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Phorbol Ester-induced Muscle Contraction
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PHORBOL ESTER-INDUCED MUSCLE CONTRACTION OF *SCHISTOSOMA MANSONI*

By

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ABSTRACT

PHORBOL ESTER-INDUCED MUSCLE CONTRACTION OF *SCHISTOSOMA MANSONI*

by

Kevin Leigh Blair

Phorbol esters are used to probe possible role(s) of protein kinase-C in excitatory neuromuscular modulation of *Schistosoma mansoni*. Phorbol esters induce tonic contraction of the longitudinal musculature that is both stereo-specific and site of esterification specific. A phospholipid and phorbol ester-dependent protein kinase is identified in the cytosol of the parasite and this kinase activity is stimulated by all of the phorbol esters that stimulate muscle contraction. Unlike several vertebrate protein kinase-C isozymes, this kinase activity is not dependent on Ca^{2+}. A high affinity and stereo-specific phorbol ester receptor in the parasite is demonstrated. The similarity between concentration-response curves of phorbol-12,13-dibutyrate-induced muscle contraction, stimulated kinase activity, and receptor binding indicate that these responses to phorbol ester are interrelated or coupled. These results are consistent with the hypothesis that protein

kinase-C is present in the schistosome and that its activation with phorbol esters leads to increased muscle tone.

The phorbol ester-induced contraction is dependent on extracellular Ca^{2+} and appears to be associated with an increase in permeability of the parasite to Ca^{2+} but not Na^{+} or H^{+}. The musculature is not depolarized during the phorbol ester-induced contraction, but surface electrical activity decreases. Threshold treatments of phorbol ester and depolarization or praziquantel produce synergistic contractions of the parasite. The contraction could not be explained by altered release of or sensitivity to putative neurotransmitters, decreased Ca^{2+} efflux, or an increase in the sensitivity of the contractile system to Ca^{2+}. These results support the hypothesis that activation of protein kinase-C in the schistosome with phorbol esters leads to muscle contraction by enhancing sarcolemmal Ca^{2+} channel activity.

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ABBREVIATIONS
Used in the Figures and Tables

DAG	1,2-diacylglycerol
DMSO	dimethylsulfoxide
nic	nicardipine
12,13-PDA	phorbol-12,13-diacetate
13,20-PDA	phorbol-13,20-diacetate
PDB	phorbol-12,13-dibutyrate
α-PDB	α-phorbol-12,13-dibutyrate
20-PDB	20-oxo-20-deoxy-phorbol-12,13-dibutyrate
PDD	phorbol-12,13-didecanoate
α-PDD	α-phorbol-12,13-didecanoate
PMA	phorbol-12-myristate-13-acetate
PS	phosphatidylserine
12,13,20-PTA	phorbol-12,13,20-triacetate
PZ	praziquantel
RPMI	Roswell Park Memorial Institute

GENERAL INTRODUCTION

A. Schistosomiasis.

Schistosoma mansoni, a blood-dwelling parasitic flatworm (Platyhelminthes, Trematoda), is one of the major etiological agents of human schistosomiasis in tropical and subtropical regions of the world. The World Health Organization ranks schistosomiasis second only to malaria in terms of socio-economic and public health importance. The World Health Organization estimates that as many as 1/2 billion people in 75 countries are infected, with a 1-2% annual mortality rate (Bergquist, 1987). While biological and ecological methods of control of this parasite have been attempted and a broad spectrum vaccine is held out as the hope for the future (Capron *et al.*, 1987), chemotherapy has been and continues to be the main tool for treating this disease (Archer, 1985).

Currently, four drugs (praziquantel, metrifonate, oxamniquine and hycanthone) are used to treat schistosomiasis in humans (Archer, 1985). Of these, praziquantel is the drug of choice for serious and life threatening infections. Only praziquantel is effective against all three of the major schistosome species. Praziquantel eliminates parasites when given as a single oral dose and produces few or no side effects while the other drugs require multiple dosings and can

induce human toxicity at therapeutic doses. Praziquantel is also the most expensive of these drugs. Consequently, distribution of praziquantel is often limited to only the sickest patients. Resistance to oxamniquine and its analog, hycanthone, has developed in South America (Bergquist, 1987). As a consequence there is the possibility that resistance to praziquantel may also develop and there may be a need for a new generation of antischistosomal agents.

