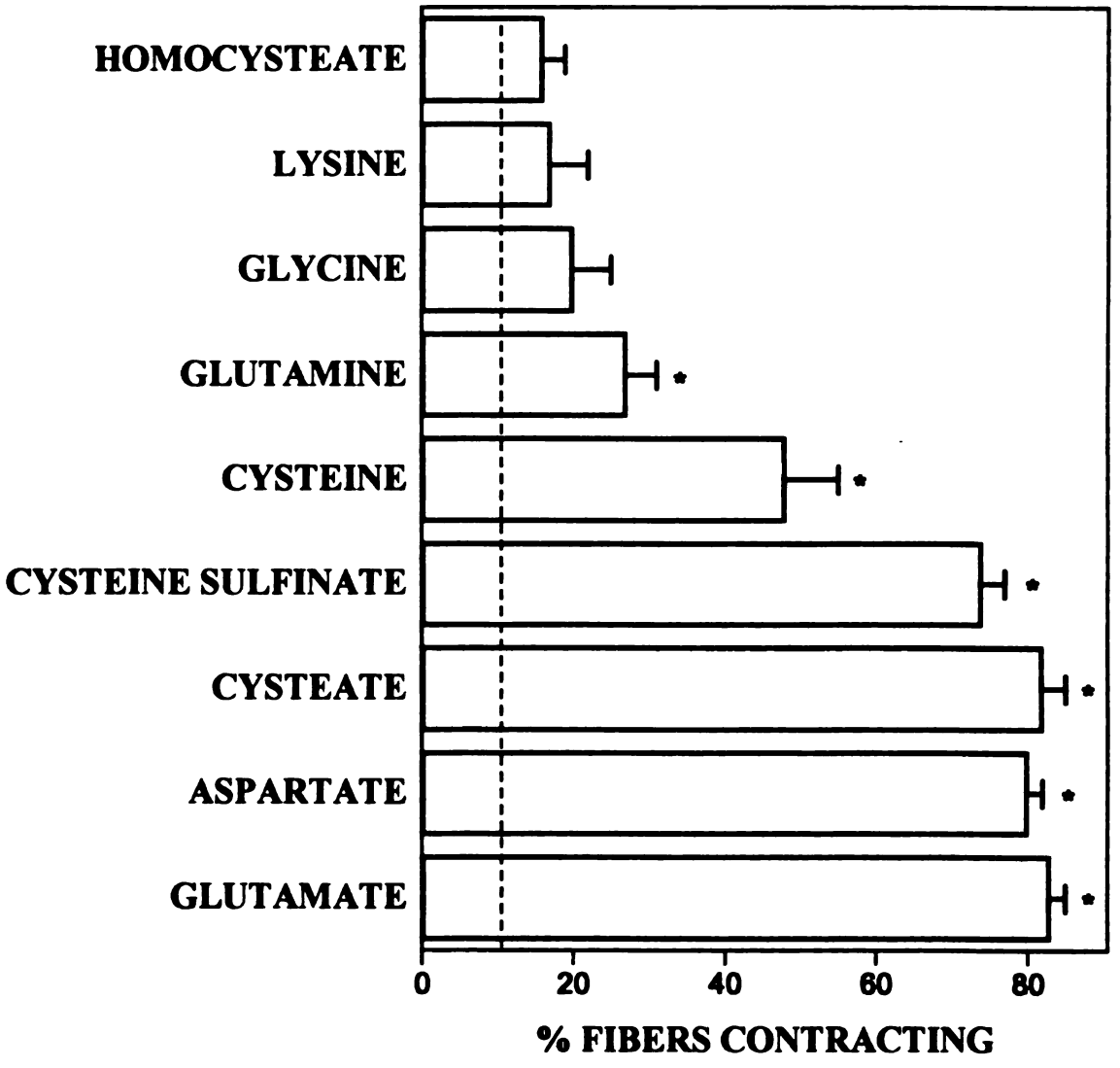
D. High-Affinity Glutamate Transporter Pharmacology

1. Transporter Substrates

If an electrogenic transporter is responsible for the contraction of the *S. mansoni* muscle fibers in response to glutamate application, then other amino acids known to be substrates of the transporter would be expected to produce a comparable contractile response. Most high-affinity amino acid transporters are known to transport L-glutamate, L- and D-aspartate, L-cysteate, and L-cysteine sulfinate quite efficiently (Kanai *et al.*, 1993). It is interesting to point out that the high-affinity glutamate transporters do not transport D-glutamate as well as L-glutamate, which is consistent with experimental observations described in section B of the results.

Selected amino acids were microperfused at a concentration of 1 mM (Figure 5). Microperfusion of L-aspartate, and L-cysteate resulted in maximal levels of contraction, which were not significantly different from the percent of fibers contracting in response to 1 mM L-glutamate. L-cysteine sulfinate, a transporter substrate and an agonist at the NMDA-type receptor, elicited 74±3% contraction.

Figure 5. Several amino acids could elicit contractions of *Schistosoma mansoni* muscle fibers. All amino acids were tested at the concentration of 1 mM. Microperfusion of L-aspartate or L-cysteate resulted in maximal percentage contraction, which was not significantly different from the percent contraction elicited by 1 mM L-glutamate. *Significantly different from I-DMEM control values, which are represent by the dashed line ($P<0.05$). All amino acids tested were levorotatory. Each bar symbolizes $N \geq 6$. Error bars are ± 1 S.E.M.


HOMOCYSTEATE
LYSINE
GLYCINE
GLUTAMINE
CYSTEINE
CYSTEINE SULFINATE
CYSTEATE
ASPARTATE
GLUTAMATE
*
0
20
40
60
80
% FIBERS CONTRACTING

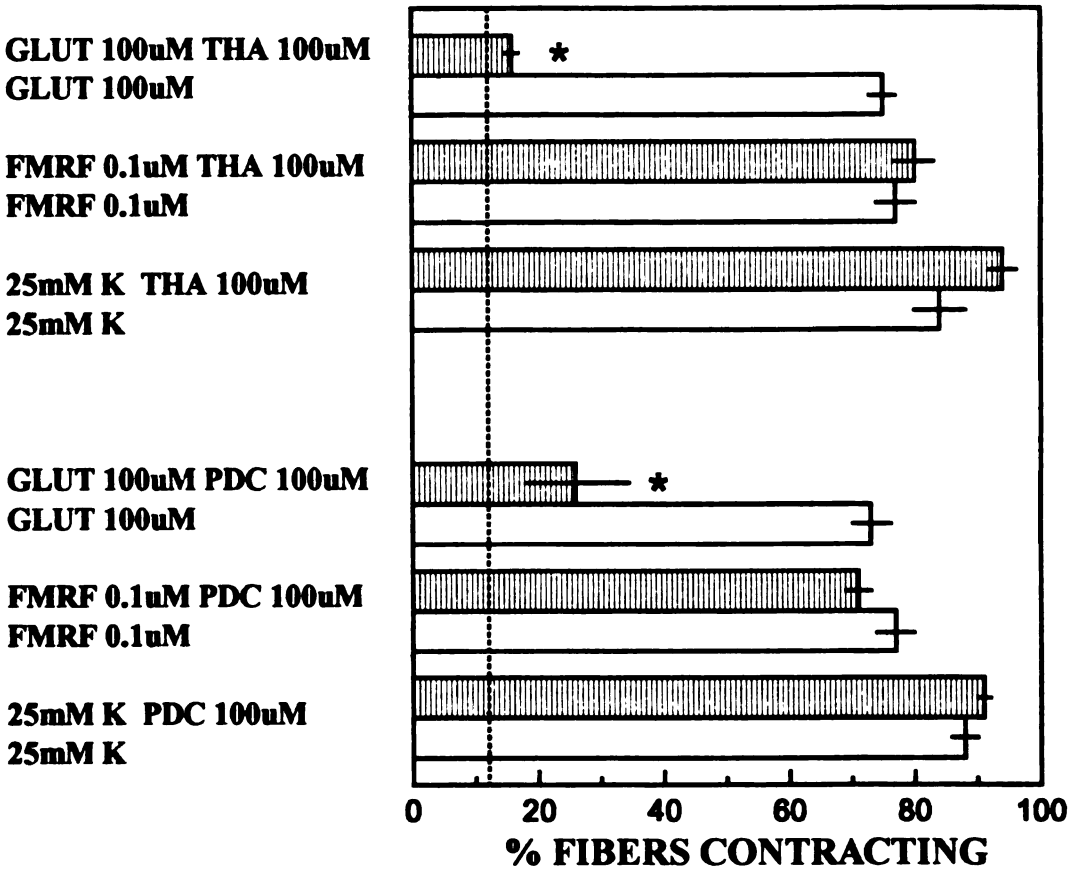
L-Cysteine and L-glutamine both produced contraction percentages that were significantly different from the I-DMEM negative controls, 48±7% and 27±4% respectively. L-Lysine, L-glycine, and L-homocysteate produced no significant percentage of contraction above control levels.

2. Transport Inhibitors

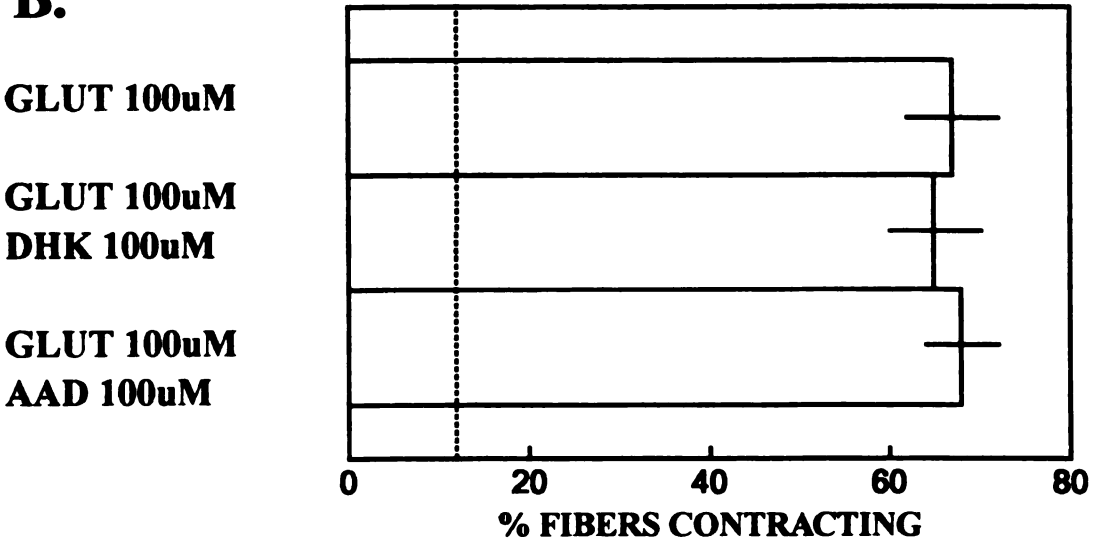
To further explore the hypothesis that a glutamate transporter is involved in the contractile response of *S. mansoni* muscle fibers, several inhibitors of the mammalian glutamate transporter were employed. All inhibitors were placed in the bath and microperfusion pipet at the concentration of 100 μM, and tested against 100 μM L-glutamate. The inhibitors L-*trans*-pyrollidine-2,4-dicarboxylic acid (PDC) and DL-*threo*-β-hydroxyaspartic acid (THA), both significantly inhibited contractions induced by 100 μM L-glutamate (Figure 6A). The percent fibers contracting in response to L-glutamate in the presence of PDC was not significantly different from the response of the fibers to microperfusion of I-DMEM control medium. The microperfusion of L-glutamate in the presence of THA produced a slightly higher percentage of contraction from I-DMEM control, 16±1% and 12±1% respectively (P=0.036). The inhibition produced by PDC and THA was reversible, because fibers which had been treated with the inhibitors and then rinsed with normal I-DMEM contracted in response to the application of 100

Figure 6. Two inhibitors of the mammalian excitatory amino acid transporter were effective at reducing the percentage of fibers contracting in response to 100 μM L-glutamate. A. *The contraction produced by 100 μM L-glutamate in the presence of both THA and PDC was significantly lower than that produced by L-glutamate alone. The contractile response induced by either FMRFamide or 25 mM K^+ was not affected by the presence of these inhibitors, suggesting that the inhibition produced by THA and PDC is specific to the L-glutamate contraction. **B.** Other classic inhibitors of the high-affinity excitatory amino acid uptake were not effective in blocking the L-glutamate-induced contraction. The contraction produced by L-glutamate, and the contraction produced by L-glutamate in the presence of the inhibitors AAD or DHK, was not significantly different. The dashed line represents the percentage of fibers contracting in response to microperfusion of I-DMEM control medium. Each bar represents $N \geq 5$. Error bars depict ±1 S.E.M. AAD, aminoadipic acid; DHK, dihydrokainic acid; FMRF, FMRFamide; GLUT, L-glutamate; PDC, L-*trans*-pyrollidine-2,4-dicarboxylic acid; THA, DL-*threo*-β-hydroxyaspartic acid; 25 mM K, elevated K^+.