B. Relevant anatomy and physiology.

1. General anatomy.

Schistosome anatomy is similar to that of most flatworms. These animals are dorso-ventrally flattened and aceolomate. The better defined and specialized tissues are near the surface of the animal while cells less specialized or differentiated are more central and are located in an area called the parenchyma (Bullock and Horridge, 1965). The adult male schistosome is approximately 1 cm in length, 1 mm wide, and 50 microns thick. It has an approximate wet weight of 440 μg (Pax *et al.*, 1987). The outer surface of the parasite, the tegument, is an anucleate, open-syncytium (Figure 1) (Silk and Spence, 1969a). The contractile elements of the circular and longitudinal muscles form discrete layers (outer and inner, respectively) below the tegument (Silk and Spence, 1969b). The nuclei of the tegument and muscle are contained in cell bodies (cytons) in the parenchyma beneath the muscle layer and

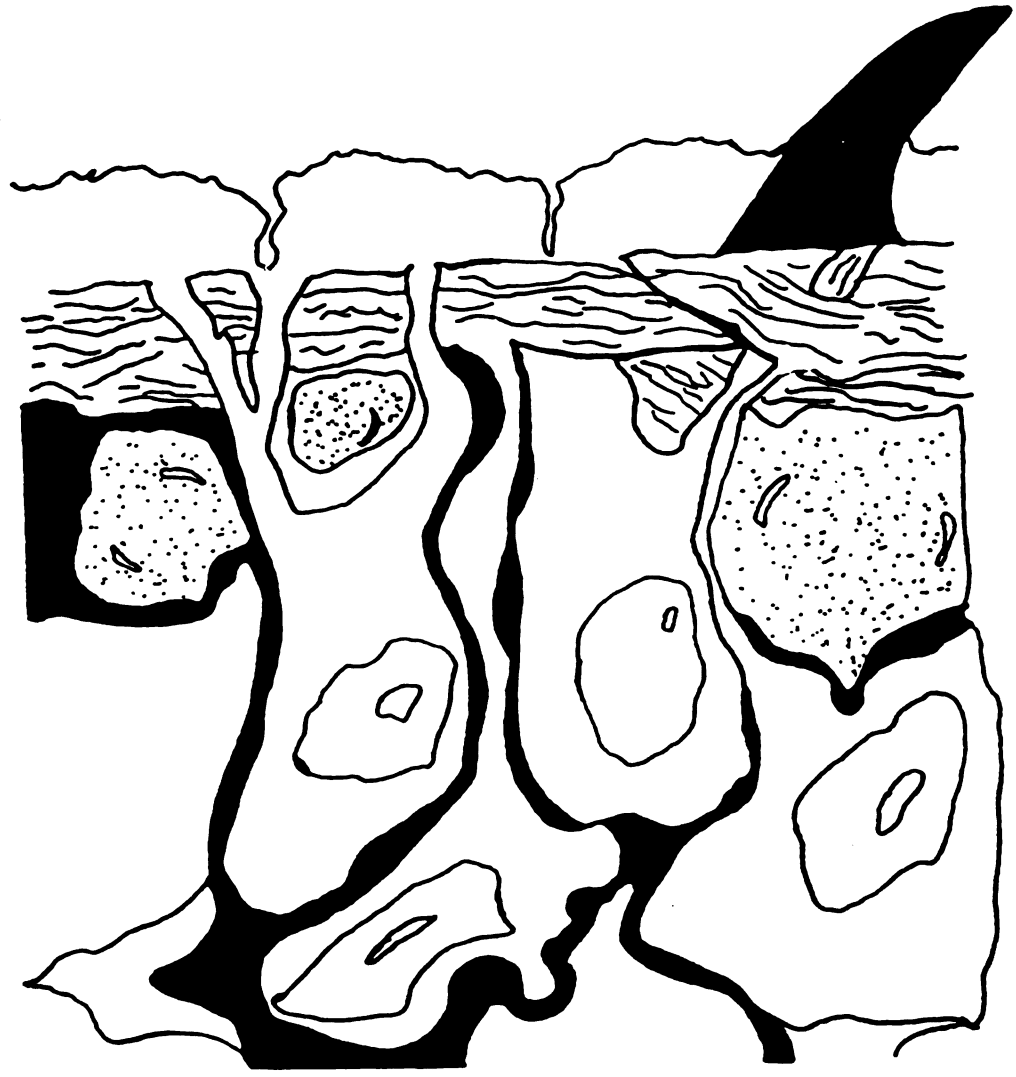

Figure 1. Diagram of the parasite in cross section. CM, circular muscle; LM, longitudinal muscle; MC, muscle cyton; n, nucleus; T, tegument; TC, tegumental cyton.

they are connected by narrow cytoplasmic processes to their respective tissues (Figure 1). The parenchymal layer surrounds a primitive gut and contains the reproductive system, protonephridial excretory system, and the somata of the neural plexus. A pair of anterior suckers give the parasite mobility.

2. The Tegument.

The tegument is the outermost layer of the parasite. It consists of a glycosylated, outer plasma membrane, a syncytial cytoplasmic layer 4-6 microns thick, and an inner plasma membrane (Silk and Spence, 1969a). There is no protective cuticle on the outer membrane. A basal lamina separates the inner tegumental plasma membrane from the circular muscle and the rest of the parasite. The tegument is electrically coupled to the longitudinal muscle (Thompson *et al.*, 1982). Actin spines, attached to the basal lamina, project through the tegument into the surrounding medium. The function of these spines is unknown.

The outer tegumental membrane is the host-parasite interface. It serves as a major nutrient uptake site and it is the site of host-immune evasion. This membrane transports hexoses (Uglem and Read, 1975; Cornford and Oldendorf, 1979), amino acids (Senft, 1968; Chappell, 1974), and purines and pyrimidines (Leavy and Read, 1975). Inhibition of nutrient uptake has been proposed as a potential target for chemotherapy (el Kouni *et al.*, 1987). Since the parasite

lives in the host's blood, the parasite must hide from and/or counter the host's immune system (Capron *et al.*, 1980). Part of this evasion is believed to be accomplished by the continuous sloughing of the outer tegumental plasma membrane as antibodies and/or complement bind to it. The efficacy of praziquantel appears to be related to its ability to bring about disruption of the tegumental membranes (Bricker *et al.*, 1983; Shaw and Erasmus, 1987) and thus allow attack by the immune system (Sabah *et al.*, 1986; Brindley and Sher, 1987; Brindley *et al.*, 1989).

The tegumental cytoplasm maintains a -60 mV potential with respect to the surrounding medium. This potential is sensitive to elevated K^+, ouabain, and temperature but not reduced Na^+ or Cl^- (Fetterer *et al.*, 1981; Pax *et al.*, 1983a). Surface electrical recordings from active parasites show a wide amplitude range (10 μV to >1 mV) of slow wave (>100 ms) electrical activity and occasional spiking (Fetterer *et al.*, 1977; Semeyn *et al.*, 1982). Microelectrode recordings from the tegument of pinned, but otherwise untreated parasites reveal spontaneous depolarizations of 4-15 mV (Fetterer *et al.*, 1980a). Current injection into the tegument of these parasites elicits non-regenerative electrical responses (Thompson *et al.*, 1982). Relaxation of the parasites with either sodium pentobarbital (1 mM), carbachol (100 μM), dopamine (100 μM), or elevated Mg^{+2} (30 mM) eliminates these electrical events. Whether they actually arise within the

tegument or are simply due to current spread from the electrically coupled muscle (Thompson *et al.*, 1982) is unknown.