A.


GLUT 100uM THA 100uM
GLUT 100uM
FMRF 0.1uM THA 100uM
FMRF 0.1uM
25mM K THA 100uM
25mM K
GLUT 100uM PDC 100uM
GLUT 100uM
FMRF 0.1uM PDC 100uM
FMRF 0.1uM
25mM K PDC 100uM
25mM K
*
*
0
20
40
60
80
100
% FIBERS CONTRACTING

B.


GLUT 100uM
GLUT 100uM
DHK 100uM
GLUT 100uM
AAD 100uM
0
20
40
60
80
% FIBERS CONTRACTING

μM L-glutamate.

The inhibitor aminoadipic acid (AAD) was not effective at blocking the contraction elicited by 100 μM L-glutamate in the *S. mansoni* muscle fiber (Figure 6B). DHK was also ineffective at blocking the L-glutamate-induced contraction. The percent fibers contracting in response to L-glutamate in the presence of either AAD or DHK was not significantly different than that of 100 μM L-glutamate alone.

To determine if the inhibition produced by THA and PDC was specific to the L-glutamate induced contraction, the frayed muscle fibers were perfused with 25 mM K^+ or FMRFamide in the presence and absence of the inhibitors PDC and THA (Figure 6A). Neither the contractions produced by 25 mM K^+ nor FMRFamide were significantly inhibited by the presence of these inhibitors at the concentration of 100 μM. From these data it appears that the inhibition produced by PDC and THA is specific for the contraction induced by L-glutamate.

E. Na^+-Dependence

If the contraction in response to L-glutamate is mediated by a high-affinity excitatory amino acid transporter, it would be expected to be dependent on extracellular Na^+, because the transporters are highly selective for Na^+. When Na^+ was replaced with N-methyl-D-glucamine (Table 5), fewer fibers contracted in

response to 100 μM L-glutamate (Figure 7). In fact, the contraction produced by L-glutamate in the presence of N-methyl-D-glucamine was not significantly different from the contraction produced by microperfusion of I-DMEM negative control medium, with or without Na^+.

Replacing Na^+ with N-methyl-D-glucamine appeared to have no deleterious effects on the fibers, as measured by their continued response to 25 mM K^+ positive control medium. In addition, the perfusion of I-DMEM containing no Na^+ was not significantly different from that of I-DMEM containing Na^+. This result clearly shows the marked dependence of the contractile effect of glutamate on the presence of extracellular sodium. Unfortunately, Na^+ dependence alone cannot be used to distinguish between an ionotropic receptor-mediated effect and a transporter-mediated mechanism.

When NaCl was replaced with LiCl, fewer fibers contracted in response to microperfusion of 100 μM L-glutamate (26±6% in the presence of Li^+, as opposed to 70±6% with Na^+ present (Figure 8)). The response of fibers to 100 μM L-glutamate in the presence of Li^+ was not significantly different from fibers microperfused with control I-DMEM containing Li^+ (26±6% and 21±3% respectively). When the fibers were microperfused with Li^+ I-DMEM control medium, a significantly higher percentage of fibers contracted than fibers

Figure 7. Replacing Na^+ with N-methyl-D-glucamine diminished the percentage of fibers contracting in response to microperfusion of 1 mM L-glutamate. The response of the fibers to 100 μM glutamate in the presence of N-methyl-D-glucamine was not significantly different from fibers perfused with I-DMEM containing sodium or N-methyl-D-glucamine. Fibers microperfused with elevated K^+ were not affected by replacing Na^+ with N-methyl-D-glucamine; in fact, slightly more fibers contracted in response to microperfusion of elevated K^+ in the presence of N-methyl-D-glucamine. The dark bars represent samples where Na^+ has been replaced with N-methyl-D-glucamine. *Significantly different from fibers microperfused with L-glutamate in the presence of Na^+. The error bars represent ±1 S.E.M. Each bar represents $N \geq 3$.

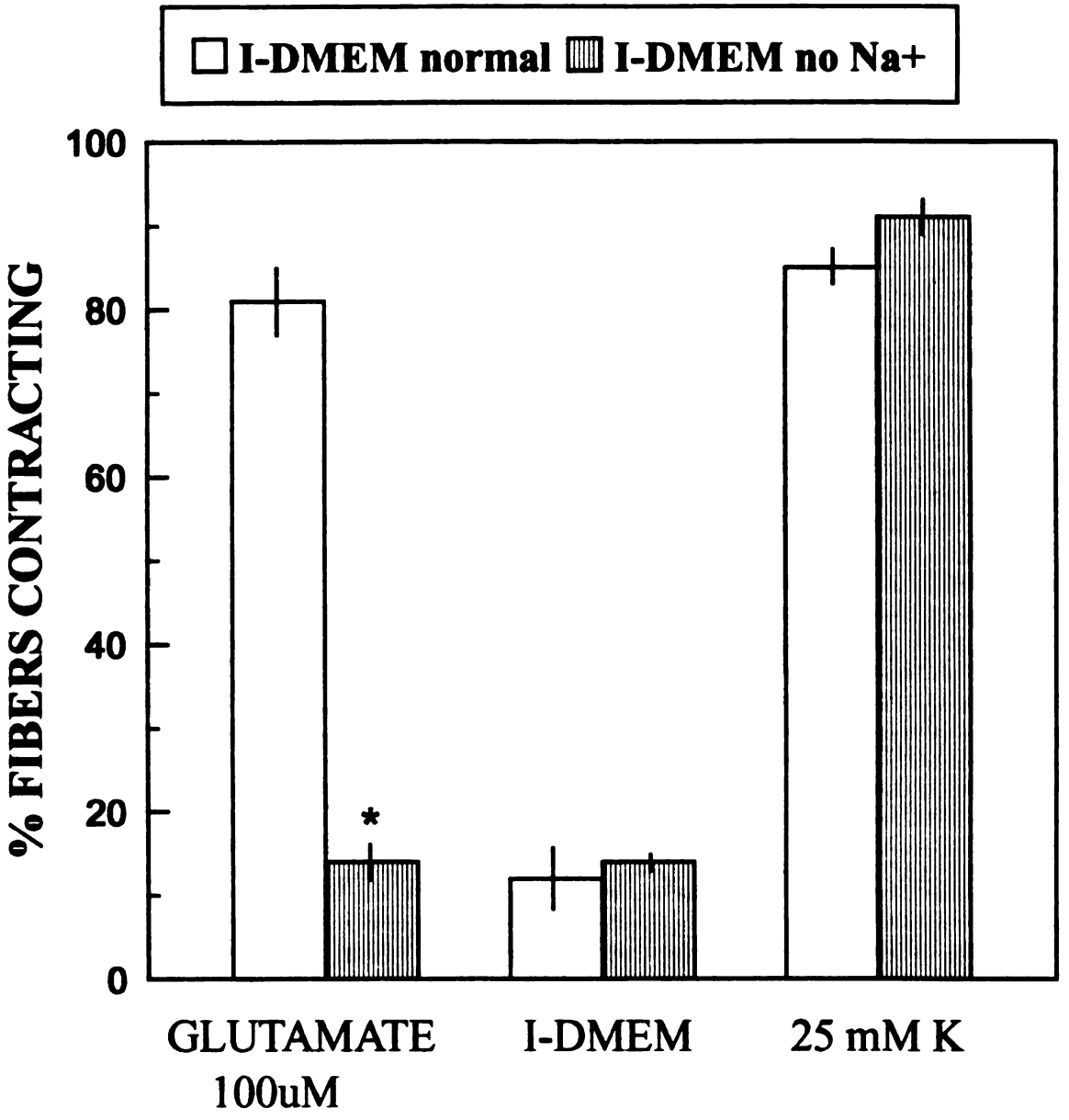
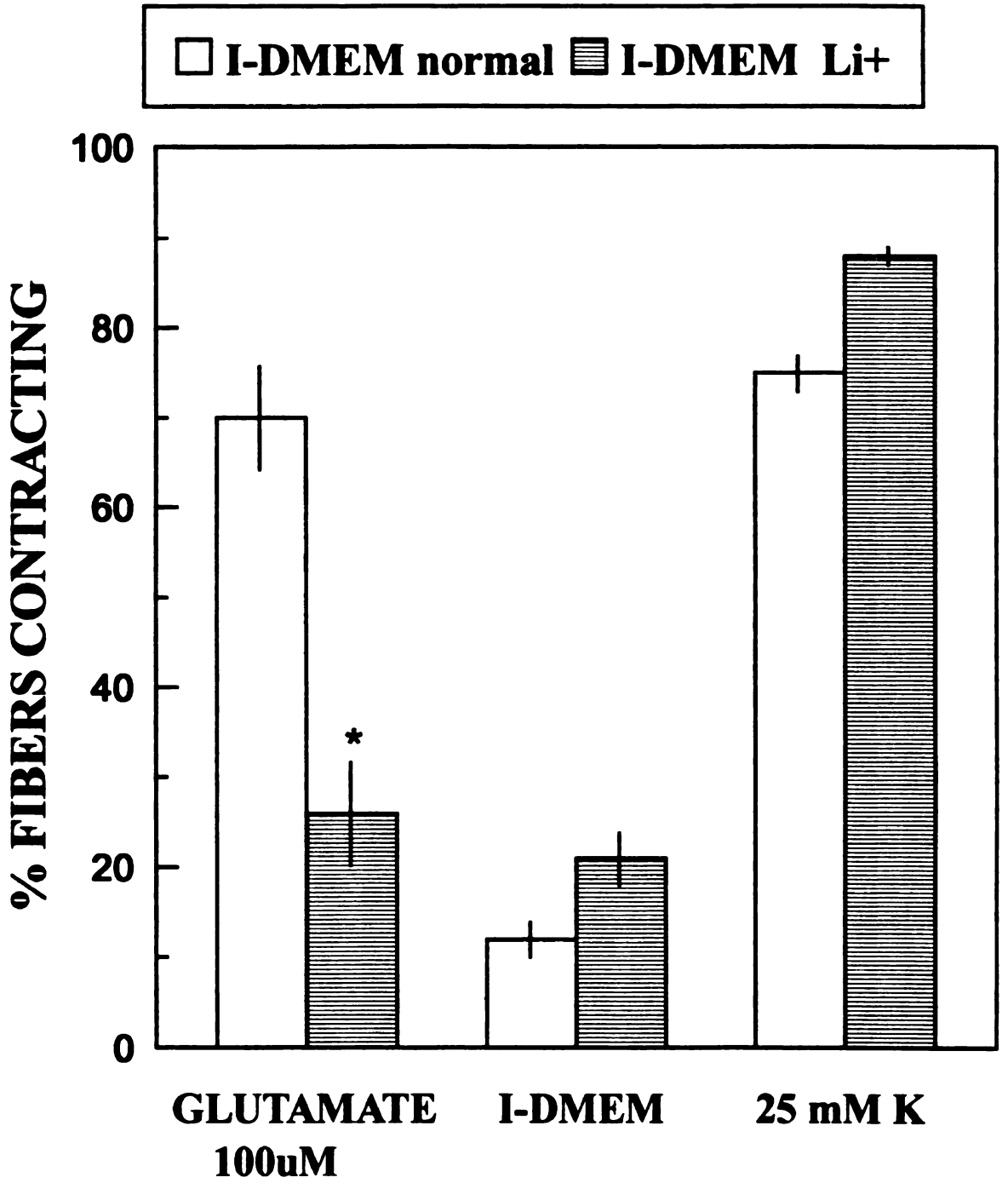

I-DMEM normal
I-DMEM no Na+
% FIBERS CONTRACTING
100
80
60
40
20
0
*
GLUTAMATE 100uM
I-DMEM
25 mM K

Figure 8. By replacing Na^+ with Li^+, fewer fibers contracted in response to microperfusion with 100 μM L-glutamate. However, both positive (25 mM K^+) and negative (I-DMEM) controls containing Li^+ responded with a significantly higher percentage of fibers contracting than did their Na^+-containing counterparts. Each bar represents $N \geq 5$. Error bars are ±1 S.E.M. Dark bars represent samples in which Li^+ has replaced Na^+.