3. The Neuromuscular system.

a. Anatomy.

The neural architecture of *S. mansoni* is similar to that of other trematodes and other flatworms in general (Bullock and Horridge, 1965). The central nervous system possesses a degree of cephalization. Two anterior ganglia joined by connectives are located on either side of the esophagus. These ganglia do not possess a discrete neuropile or outer cell body rind as seen in more developed polyclad flatworms (Koopowitz, 1986). A ventral pair and a dorsal pair of nerve cords project posteriorly from the ganglia through the parenchyma. They are connected via commissures at regular intervals along their length. Cell bodies and nerve-nerve synapses are located along this primitive central nervous system. Peripheral nerves project into the surrounding tissues, i.e., muscles, gut and gonads (Silk and Spence, 1969c). Surface structures which may be sensory receptors have been observed (Silk and Spence, 1969c; Gustafsson, 1987) but their specific innervation or physiological functioning has not been demonstrated. While spiking neurons have been demonstrated in the polyclad flatworm *Notoplana actiola* (Keenan and Koopowitz, 1981, 1984), they have not been demonstrated in schistosomes or other trematodes.

The musculature is of the smooth type and is arranged into well defined outer circular and inner longitudinal layers as well as random oblique (dorso-ventral) fibers (Silk and Spence, 1969b). The myofibrils consist of thick and thin filaments but they are not arranged into sarcomeres. The sarcoplasmic reticulum is poorly developed and there is no transverse tubule network. Junctional complexes (gap junctions) are believed to couple the contractile fibers together.

Neuromuscular junctions are present and the presynaptic terminals contain both light and dense core vesicles (Silk and Spence, 1969c) indicating co-transmission may occur. The identity of the transmitters involved or their mechanism of action at these junctions is unknown.

b. Physiology and pharmacology.

i. General. When the longitudinal muscle of the parasite is subjected to a load, the muscle often exhibits rhythmic phasic contractions superimposed upon some degree of tone (Fetterer *et al.*, 1977). Not all of the parasites exhibit the phasic contractions but muscle tone is always present. Both the phasic and tonic components of muscle contraction are sensitive to a variety of treatments.

ii. Membrane potential. In parasites which have been paralyzed with carbachol the muscle has a resting membrane potential of -25 to -30 mV (Bricker *et al.*, 1982). Exposure to elevated K^+ media depolarizes the tegument and

muscle and elicits a tonic contraction of the longitudinal muscle (Fetterer *et al.*, 1978, 1980a). Lack of Ca^{2+} in the incubation medium prevents the contraction but not the depolarization produced by high K^{+} (Wolde Mussie *et al.*, 1982). The contraction is also blocked by putative Ca^{+2} channel blockers such as Co^{+2}, La^{+3}, and D-600 but not by elevated Mg^{+2} (Fetterer *et al.*, 1980b). The tegument is not essential for this response since depolarization of the muscle and tonic contraction are also observed in parasites in which the tegument has been disrupted by exposure to the detergent Triton X-100 (Depenbusch *et al.*, 1983).

One reason why lack of Ca^{2+} in the incubation medium prevents tonic muscle contraction may be that the muscle is directly dependent for its contraction on extracellular Ca^{2+}. The absence of Ca^{2+} in the incubation medium could deplete extracelluar Ca^{2+}. In these whole animal studies, however the effect might also be traceable to an indirect effect due to a lack of depolarization-evoked transmitter release in the absense of extracellular Ca^{2+}.

Recently it has been shown that schistosome muscle fibers in isolation also contract rapidly when exposed to elevated K^{+} (25-130 mM K^{+}, 0.4 mM Ca^{2+}) (Lewis, personal communication). This contraction is associated with a rise in Ca^{2+}_{i} as determined with Ca^{2+}-dependent fluorescence. The contracted fibers slowly relax as K^{+} apparently diffuses away. The contraction is prevented when Ca^{+2} is omitted from the

ejection medium or when the putative Ca^{+2} channel blocker Co^{+2} (5 mM) is included in the high K^+ ejection media. The Co^{2+} block is readily reversible upon re-exposure to a high K^+ medium that does not contain Co^{2+}. These results indicate that extracellular Ca^{+2} and voltage-sensitive Ca^{2+} channels are involved in the depolarization-contraction coupling of schistosome muscle.

iii. Neurotransmitters. Several agents (serotonin, acetylcholine, catecholamines and peptides) have been proposed as possible neurotransmitters in schistosomes. Several criteria must be met before a compound can be considered a neurotransmitter (Cooper *et al.*, 1986). The putative transmitter must be present in the presynaptic terminal in its native state or as a pre-transmitter with the appropriate enzymes for conversion upon release. Specific enzymes for the synthesis of the putative transmitter should be demonstrable. The putative transmitter should be released specifically upon stimulation to initiate communication and then be rapidly removed to terminate that communication. The putative transmitter must elicit some specific postsynaptic response, a comparable response should be evocable with either exogenous transmitter or agonists and be blockable by antagonists. At this time, only a few of these criteria have been met by any of the putative transmitters in *S. mansoni*.

iv. Serotonin. Serotonin was first demonstrated in the parasite by Bennett *et al.*, (1969). It

is present in the central ganglia, the plexus associated with the musculature, and the major nerve trunks as demonstrated by formaldehyde fluorescence histochemistry (Bennett and Beuding, 1971) and by monoclonal antibody immunocytochemistry (Gustaffson, 1987). The parasite lacks tryptamine hydroxylase and is unable to synthesize serotonin from tryptamine (Bennett and Bueding, 1973). However a high affinity saturable serotonin uptake system is present (Bennett and Beuding, 1973; Wood and Mansour, 1986). This uptake system is linear during the initial minutes of uptake and has a K_m of 1.7 μM (Wood and Mansour, 1986) but it accounts for only 50-60 % of the total serotonin uptake. This saturable uptake is still present in detegumented parasites. Serotonin has not been demonstrated in presynaptic neuromuscular terminals nor has evoked release been demonstrated.

Exogenous serotonin (1 μM to 1mM) increases the rate and amplitude of contractions of the longitudinal and circular muscle (Pax *et al.*, 1984) but does not alter longitudinal muscle tone of the intact parasite. In worm slices, serotonin still increases contractile activity of longitudinal and circular muscle but increases muscle tone of longitudinal muscle while it decreases tone of circular muscle (Pax *et al.*, 1984). Serotonin also increases surface electrical activity of the parasite (Semeyn *et al.*, 1982). While a serotonin-sensitive adenylate cyclase is present in isolated membranes of the parasite (Estey and Mansour, 1987, 1988) and

antagonists such as metergoline inhibit both serotonin-dependent adenylate cyclase activity (Estey and Mansour, 1988) and muscle contraction (Pax *et al.*, 1984), a direct link between adenylate cyclase activity and muscle contraction has not been demonstrated.

v. Acetylcholine. Acetylcholineesterase-like activity is associated with nerve tissues in the parasite (Fripp, 1967) and choline acetyltransferase activity is present in extracts of the schistosome (Bueding, 1952). A substance with physiological actions on isolated guinea pig ileum analogous to that of acetylcholine has been isolated from schistosomes (Barker *et al.*, 1966) but presynaptic localization and evoked release of acetylcholine has not been demonstrated.

Cholinergic agonists relax the parasite's muscle (Fetterer *et al.*, 1977; Mellin *et al.*, 1983; Pax *et al.*, 1984; Semeyn, 1982, 1987), decrease surface electrical activity (Semeyn *et al.*, 1982) and inhibit contractions evoked by electrical stimulation (Pax *et al.*, 1981). Acetylcholine esterase inhibitors also relax the parasite and their effect is enhanced by the co-administration of exogenous acetylcholine. Cholinergic antagonists increase muscle tone (Mellin *et al.*, 1983; Pax *et al.*, 1984; Semeyn, 1987). These results indicate that the muscle is under tonic inhibition as well as tonic excitation from the nervous system.