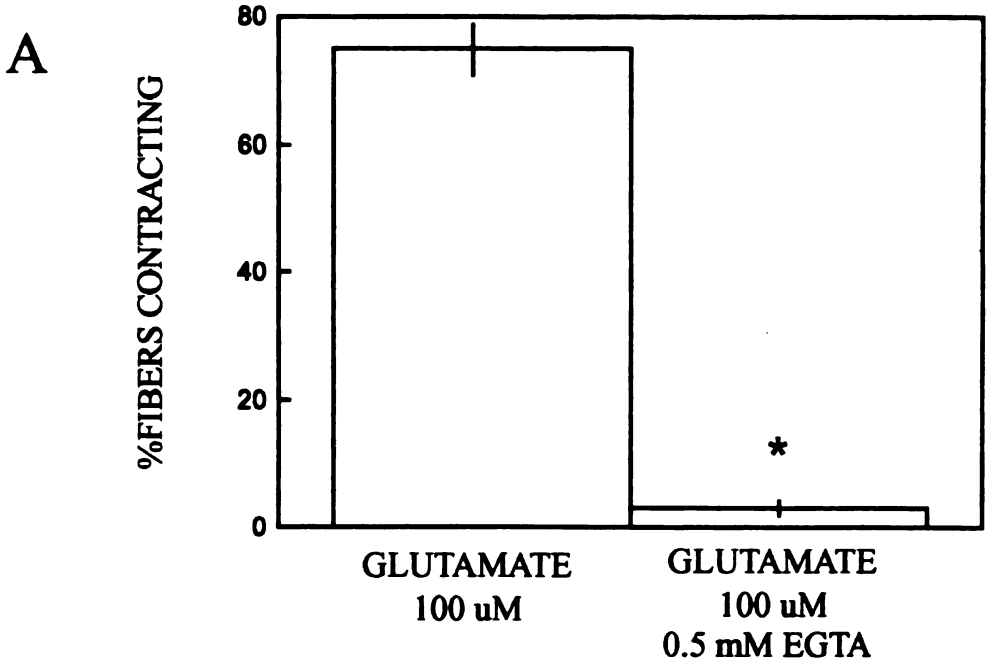

I-DMEM normal
I-DMEM Li+
% FIBERS CONTRACTING
100
80
60
40
20
0
*
GLUTAMATE 100uM
I-DMEM
25 mM K

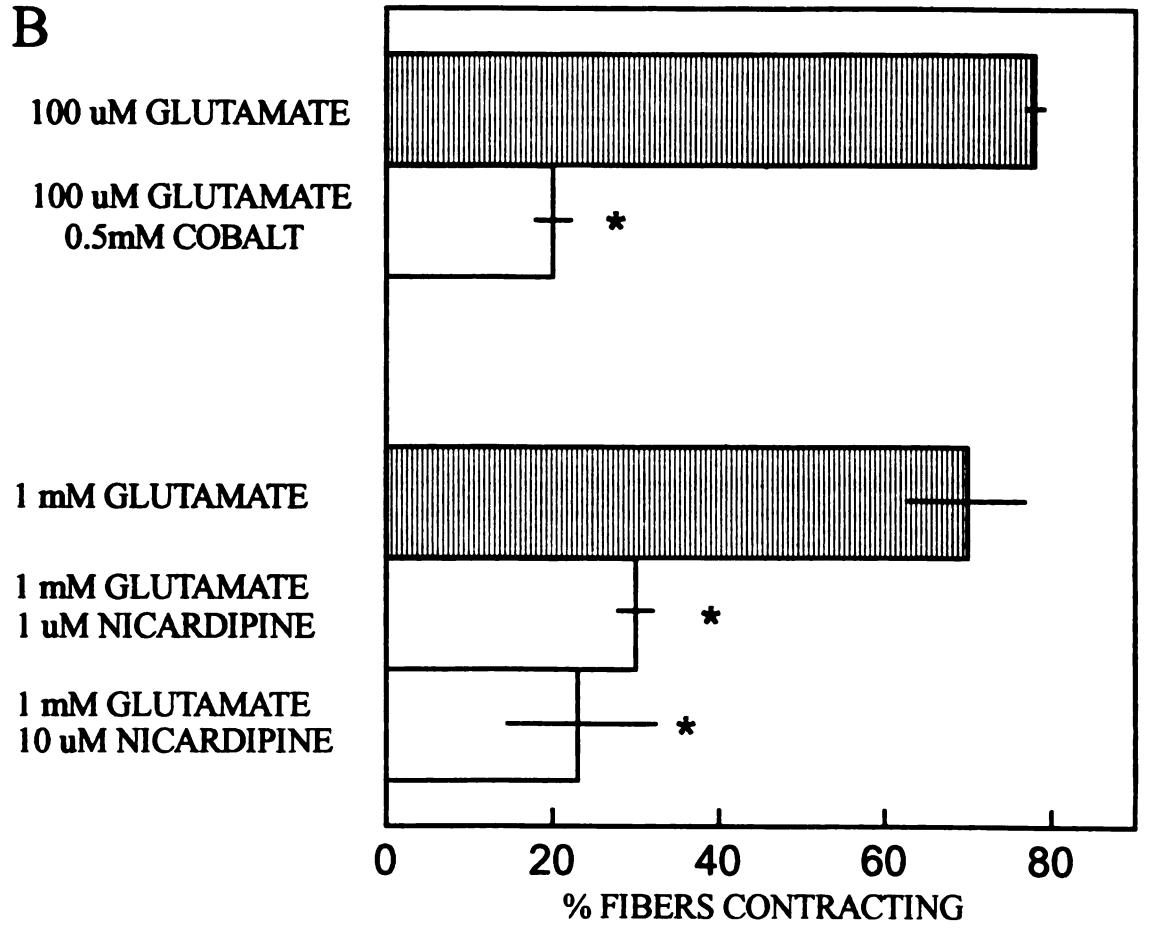
microperfused with I-DMEM control containing Na^+ (21±3% and 13±1% respectively). Both positive (25 mM K^+) and negative (I-DMEM) controls containing Li^+ responded with a significantly higher percentage of fibers contracting than their Na^+-containing counterparts.

F. Ca^{++} Dependence

If a L-glutamate transporter is responsible for the contraction of the muscle fibers due to the depolarizing electrogenic current of the transporter, then this depolarization must somehow trigger an increase in intracellular Ca^{++} needed to initiate contraction. This source of Ca^{++} could be from release of internal stores of Ca^{++}, or influx of extracellular Ca^{++}. To determine if influx of extracellular Ca^{++} was responsible for a rise in intracellular Ca^{++} leading to contraction, normal I-DMEM was replaced with Ca^{++}-free I-DMEM containing 0.5 mM EGTA directly preceding the 10 minute incubation (Table 5). EGTA remained present throughout the experiment, and all agonists tested were dissolved in the same I-DMEM containing 0.5 mM EGTA. When L-glutamate was microperfused onto the fibers in the presence of EGTA, the muscle fibers contracted significantly less than control fibers, 3±1% and 75±4% respectively (Figure 9). The presence of EGTA in the bath lowered the amount of spontaneous contractions observed when fibers were microperfused with negative control I-DMEM.

Figure 9. The contraction elicited by L-glutamate is Ca^{++}-dependent. A. By using medium containing no Ca^{++} and 0.5 mM EGTA, less fibers contract in response to L-glutamate. **B.** Ca^{++} appears to be flowing through a channel that can be blocked by nicardipine, a L-type voltage-gated Ca^{++} channel blocker *Significantly different from glutamate positive control values. Each bar depicts $N \geq 4$. The error bars represent ±1 S.E.M.


A
%FIBERS CONTRACTING
80
60
40
20
0
*
GLUTAMATE
100 uM
GLUTAMATE
100 uM
0.5 mM EGTA


B
100 uM GLUTAMATE
100 uM GLUTAMATE
0.5mM COBALT
*
1 mM GLUTAMATE
1 mM GLUTAMATE
1 uM NICARDIPINE
*
1 mM GLUTAMATE
10 uM NICARDIPINE
*
0
20
40
60
80
% FIBERS CONTRACTING

The loss of the ability of the fibers to contract in the presence of EGTA did not appear to be due to irreversible damage, because the EGTA-treated fibers retained their ability to contract in response to elevated K^+ solution containing the normal amount of Ca^{++}. The percent contraction produced by elevated K^+ in the presence of 0.5 mM EGTA was not significantly different from that of elevated K^+ microperfused onto normally treated fibers, suggesting that 0.5 mM EGTA is not permanently damaging the fibers.

If 0.5 mM EGTA is present in the bath, and the microperfusion pipet contains normal amounts of Ca^{++}, then an increased percentage of fibers will contract in response to L-glutamate. Fibers contracted 3±1% in response to glutamate with EGTA in the bath and the microperfusion pipet, while fibers contracted 30±9% when EGTA was present in just the bath and absent in the microperfusion pipet. The Ca^{++} present in the microperfusion pipet alone was enough to allow contraction in response to glutamate, even when 0.5 mM EGTA was present in the bath.

It was not possible to asses the effect of medium made with no Ca^{++}, because this medium independently produced spontaneous contractions in the frayed fibers. Consequently, the measurement of contraction could no longer be employed. This phenomenon may be due to Ca^{++} leaching from internal stores, causing transient increases in the intracellular Ca^{++} levels.

If Ca^{++} is flowing into the fibers by way of a Ca^{++} channel, it would be possible to block this action by the addition of cobalt chloride, a non-specific competitive Ca^{++} channel blocker. When normal I-DMEM was replaced with I-DMEM containing 0.5 mM cobalt in the bath and in the microperfusion pipet, 100 μM glutamate had a significantly reduced ability to contract the fibers compared to control values, 21±2% and 78±1% respectively. The ability of Co^{++} to block contraction in response to 100 μM L-glutamate was lost when the microperfusion pipet did not contain cobalt. Also, microperfusion of elevated K^+ medium containing no Co^{++} caused the fibers bathed in medium containing cobalt to contract in a normal fashion; again suggesting that the effect of Co^{++} was both rapidly reversible and not damaging to the fiber's ability to contract.

In the *S. mansoni* frayed muscle fibers, the Ca^{++} needed to produce contraction in response to microperfusion of elevated K^+ appears to be extracellular (Day *et al.*, 1994a). This was demonstrated by blocking 25 mM K^+ contractions with the dihydropyridine voltage-gated Ca^{++} channel blocker, nicardipine. Nicardipine significantly reduced the percentage of fibers contracting in response to 25 mM K^+ microperfusion, at the concentration of 1 and 10 μM (Day *et al.*, 1994a). To examine if Ca^{++} was passing through voltage-gated Ca^{++} channels to cause contraction in response to glutamate, nicardipine was placed in the bath of frayed fibers microperfused with 1 mM L-glutamate. Nicardipine at

the concentrations of 1 and 10 μM significantly reduced the percentage of fibers contracting in response to 1 mM L-glutamate (Figure 9B). L-Glutamate at the concentration of 1 mM produced 70±% contraction, and 1 mM L-glutamate with 10 μM nicardipine produced 23±9% contraction. This is consistent with the hypothesis that Ca^{++} may be flowing through voltage gated Ca^{++} channels, to produce contraction.