Activation of a Ca^{2+}/Mg^{2+} ATPase has been proposed to be the mechanism by which acetylcholine stimulates relaxation of the parasite's muscles (Semeyn, 1987). Cholinergic agonists stimulate a Ca^{2+}/calmodulin-sensitive, Mg^{2+} ATPase in isolated membranes. Compounds that stimulate this ATPase also relax the parasites and stimulate $^{45}Ca^{2+}$ efflux from whole animals. Cholinergic antagonists that stimulate muscle contraction also inhibit the cholinergic-stimulated ATPase activity. The pharmacological nature of these cholinergic responses does not fit either nicotinic or muscarinic classification. No specific receptor binding studies have been reported.

vi. Catecholamines. Catecholamines have been demonstrated to be present by fluorescence histochemistry (Bennett and Bueding, 1971; Chou *et al.*, 1972) and schistosomes are able to synthesize dopamine from the precursor L-DOPA. Presynaptic localization and evoked release of catecholamines has not been demonstrated.

Exogenous dopamine produces a relaxation of the longitudinal muscle (Pax *et al.*, 1984; Semeyn *et al.*, 1982) and reduces surface electrical activity (Semeyn *et al.*, 1982) but does not prevent electrically-stimulated contractions (Pax *et al.*, 1981). No specific biochemical or biophysical mechanism has been proposed for the action of dopamine but the inability of dopamine to inhibit electrically-evoked contractions indicates that dopamine may not inhibit the muscle directly. Exogenous epinephrine, norepinephrine and

octopamine are without effect on muscle contraction (Pax *et al.*, 1984).

vii. Peptides. Positive reactivity to monoclonal antibodies raised against vertebrate neuropeptides indicates the presence of FMRF-amide, FR-amide, substance-P, leu-enkephalin, and growth hormone releasing factor in schistosomes (Gustafsson, 1987). The FMRF-amide and substance-P immunoreactivity is associated with the muscle layer. No immunoreactivity was observed for bombesin, ACTH, ß-endorphin, neurotensin, urotensin I, oxytocin, and vasotocin. No physiological activity or potential role for any of these putative neuropeptides has been demonstrated.

C. Praziquantel.

Praziquantel is a selective anthelmintic with trematocidal (Campbell, 1986) and cestocidal activity (Thomas and Andrews, 1977; Thomas and Gonnert, 1977) but no activity against turbellarians or higher organisms such as nematodes. Among the insensitive parasites are *Fasciola hepatica* and *Echinococcus multilocularis* (Campbell, 1986). Praziquantel is currently the drug of choice for treating human schistosomiasis.

Pharmaceutical praziquantel is a racemic mixture, with the *in vivo* activity residing in the levo-praziquantel isomer (Andrews, 1985; Xiao and Catto, 1989). Exposure of the parasites to praziquantel causes two major responses *in vivo*

and *in vitro*: blebbing and disruption of the tegumental membranes (Bricker *et al.*, 1983; Shaw and Erasmus, 1987; Xiao *et al.*, 1984) and contraction of the musculature (Pax *et al.*, 1978). These responses are specific to the levo-isomer (Andrews, 1985; Xiao and Catto, 1989). Laudron (1988) suggests that stereospecificity of a drug's physiological response is the most stringent evidence that the drug is interacting with a specific receptor. From this it appears likely that there is a specific receptor site for praziquantel present in the schistosome.

Despite years of study, a specific site and mechanism of action for praziquantel have yet to be demonstrated, though a praziquantel-induced loss of Ca^{2+} homeostasis may be responsible. Praziquantel increases muscle contraction at concentrations below 100 nM and complete tonic contraction (spastic paralysis) by 1 μM (Pax *et al.*, 1978). The muscle is not immediately depolarized by praziquantel (10 μM) but it is slowly depolarized over a 10 min period of exposure (Fetterer *et al.*, 1980a). This praziquantel-induced tonic contraction is dependent on Ca^{+2} in the external medium since the contraction becomes phasic when Ca^{+2} is omitted from the medium (Wolde Mussie *et al.*, 1982).