Although the concentrations of nicardipine used in these experiments may seem high, few toxic effects were observed. FMRFamide, a platyhelminth peptide which causes schistosome muscle fibers to contract, is not inhibited by these same concentrations of nicardipine (Day *et al.*, 1994b). However, this concentration of nicardipine may not be specifically blocking L-type voltage-gated Ca^{++} channels. Verapamil, a phenylalkylamine voltage-gated Ca^{++} channel blocker, at the concentration of 10 μM produced no significant inhibition of the L-glutamate induced contraction. Fibers contracted 75±3% in response to 100 μM L-glutamate in the presence of 10 μM verapamil, and 77±1% in response to 100 μM L-glutamate alone. It is interesting to note that verapamil also had no effect on elevated K^+ and FMRFamide-induced contractions. These data imply that the Ca^{++} needed for contraction in response to L-glutamate is extracellular, and may be passing through a voltage-gated Ca^{++}channel. Therefore, if an electrogenic glutamate transporter is mediating this effect, then the current produced is

sufficient to cause depolarization of the fiber membrane leading to opening of voltage-gated Ca^{++} channels. To support this hypothesis, additional evidence of glutamate transport is needed.

II. Glutamate Uptake Experiments

A. Time-Dependence

If a high-affinity glutamate transporter is mediating the *S. mansoni* muscle fiber contraction, it should be possible to observe the uptake of [^{3}H]-L-glutamate into the isolated fiber preparation. To demonstrate the presence of an excitatory amino acid transporter, the preparation was incubated with [^{3}H]-L-glutamate, (1 μCi/60 mM) at 37°C. The samples were all pre-incubated for 10 minutes at 37°C to acclimate the muscle fibers to this temperature. [^{3}H]-L-Glutamate was taken up in a time-dependent manner, reaching maximal rate of uptake between 20 and 30 minutes (Figure 10). In subsequent experiments, all samples were incubated for 30 minutes.

B. Dose-Dependence

Increasing concentrations of unlabeled L-glutamate inhibited uptake of 100 nM [^{3}H]-L-glutamate (1 μCi/60 mM) in a dose-dependent manner (Figure 11). Maximal inhibition of the uptake of [^{3}H]-L-glutamate was observed in the

Figure 10. [^{3}H]-L-Glutamate is taken up in a time-dependent manner. The rate of transport appears to be linear between 0 and 20 min, and reaches a maximum between 20 and 30 minutes. Error bars represent ±1 S.E.M. Each data point represents an average of three or more samples.

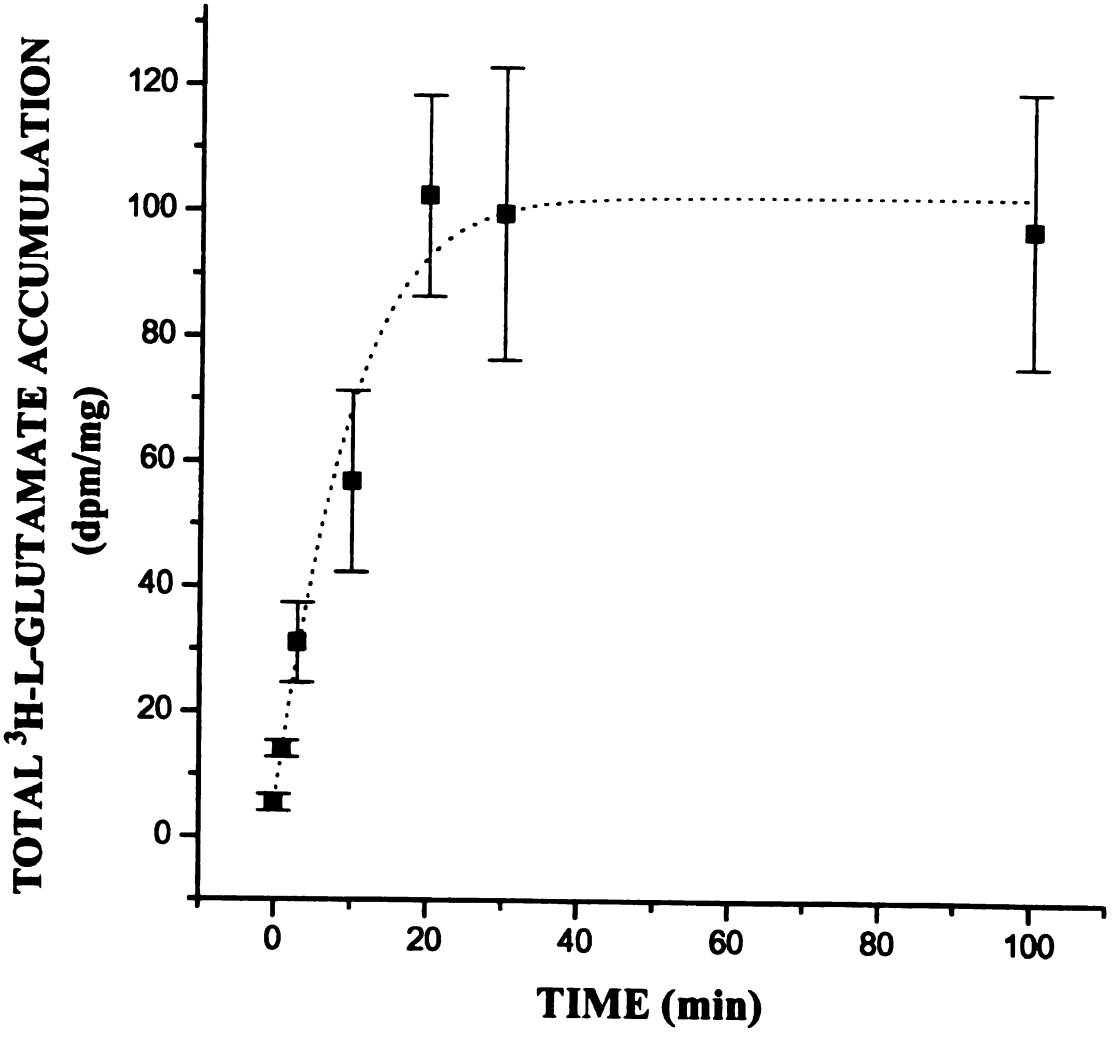
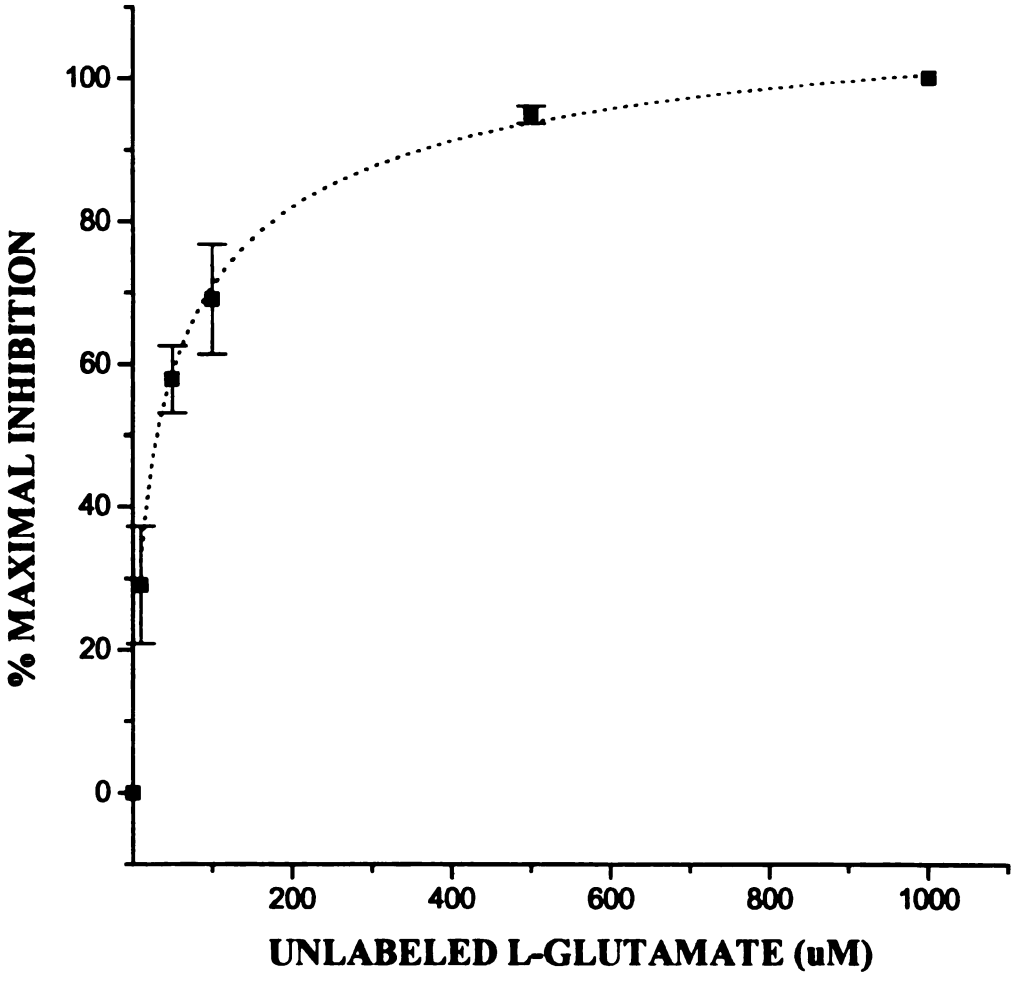

TOTAL ³H-L-GLUTAMATE ACCUMULATION
(dpm/mg)
120
100
80
60
40
20
0
0
20
40
60
80
100
TIME (min)

Figure 11. [^{3}H]-L-Glutamate is transported in a dose-dependent manner. Increasing concentrations of unlabeled L-glutamate and 100 nM [^{3}H]-L-glutamate were used to define the transport affinity. The EC_{50} value estimated from these data is 52 μM using the least squares analysis. 1 mM of unlabeled L-glutamate completely inhibited the transport of 100 nM [^{3}H]-L-glutamate, and this value was not significantly different from samples which were sonicated. Each data point depicts an average of four samples, and error bars are ±1 S.E.M..


% MAXIMAL INHIBITION
100
80
60
40
20
0
200
400
600
800
1000
UNLABELED L-GLUTAMATE (uM)

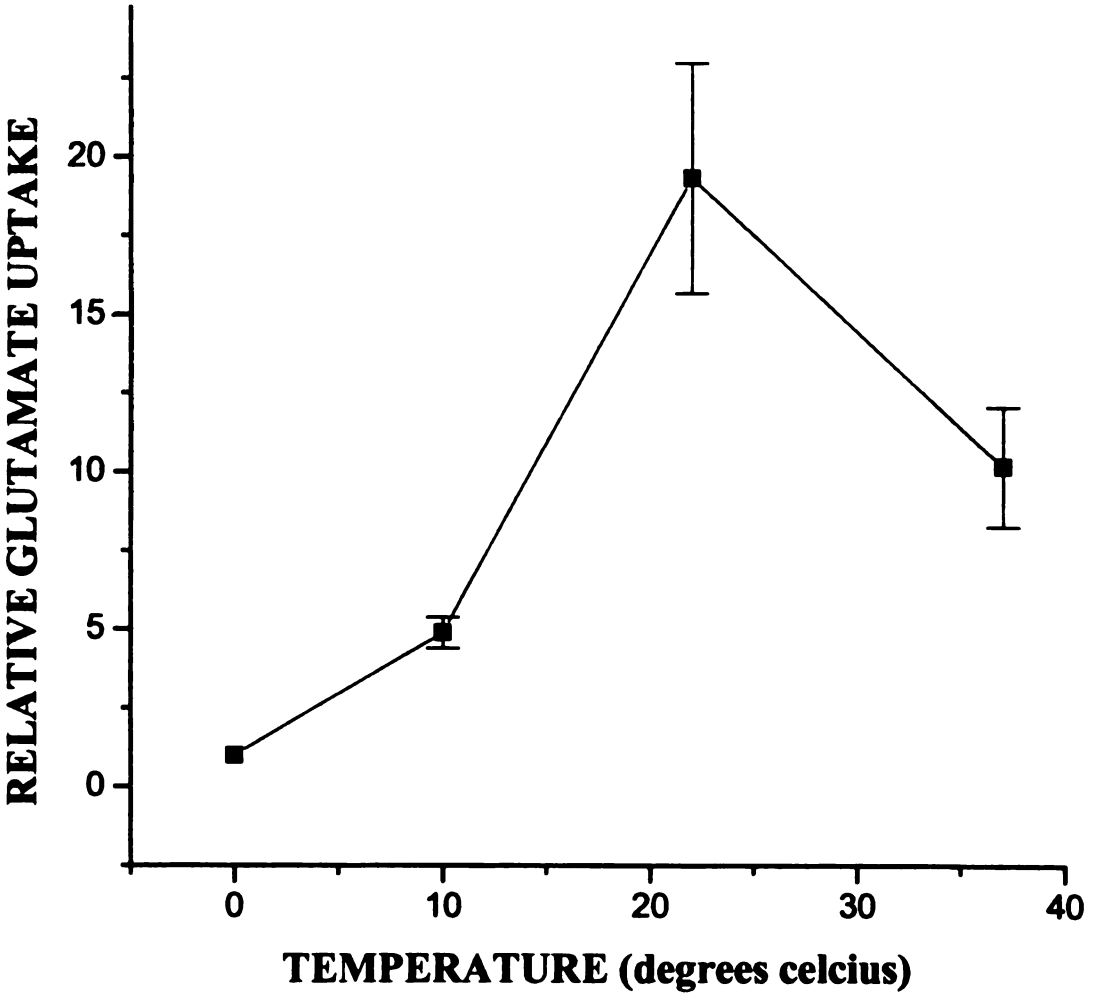
presence of 1000 μM unlabeled glutamate. This level of uptake was equivalent to that associated with samples which were sonicated prior to incubation with [^{3}H]-L-glutamate, (P=0.69). When samples are sonicated, there is no intracellular space available into which [^{3}H]-L-glutamate can be sequestered by a transport mechanism. Therefore, sonicated samples represent binding of [^{3}H]-L-glutamate to the muscle fiber preparation and not transport.

These data were analyzed using non-linear regression analysis, and the IC_{50} was determined to be 52±19 μM. The normalized data plotted in Figure 11 is fit with a rectangular hyperbola which predicts the inhibition at 50% to be 48 μM. These estimated values of 52 and 48 μM are on the upper limit of what has been traditionally described for the high-affinity, Na^+-dependent excitatory amino acid transporter, which is defined by a K_m below 50 μM (Cox *et al.*, 1977). However, these traditional defining characteristics are of less importance now that molecular sequence data are available. From this new cloned family of receptors, it has been shown that the K_m's of the expressed transporter range from 2.0 to 97.0 μM, depending on the specific clone, the expression system, and method of measurement (Kanai *et al.*, 1993; Arriza *et al.*, 1994).

C. Temperature-Dependence

The uptake of [^{3}H]-L-glutamate was temperature-dependent (Figure 12).

Figure 12. [^{3}H]-L-Glutamate transport is temperature-dependent. Samples were pre-incubated for ten minutes at the designated temperatures, 1 μCi [^{3}H]-L-glutamate was then added and incubation continued at the respective temperatures for 30 minutes. Each data point is an average of at least 3 samples and error bars are ±1 S.E.M.


RELATIVE GLUTAMATE UPTAKE
0
5
10
15
20
0
10
20
30
40
TEMPERATURE (degrees celcius)

Frayed fiber preparation samples tested at 22°C most efficiently transported [^{3}H]-L-glutamate. Because these data were highly variable, the uptake observed at 0°C was normalized to 1. The observed variability is inherent to the difficulties of keeping samples at a designated temperature throughout the experiment. [^{3}H]-L-Glutamate transport was most efficient at 22°C, however, 37°C was chosen for the standard temperature to allow comparison of these data to the microperfusion experiments which were also performed at 37°C. The Q_{10} value of glutamate uptake was measured to be 3.3 using the equation $Q_{10}=(K_1/K_2)^{10/(t1-t2)}$ where K_1 and K_2 represent the velocity constants of the transport of glutamate (dpm/mg/hr), and t_1 and t_2 represent temperature in Celsius at 10 and 22 degrees. The Q_{10} value is a measure of the increase in reaction velocity over a temperature rise of 10 °C (Prosser, 1973).

D. Transport Pharmacology-Transport Inhibitors

If L-glutamate transport is mediating the contraction observed when fibers are microperfused with L-glutamate, then it would be expected that the pharmacology observed for the uptake of [^{3}H]-L-glutamate would be similar to the pharmacology of the contractile response. Since the inhibitors PDC and THA effectively lowered the percentage of fibers which contracted in response to microperfusion of L-glutamate, then it would be expected that these inhibitors

would also block uptake, which was observed. PDC inhibited the transport of [^{3}H]-L-glutamate into the preparation in a dose dependent manner (Figure 13). The logistic sigmoidal curve fit to these data predicts the IC_{50} to be 3.2 μM PDC, which indicates that PDC is quite potent and maximally effective.

Both PDC and THA completely block uptake of [^{3}H]-L-glutamate at the concentration of 100 μM. When 100 μM PDC or THA was added to the samples, the percentage of [^{3}H]-L-glutamate uptake was 11±1%, and 16±2%, respectively (Figure 14). These percentages are not significantly different from the percentage of [^{3}H]-L-glutamate measured in samples that were sonicated, representing non-specific binding of [^{3}H]-L-glutamate. This indicates that these inhibitors at the concentration of 100 μM were completely blocking [^{3}H]-L-glutamate uptake. Because these inhibitors are thought to be quite specific for the high-affinity glutamate transporters, these data provide further support of the hypothesis that there is a glutamate transporter in *S. mansoni*.

E. Na^+-Dependence

An inherent quality of high-affinity glutamate transport is Na^+ dependence. Therefore, an integral piece of data, in addition to the observed Na^+ dependence of glutamate-induced contraction, is Na^+ dependence of [^{3}H]-L-glutamate uptake. To demonstrate Na^+-dependence, samples were incubated with the normal amount of

Figure 13. *trans*-Pyrollidine-2,4-dicarboxylic acid (PDC), a high-affinity glutamate transport inhibitor, inhibits uptake of [^{3}H]-L-glutamate in a dose-dependent manner. The estimated IC_{50} is 3.2 μM. [^{3}H]-L-Glutamate transport was inhibited 100% by 100 μM PDC. Error bars depict ±1 S.E.M.

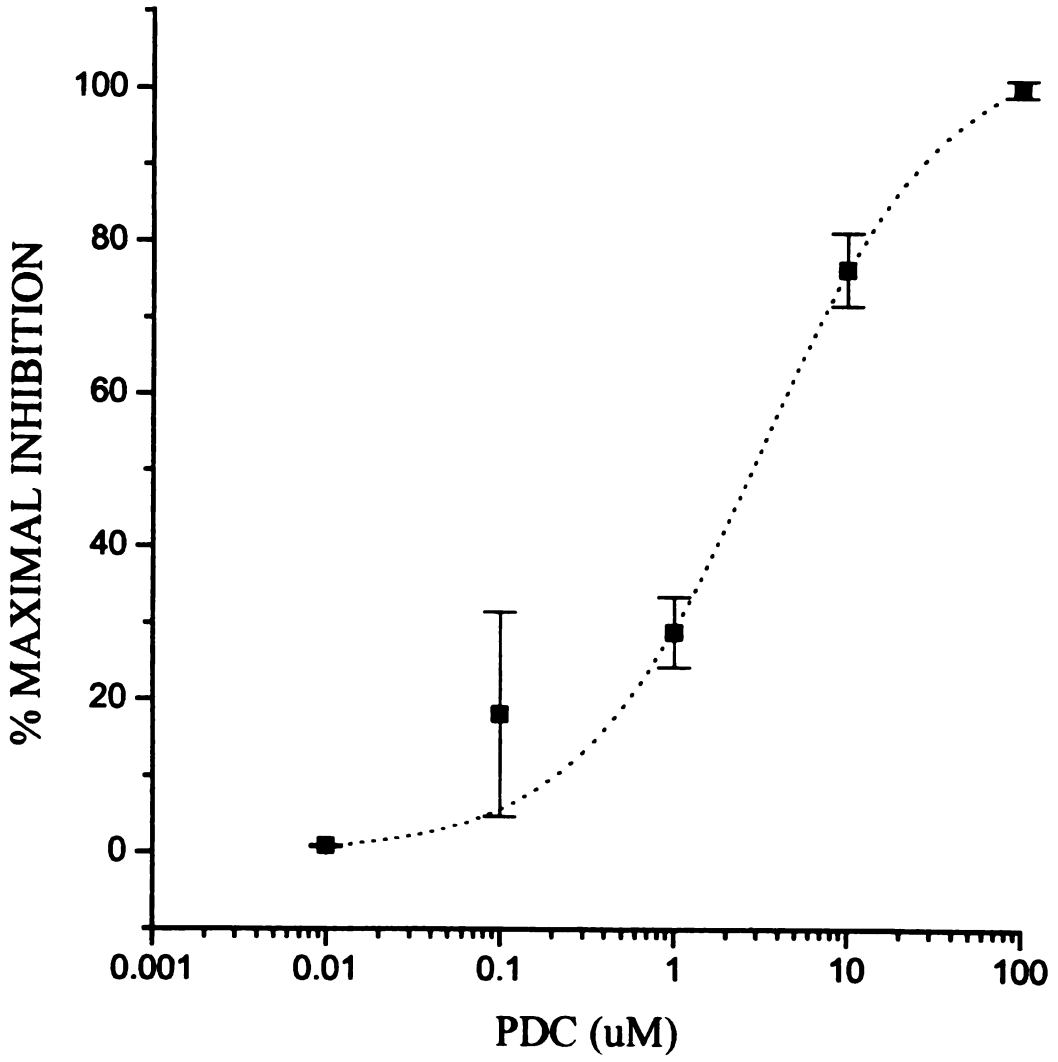
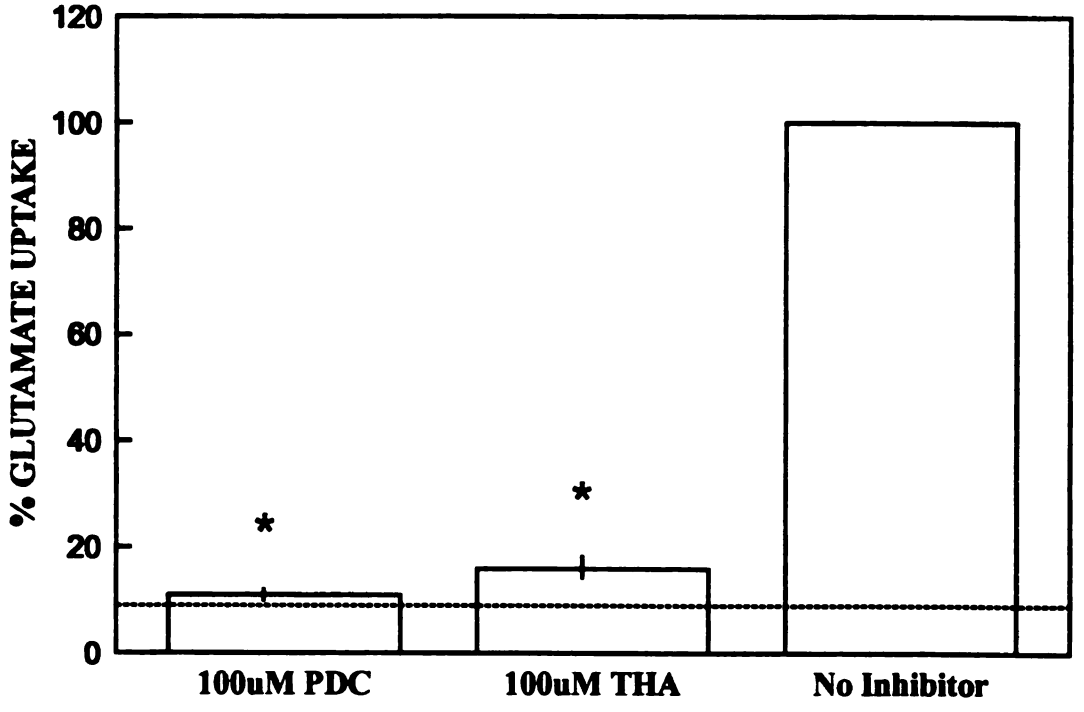
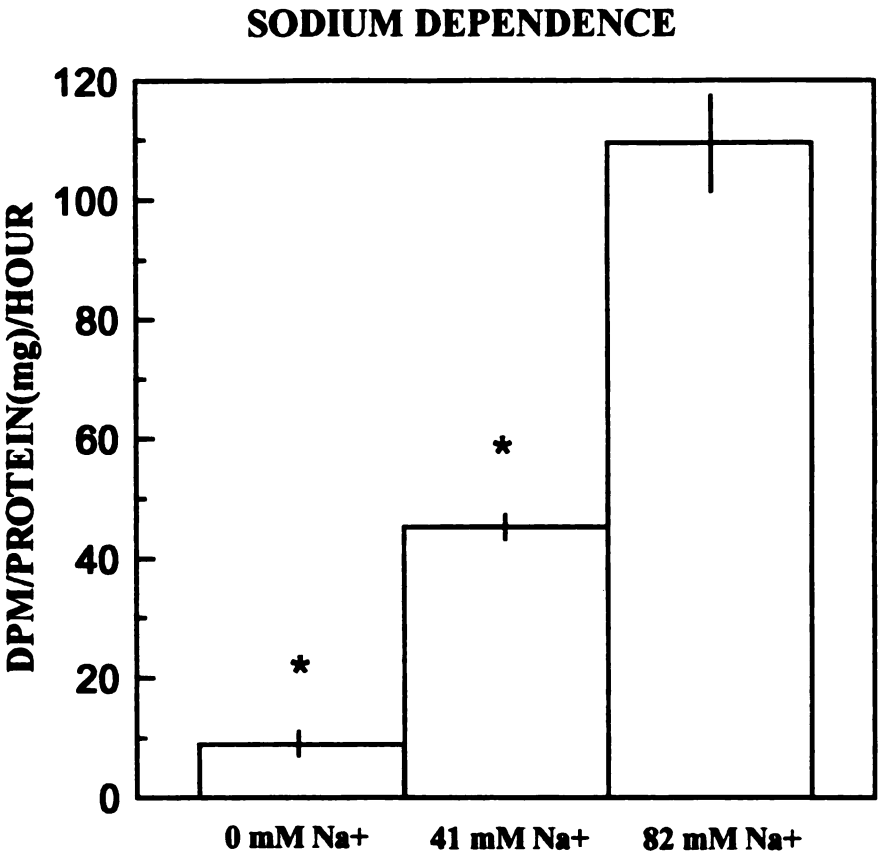

% MAXIMAL INHIBITION
100
80
60
40
20
0
0.001
0.01
0.1
1
10
100
PDC (uM)

Figure 14. The addition of 100 μM PDC or THA inhibits the transport of [^{3}H]-L-glutamate. *Significantly different from samples which were incubated without inhibitor. The percent [^{3}H]-L-glutamate in samples incubated with [^{3}H]-L-glutamate and 100 μM inhibitor was not significantly different from samples that were sonicated prior to incubation with 83.5 nM [^{3}H]-L-glutamate. Samples which have been sonicated represent non-specific binding of [^{3}H]-L-glutamate (9±1% of total uptake), and serve as the negative control represented by the dashed line. Error bars are ±1 S.E.M.. Each bar symbolizes N≥4. PDC, *trans*-pyrollidine-2,4-dicarboxylic acid; THA, DL-*threo*-β-hydroxyaspartic acid


120
100
80
60
40
20
0
% GLUTAMATE UPTAKE
*
*
100uM PDC
100uM THA
No Inhibitor

Na^+ (82 mM), 41 mM Na^+, and zero Na^+ (Figure 15). N-methyl-D-glucamine was used to replace the Na^+ in a equimolar fashion. Reducing the Na^+ concentration reduced the transport of [^{3}H]-L-glutamate. Samples containing zero Na^+ were not significantly different from samples which were sonicated, demonstrating non-specific binding 8±1% and 9±1%, respectively. These results support the hypothesis that there is a high-affinity, Na^+-dependent glutamate transporter on the membrane of the *S. mansoni* muscle fiber.

Figure 15. [^{3}H]-L-Glutamate uptake is Na^+-dependent. *Samples with no Na^+, and those with ½ the normal amount of Na^+, were significantly different from the samples containing 82 mM Na^+ ($P<0.05$). When Na^+ was completely replaced with N-methyl-D-glucamine, the amount of [^{3}H]-L-glutamate uptake was not significantly different from the amount of [^{3}H]-L-glutamate in samples which were sonicated to represent non-specific binding (8.2±1.3% and 8.8±1.3% respectively). Each bar represents an average (±1 S.E.M.) of 6 samples.


SODIUM DEPENDENCE
DPM/PROTEIN(mg)/HOUR
120
100
80
60
40
20
0
*
*
0 mM Na+
41 mM Na+
82 mM Na+

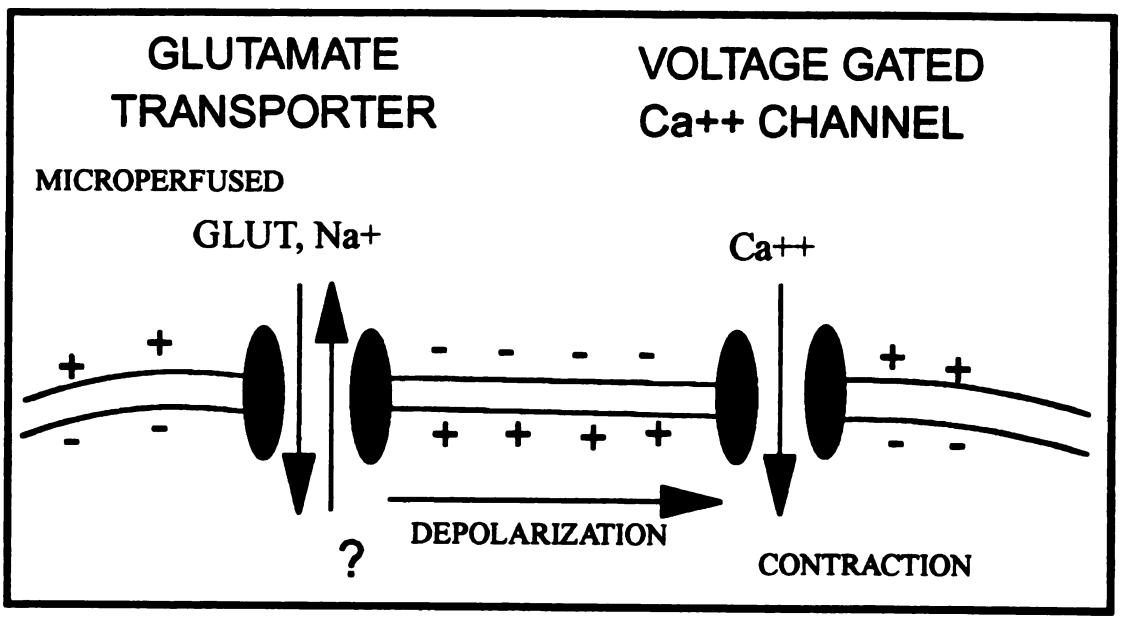
DISCUSSION

I. Pharmacology

A. Glutamate

The results from both the microperfusion experiments and the [^{3}H]-L-glutamate uptake experiments support the hypothesis that there is a transporter on the isolated *S. mansoni* muscle fiber membrane, and that it is most likely responsible for the observed contraction of the isolated muscle fibers in response to microperfusion of L-glutamate. The model proposed to describe the mechanism is depicted in Figure 16. In this model, L-glutamate and other transport substrates are taken up by an electrogenic, high-affinity glutamate transporter into the frayed fiber. This Na^+-dependent transport produces a depolarizing current, which in turn opens nicardipine-sensitive voltage-gated Ca^{2+} channels allowing $[Ca^{2+}]_o$ to enter the fiber, initiating contraction. The estimated EC_{50} of transport for [^{3}H]-L-glutamate into *S. mansoni* muscle fibers is approximately 52 μM, which is similar to the EC_{50} of the contractile response which was estimated to be 11 μM. Even if the transport of glutamate was solely responsible for the contraction induced by microperfusion of glutamate, the EC_{50} values of these processes may not be equal for several reasons. For instance, the mechanism leading to contraction may

Figure 16. Proposed mechanistic model of the contraction induced by L-glutamate in the *S. mansoni* muscle fiber. When glutamate is microperfused onto the frayed muscle fiber, it may be transported by a Na^+-dependent high-affinity glutamate transporter. The question mark indicates that the exact stoichiometry of the transporter is unknown. Currently it is thought that two Na^+ ions are co-transported with each molecule of glutamate (Kanai et al,. 1993). However, unpublished research from the laboratory of M. Kavanaugh suggests that there may be three Na^+ ions co-transported with each molecule of glutamate (personal communication). It is thought that one K^+ ion is counter-transported with each molecular of glutamate, as measured by K^+-sensitive electrodes (Bouvier et al,. 1994). In addition, a pH-changing ion is transported; it is still unknown, however, whether this effect is mediated by co-transport of a H^+, or counter-transport of a OH^-. In retinal cells evidence from anion substitutions supports counter-transport of a OH^- or HCO_3^- (Bouvier et al,. 1994). Regardless of the exact stoichiometry, inward transport of substrate through a high-affinity glutamate transporter produces a depolarizing current. It is possible that this depolarization is sufficient to open nicardipine-sensitive voltage-gated Ca^{++} channels. The flow of Ca^{++} down its electrochemical gradient into the fiber may be sufficient to initiate contraction of the *S. mansoni* muscle fiber.


GLUTAMATE
TRANSPORTER
VOLTAGE GATED
Ca++ CHANNEL
MICROPERFUSED
GLUT, Na+
Ca++
DEPOLARIZATION
?
CONTRACTION

consist of one or more amplification steps. This would result in an EC_{50} value for contraction that is lower than the EC_{50} value for glutamate transport, which is consistent with the results of this study. In addition, the amount of depolarization required to produce a detectable contraction of the muscle fiber in our assay is unknown. The transporter may not need to be maximally activated to observe maximal percentage contraction in our assay. Because the EC_{50} values for glutamate transport and the glutamate-induced contraction are similar, it suggests that glutamate transport could account for the contractile effect. However, it would be premature to conclude that a glutamate receptor is not also present on the schistosome muscle membrane.

B. Stereospecificity

When isolated fibers are microperfused with enantiomers of both glutamate and aspartate, the resulting stereospecificity pattern is characteristic of the preferences of the high-affinity excitatory amino-acid transporter (Kanner & Schuldiner, 1987; Arriza *et al.*, 1994; Klockner *et al.*, 1994). L-glutamate, and both L- and D-aspartate, produce maximal contraction of the isolated muscle fibers; however D-glutamate is not as potent. D-isomers are not generally potent agonists for glutamate receptors. In fact, the crayfish neuromuscular glutamate receptor is 250-fold more sensitive to L-glutamate than D-glutamate (Bishop *et al.*,

1987). Because D-aspartate elicited maximal contraction of the isolated muscle fibers, this effect is probably not mediated by a glutamate receptor, which is consistent with the hypothesis supporting a glutamate transporter-mediated mechanism.

C. Receptor Agonists

Microperfusion of selected glutamate receptor agonists onto the muscle fibers produced little or no contraction of the isolated muscle fibers, which provides further evidence that the contractile response induced by glutamate is not being mediated by a mammalian-like glutamate receptor. However, it is difficult to characterize an invertebrate glutamate receptor using mammalian receptor agonists, because often the pharmacological profile of invertebrate receptors is different from that of the well-described and cloned mammalian subtypes (Shinozaki & Ishida, 1992). Although both mammalian metabotropic receptor agonists produced statistically significant contraction of the isolated muscle fibers, this response may not represent a specific action on a metabotropic receptor. However, the low efficacy and potency of these metabotropic agonists could be due to inherent differences between a schistosome glutamate receptor and the mammalian metabotropic subtypes. It is possible that there may be a subset of the frayed fibers that contract in response to the metabotropic agonists. If this is true,

then the contractile response induced by L-glutamate may be mediated by more than one mechanism.

It is also possible that the metabotropic agonists, L-AP-4 and ACPD, are functioning as substrates for a glutamate electrogenic transporter. Presently, a thorough study of the mammalian glutamate receptor agonists that are also substrates for the mammalian high-affinity transporter is incomplete. However, it is known that NMDA is either poorly transported or not transported at all (Johnston *et al.*, 1979; Garthwaite, 1985; Rosenberg *et al.*, 1992; Wadiche *et al.*, 1995b). This is consistent with data presented in this study which show that NMDA does not cause the muscle fibers to contract. In addition, kainate is not transported by the mammalian high-affinity transporters, and actually functions as an antagonist in some transporter subtypes (Wadiche *et al.*, 1995b). Kainate did not elicit contraction in the frayed fibers, suggesting that it is neither transported, nor does it bind to an excitatory kainate receptor.

D. Receptor antagonists

Glutamate receptor antagonists have been instrumental in the early process of defining the mammalian glutamate receptor subtypes. However, in invertebrate systems few of these inhibitors are effective (Walker & Holden-Dye, 1989; Shinozaki & Ishida, 1992). Therefore it is difficult to use the antagonists as a tool

to define the nature of an invertebrate receptor, especially in the evolutionarilly distant schistosome. The antagonists tested did not inhibit the contraction of the muscle fibers induced by L-glutamate, even at relatively high concentrations known to block mammalian receptor responses (Hoehn & White, 1990). This result is consistent with the hypothesis that an electrogenic transporter is mediating contraction. However, it is not reasonable to eliminate the possibility that there may be a glutamate receptor on the schistosome muscle because an active mammalian glutamate receptor antagonist has not yet been identified.

E. Transporter substrates

All commonly-transported amino acids tested (L-glutamate, L-and D-aspartate, L-cysteate, and L-cysteine sulfinate) caused contraction of the *S. mansoni* muscle fibers. If an electrogenic transporter is depolarizing the membrane, then presumably the amount of current produced by the transporter is the factor which determines if the fibers contract. The amount of current measured in response to a transport substrate is related to the amount of substrate transported, although it is not a direct or straight-forward relationship (Wadiche *et al.*, 1995a). These transporter substrate data are consistent with the proposed model for glutamate-induced contraction mediated by a high-affinity glutamate transporter.

L-Homocysteate did not produce contraction of the *S. mansoni* frayed muscle fibers. It is thought that L-homocysteate is transported by the low-affinity glutamate transporter and is a poor substrate for the high-affinity glutamate transporter (Cox *et al.*, 1977; Tanaka, 1994). In addition, L-homocysteate is an agonist for the NMDA mammalian glutamate receptor. Since homocysteate did not produce a significant percentage of contraction in the frayed fibers, it seems unlikely that the contractile response is mediated by a NMDA receptor subtype, or by a low-affinity transporter. The amino acid glycine also did not produce a significant percentage of contraction in the isolated fibers, which suggests that glycine is not transported into the isolated muscle fibers. Like homocysteate, glycine is a relatively selective substrate for the low-affinity glutamate transporter (Webb, 1986). Recently, it has been shown that L-cysteine is a substrate of the high-affinity EAAT3 transporter subtype (K_m = 190 μM), although it does not produce maximal current when transported (Zerangue & Kavanaugh, in press). This is consistent with the finding that 1 mM L-cysteine did not cause maximal contraction of the fibers. These data support the proposed model where the contractile effect is produced by electrogenic transport in the isolated muscle fibers.

F. Transport Inhibitors

trans-Pyrollidine dicarboxylic acid (PDC) and *threo*-hydroxyaspartic acid (THA) are a potent inhibitors of glutamate high-affinity transport. PDC is specific for the high-affinity transporters, and it does not bind mammalian glutamate receptors (Freund *et al.*, 1995). PDC inhibits [^{3}H]-L-glutamate uptake into the muscle fiber preparation in a dose-dependent manner. PDC and THA (100 μM) completely blocked [^{3}H]-L-glutamate uptake, and inhibited the contraction elicited by 100 μM L-glutamate in the isolated muscle fibers. Although 100 μM of inhibitor may seem quite high, these concentrations and higher are commonly employed (Tanaka, 1993; Arriza *et al.*, 1994).

Most inhibitors of glutamate high-affinity transport are competitive, and are themselves transported. When PDC (1 mM) was microperfused as a substrate, it caused 43±7% of the fibers tested to contract (N=4). This suggests that the transport of PDC is producing a depolarizing current. PDC also produces a depolarizing current in patch-clamped oocytes that were injected with cloned glutamate transporter message (Arriza *et al.*, 1994). However, the current produced by PDC is only 34-52% of the current produced by L-glutamate (Arriza *et al.*, 1994). This explains why PDC did not produce maximal levels of contraction in *S. mansoni* muscle fibers (even at the high concentration of 1 mM), and why PDC can also serve as an inhibitor of glutamate-induced contraction.

If the *S. mansoni* muscle fiber contained an excitatory glutamate receptor that was responsible for mediating contraction in addition to a high-affinity glutamate transporter, then the co-application of PDC and glutamate should cause more fibers to contract than application of glutamate alone. If the transporter is blocked by PDC, then less glutamate is sequestered which in turn would increase the concentration of glutamate available to bind the excitatory glutamate receptor. This paradigm is typically observed in brain slice preparations which contain both transporters and receptors for glutamate. However, fewer *S. mansoni* fibers contracted in response to co-applied of PDC and glutamate, suggesting that the contractile effect is not mediated by a glutamate receptor.

II. Ion Specificity

A. Na^+-Dependence

Both the contractile response of the muscle fibers elicited by L-glutamate, and the uptake of [^{3}H]-L-glutamate by the muscle fiber preparation were found to be Na^+-dependent processes. In the absence of Na^+, glutamate-induced contraction and [^{3}H]-L-glutamate uptake were reduced to control levels, suggesting that these processes are highly Na^+-dependent. With this information, those glutamate-mediated processes that do not involve a Na^+ current can be eliminated, such as glutamate metabotropic receptor-mediated IP_3 release of Ca^{++} from internal stores,

or other such mechanisms.

When Na^+ was replaced with Li^+, no significant contraction of the isolated muscle fibers was observed in response to microperfusion of L-glutamate. The high-affinity transporters are very selective for Na^+, and Li^+ does not substitute for Na^+ in the transport of glutamate. However, Li^+ is a suitable ion candidate for the NMDA and non-NMDA glutamate receptor channels and other Na^+-conducting channels (Schwartz & Tachibana, 1990; Yamaguchi & Ohmori, 1990; Barbour *et al.*, 1991; Wyllie *et al.*, 1991). Since Li^+ did not functionally substitute for Na^+, the contraction was probably not mediated by a glutamate ionotropic receptor. These data are, however, consistent with the hypothesis that contraction is being mediated *via* a high-affinity, Na^+-dependent excitatory amino acid transporter.

It is interesting to note that both positive (25 mM K^+) and negative (I-DMEM) controls containing Li^+ responded with a significantly higher percentage of fibers contracting than did their Na^+-containing counterparts. This could be explained by an increased amount of spontaneously-contracting fibers (although this was not apparent during the time of data collection), or the membrane could be more permeable to Li^+, causing the fibers to become depolarized. It is also possible that Li^+ cannot adequately substitute for Na^+ in the Na^+/K^+ ATPase, which would result in depolarization of the membrane and lead to its increased excitability. It would be interesting to know if ouabain would mimic this effect.

B. Ca^{++}-Dependence

The L-glutamate-induced contraction appears to be dependent on the presence of extracellular Ca^{++} and is blocked by the dihydropyridine L-type voltage-gated Ca^{++} channel blocker nicardipine; however the response was not inhibited by the phenylalkylamine verapamil. Although verapamil blocks the same type of voltage-gated Ca^{++} channels, it is not as potent as nicardipine at relaxing vascular smooth muscle. In addition, dihydropyridines produce a state-dependent block of voltage-gated Ca^{++} channels, and are thought to bind best to the inactivated state of the Ca^{++} channel. As a result, dihydropyridines are more potent at depolarized potentials. From the results of early microelectrode studies, it is thought that the resting potential of schistosome muscle is approximately -28 mV (Thompson *et al.*, 1982). This relatively depolarized value would increase the ability of dihydropyridines to block voltage-gated Ca^{++} channels. In addition, dihydropyridines are known to inhibit cyclic nucleotide phosphodiesterases, which may increase cyclic nucleotide concentrations. These factors may play a role in the ability of the nicardipine, but not verapamil to inhibit the contractile response.

The contraction induced by 25 mM K^+ is also blocked by nicardipine and not verapamil (Day *et al.*, 1994a). This suggests that depolarization and Ca^{++} entry through voltage-gated Ca^{++} channels are common mechanisms to both the 25 mM

K^+-induced contraction and the glutamate-induced contraction. This is consistent with the hypothesis that the Ca^{++} needed to initiate contraction is extracellular and may be flowing through nicardipine-sensitive voltage-gated Ca^{++} channels.

III. Glutamate Transport

The observation that [^{3}H]-L-glutamate is incorporated into the muscle fiber preparation is strong evidence for the existence of a glutamate transporter. The estimated EC_{50} of [^{3}H]-L-glutamate transport into the muscle fiber preparation is 51.7 μM. This is a typical value for a high-affinity glutamate transporter. *S. mansoni* glutamate transport saturates at approximately 30 minutes. The frayed fibers are part of a primary preparation, and are known to be quite temperature-sensitive. Fibers heated to 37°C retain their ability to contract only for a limited amount of time. Therefore, at time points of 30 minutes and greater, the muscle fiber preparation is deteriorating, adding to the observed effect of transport saturation.

[^{3}H]-L-Glutamate is taken up in a time-dependent manner which is similar to that of other transporters (Pines *et al.*, 1992). Transport of [^{3}H]-L-glutamate into the frayed fiber preparation is also temperature-dependent. Temperature dependence is quite common in amino acid transporters (Lerner, 1978). The calculated Q_{10} value of 3.3 for the transport of glutamate into the fiber preparation

is higher than that expected for metabolic processes which typically have Q_{10} values between 2 and 2.5 (Prosser, 1973). This is additional evidence for a transport-mediated process, rather than a ionotropic receptor-mediated response which would be less temperature-dependent.

IV. Transporter Subtype

Based on the data as a whole, it is difficult to assign the putative *S. mansoni* glutamate transporter to a particular transporter category. It is important to note that the categorization constructed for this dissertation for the cloned high-affinity glutamate transporters into EAAC-like, GLAST-like and GLT-like groups is most likely an oversimplification of the actual number of categories of these transporters (Arriza *et al.*, 1994).

The transport EC_{50} of the schistosome transporter has been estimated at approximately 52 μM, and the EC_{50} of contraction in response to L-glutamate is approximately 11 μM. However, it does not seem possible to delineate between transporter categories based on these values, because the K_m values for the cloned transporters range from 2.0 to 97.0 μM (Tables 2-4). This variation is due to the specific transporter clone being characterized, the expression system used, and method of measurement (either transport of [^{3}H]-L-glutamate or current produced by L-glutamate).

It would seem reasonable that the schistosome transporter would be similar to a subtype that is found in several tissues, and not a subtype that is restricted to the brain. In this case, the EAAC-like transporters seem a likely choice, because they have been found in broad range of tissues including the heart and skeletal muscle (Kanai & Hediger, 1992; Arriza *et al.*, 1994; Freund *et al.*, 1995). Some of the GLAST-like transporters have also been immunohistologically localized to muscle tissue (Tanaka, 1993; Arriza *et al.*, 1994; Wadiche *et al.*, 1995b), but the GLT-like transporters, to date, have only been found in the brain (Pines *et al.*, 1992; Arriza *et al.*, 1994; Freund *et al.*, 1995).

The pharmacology of the different high-affinity glutamate transporter categories is overlapping, but some generalizations can be made. Aminoadipic acid (AAD) and dihydrokainic acid (DHK) are effective inhibitors of the GLT-like transporters, but do not block uptake by the GLAST-like or EAAC-like transporters. Neither AAD or DHK were able to inhibit the glutamate-induced contraction of the muscle fibers. In addition, the amino acid cysteine, which produced contraction of the isolated muscle fibers, is a transport substrate for the EAAT3 transporter which is a GLAST-like transporter. If these generalizations hold true, then the putative transporter on the *S. mansoni* muscle membrane is most likely not a GLT-like transporter, but could be a GLAST-like or EAAC-like transporter. Of course, this question could be addressed directly by cloning and

expressing the schistosome glutamate transporter. Preliminary sequence alignment of cloned transporters reveals that it would be possible to design PCR primers or an oligonucleotide probe.

V. Transporter Function

A. Modulation of Membrane Potential

The data collected in this study support the hypothesis that there is a glutamate transporter on the *S. mansoni* muscle fiber, and that it is most likely responsible for the glutamate-induced contraction observed in the isolated muscle fibers. Glutamate transporter activation has never before been associated with a contractile effect. However, glutamate transporters are known to cause depolarization and increased levels of $[Ca^{2+}]_i$. From literature concerning glutamate's actions on rat brain synaptosomes, it was observed that externally-applied L-glutamate was causing the synaptosomes to release adenosine (Hoehn & White, 1990). It was shown that a high-affinity glutamate transporter was causing the synaptosomes to depolarize, and that this depolarization was opening voltage-gated Ca^{++} channels normally involved in transmitter release. The authors suggest that the glutamate transporter on the synaptosome may be involved in modulation of a feedback mechanism involving glutamate and adenosine by means of altering the membrane potential. This ability of glutamate transporters to modulate the

membrane potential appears to be a recurring theme to proposals of their physiological relevance *in vivo*.

Glutamate transport has also been shown to raise cytosolic Ca^{++} in GH_3 pituitary cells *via* a high-affinity transporter (Villalobos & Garcia-Sancho, 1995). GH_3 cells are responsible for hormone release, which is controlled by oscillations of intracellular Ca^{++} concentrations. The authors suggest that the ability of the glutamate transporter to modulate the GH_3 cell membrane potential may cooperate with other regulatory mechanisms, such as electrical activity and hypothalamic releasing factors, to alter hormone secretion.

Glutamate transporters play an important role in the visual system of several vertebrates. The transporters are located in rods and cones of the goldfish retina (Marc & Lam, 1981), and salamander retinal glial cells (Müller cells) (Bouvier *et al.*, 1992; Eliasof & Werblin, 1993). It has been suggested that the cellular acidification caused by the counter-transport of OH^- may modulate intracellular messengers (Bouvier *et al.*, 1992). It is known that activation of the high-affinity glutamate transporter in Müller cells raises $[K^+]_o$. When light activates photoreceptors, glutamate release is suppressed, which in turn reduces the K^+ efflux from the Müller cells, and may contribute to shaping the C-wave of the electroretinogram (Amato *et al.*, 1994).

As the mammalian high-affinity glutamate transporters were cloned, it was

found that several of the sequences were expressed in areas outside the central nervous system, such as intestine, kidney, heart, placenta, and skeletal muscle. In fact, the rabbit EAAC1 transporter was cloned on the premise that the intestinal transporters, presumably being employed for nutrient absorption, were similar to the transporters in the CNS which are responsible for terminating the action of glutamate by reuptake. Although the immunohistochemistry was explored, surprisingly little was said about the appearance of these transporters and their function outside the central nervous system. For instance, a high-affinity glutamate transporter has been described in frog red blood cells; however no attempt to formulate a physiological relevance was made (Gallardo *et al.*, 1994). There is evidence for high-affinity glutamate transporters in muscle tissue (Arriza *et al.*, 1994; Shashidharan *et al.*, 1994), but little is known about their function. The physiological function of the schistosome transporter is also unknown.

B. Glial Cell Theory

Because flatworms are not reported to have glial cells, It has been proposed that other cell types might play the role of a glial cell by transporting glutamate out of the extracellular space (Webb, 1986). Glial cells in the mammalian CNS contain high-affinity glutamate transporters and keep levels of glutamate in the extracellular space below toxic levels and provide a concentration gradient to draw

glutamate out of the synapse. It is possible that the transporter on the schistosome muscle membrane is present to transport excess glutamate released from the nervous system in a fashion similar to the extraneuronal norepinephrine uptake sites found in guinea pig tracheal smooth muscle. These transporters have a lower affinity (K_m = 156 μM) for glutamate and have been named uptake-2 (O'Donnel & Saar, 1978).

C. Post-Junctional Transport

In the flatworms, glutamate immunofluorescence is concentrated in the neural tissue (Solis-Soto & Brink, 1994), and glutamate is proposed to be an excitatory neuromuscular transmitter (Webb & Eklove, 1989). However, the studies presented here with isolated muscle fibers revealed little evidence of glutamate receptors on the muscle fibers. There are several reasons why this conclusion may be flawed. First, only one type of muscle fiber in the preparation was studied (frayed fibers), and it is possible that other schistosome fiber types contain glutamate receptors. It may be possible that only a subset of the muscle fibers contact the neural tissue, and the remaining fibers are controlled by electrical coupling (Thompson *et al.*, 1982).

It is possible that the frayed fibers studied contain glutamate receptors that were functionally damaged by the enzymatic portion of the isolation procedure,

yielding receptors that produce no excitatory activity upon application of glutamate. It is also important to consider that the frayed fibers have lost their nucleus and possibly their sarconeural arms. The schistosome muscle cell nucleus is located on a cytoplasmic stalk. Presumably, it is sheared during the isolation procedure, because they are not normally observed to be attached to the frayed fibers in our preparation. In addition, the sacroneural arm, which is an extension of the muscle fiber that contacts the nerve, may also have been sheared in the isolation procedure. Therefore, if the receptors for glutamate are localized to one of these structures, then no receptor-mediated response for glutamate would be observed in the frayed fibers.

Mammalian high-affinity transporters are present at glutamatergic synapses, and are thought to quickly sequester glutamate to terminate its action. If an excitatory glutamate receptor is normally present on the frayed fibers, then the role of the glutamate transporter on the frayed fiber membrane may be to sequester glutamate released from the neuronal tissue to terminate its action.

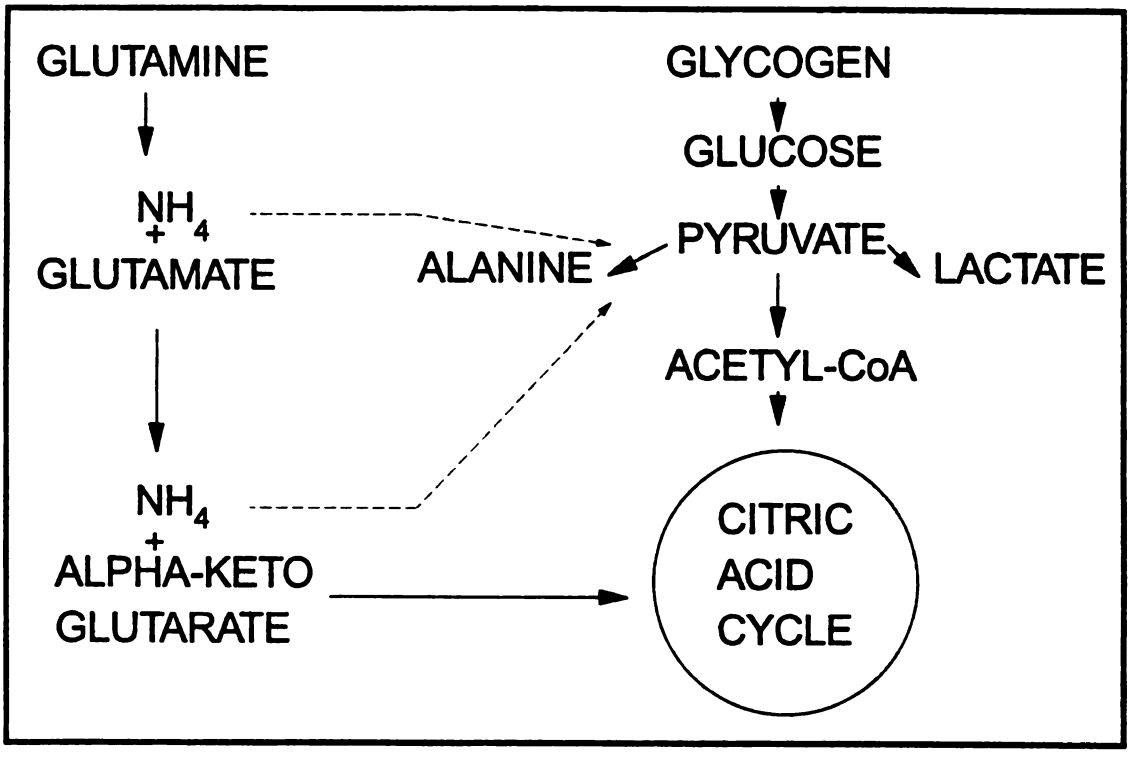
C. Metabolic Theory

L-glutamate is known to be involved in energy metabolism by functioning as a metabolic intermediate in invertebrates (Webb, 1986). The glutamate transport detected in the *S. mansoni* muscle fibers may serve the function of

transporting amino acids for metabolic purposes (Figure 17). In support of this theory, whole schistosomes have been shown to take up radiolabeled L-glutamate and L-glutamine from bath medium *in vitro*, and metabolites of [^{14}C]-L-glutamine have been traced. Results reveal that L-glutamine is metabolized to L-glutamate, α-ketoglutaramate and α-ketoglutarate (Foster *et al.*, 1989). α-Ketoglutarate can be directly incorporated into the citric acid cycle, an aerobic metabolic pathway (Figure 17).

Although it is relatively well-accepted that schistosomes are predominately lactate producers, it has been shown that they have the ability to use an aerobic metabolic pathway, especially when in an *in-vitro* environment (Tielens & VanDenBergh, 1987). This is supported by the observation that worms incubated *in vitro* release relatively large amounts of alanine (Foster *et al.*, 1989). When glutamate is converted to either α-ketoglutaramate or α-ketoglutarate, an amino group is lost, pyruvate may serve as the amino recipient in this transamination. The product of this reaction is alanine. In addition, it has been shown that schistosomes metabolize [^{14}C]-L-glutamine and [^{14}C]-L-glutamate to $^{14}CO_2$, which is the product expected if metabolism occurs *via* the citric acid cycle (Foster *et al.*, 1989). It is possible that glutamate metabolism in the schistosome muscle is a back-up energy pathway, employed predominantly during times of stress.

Figure 17. Proposed metabolic pathways of the schistosome. Normally schistosomes produce ATP in the muscle through glycogenolysis of glycogen storage granules, which are converted to pyruvate by glycolysis. The parasites release lactate as a waste product. Recently, it has been shown that *S. mansoni* will take up radiolabeled glutamine and metabolize it to glutamate, alpha-ketoglutarate, and CO_2. These *in-vitro* experiments provide evidence for aerobic metabolism. It may be that glutamine is taken up through the tegument and converted to glutamate, which is transported by the high-affinity glutamate transporter into the muscle. This glutamate could then be transaminated to alpha-ketoglutarate which can be shunted into the citric acid cycle and used as a source of energy for the muscle. The NH_4 group produced by the transamination reactions could be combined with pyruvate to be converted to alanine. This may explain why parasites incubated *in vitro* (whose glycogen stores have been depleted) produce large amounts of alanine.


GLUTAMINE
GLYCOGEN
GLUCOSE
NH4
+
GLUTAMATE
ALANINE
PYRUVATE
LACTATE
ACETYL-CoA
NH4
+
ALPHA-KETO
GLUTARATE
CITRIC
ACID
CYCLE

SUMMARY

1. Isolated frayed fibers contract in a dose-dependent manner in response to L-glutamate microperfusion, and pharmacological characterization of this response suggests that it is not mediated by a glutamate receptor.

2. The frayed muscle fiber preparation transports [^{3}H]-L-glutamate in a dose-dependent manner, which is also temperature- and time-dependent.

3. Both the contractile response of the frayed fibers and the [^{3}H]-L-glutamate transport was Na^+-dependent, and could be blocked by high-affinity glutamate transport inhibitors.

4. Other substrates of the high-affinity glutamate transporters could mimic the contractile response in isolated muscle fibers.

5. The presence of a high-affinity glutamate transporter on the membrane of the *S. mansoni* isolated frayed muscle fiber may be responsible for the contraction observed in response to microperfusion of glutamate.

6. The high-affinity glutamate transporter on the *S. mansoni* muscle fiber may play a role in modulating the membrane potential of the muscle.

7. Because flatworms do not contain glial cells, it is possible that the high-affinity transporter on the frayed fiber serves this purpose.

8. The *S. mansoni* high-affinity glutamate transporter could transport glutamate and other amino acids to be used in energy metabolism for the muscle.

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