The praziquantel-induced tonic contraction also becomes phasic when the parasites are incubated in a medium in which the Mg^{+2}:Ca^{+2} ratio is elevated to 30:0.4 (Fetterer *et al.*, 1980c; Pax *et al.*, 1978, 1983b). Once praziquantel-stimulated

schistosomes relax in this elevated-Mg^{+2} medium, they cannot be stimulated to contract (flaccid paralysis) with 60 mM K^{+} (Fetterer *et al.*, 1980c) or the benzodiazepine RO 11-3128, another drug which induces sustained contractions in this animal (Pax *et al.*, 1983b). The tegument is not essential for producing this flaccid paralysis since flaccid paralysis is also observed in detegumented parsites exposed to praziquantel under the same conditions (Blair, unpublished observations). Praziquantel-induced tegumental damage is also prevented in this elevated Mg^{2+} (Bricker *et al.*, 1983) but not in the "0"-Ca^{2+} medium. The Ca^{+2} channel blockers La^{3+} or Co^{2+} (10 mM) and 100 μM D-600 do not block praziquantel-induced contraction nor do they prevent tegumental damage (Bricker *et al.*, 1983).

Praziquantel (2-10 μM), when pressure ejected onto dissociated muscle fibers, reversibly stimulates them to contract (Lewis, personal communication). The fibers contract rapidly after a 5-10 sec delay and generally stay contracted for the duration of the exposure. Once the ejection of praziquantel stops, the contracted fibers relax as the praziquantel apparently diffuses away. Fibers that do relax while praziquantel is still being ejected fail to respond to subsequent treatment with either praziquantel or high K^{+}. These induced contractions do not occur in the absense of Ca^{2+} in the ejection medium but they are not blocked when 5 mM Co^{2+} is included. This indicates that the muscle itself may contain a praziquantel target site.

Several possible mechanisms of disrupting Ca^{2+} metabolism have been shown to be inadequate to explain praziquantel's mechanism(s) of action. Praziquantel does not act as an ionophore, translocating Ca^{2+} directly (Fetterer *et al.*, 1980b). Praziquantel does not alter ATPase activities in the parasite (Nechay *et al.*, 1980) indicating that praziquantel does not interact directly with the contractile elements associated with the Mg^{2+} ATPase responsible for muscle contraction. Schistosome Ca^{2+}/calmodulin-dependent phosphodiesterase activity is not altered by praziquantel (Thompson *et al.*, 1986). Harder *et al.* (1988) and Schepers *et al.* (1988) have postulated a direct phospholipid bilayer perturbation as the praziquantel mechanism of action but the effects reported are insufficient to explain the selectivity and stereo-specificity of praziquantel.

Praziquantel appears to be acting through a receptor mediated alteration in Ca^{2+} regulation. The muscle is a target site but others (e.g., the tegument) may exist. This would explain both the muscle contraction and tegumental damage produced by exposure to praziquantel. Praziquantel and elevated K^{+} depolarization-induced contractions are dependent on the same Ca^{2+} pool, but that pool apears to be mobilized in different ways since these induced contractions are not sensitive to the same Ca^{2+} channel blockers.

D. Protein kinase-C: The phorbol ester receptor.

Transduction of extracellular signals into cells by way of receptor-mediated hydrolysis of inositol phospholipids was becoming increasingly recognized as a mechanism common to many cells (for review see Nishizuka, 1984, 1986). The two major classes of products of this hydrolysis are inositol phosphates and the lipid 1,2-diacylglycerol. These two classes of compounds act as second messengers; inositol phosphates modulate intracellular Ca^{2+} release and 1,2-diacylglycerol stimulates protein kinase-C (Majerus *et al.*, 1986). Direct measurement of the possible involvement of inositol phosphates is difficult since there are no membrane permeable analogs available. However, the possible involvement of protein kinase-C can be readily determined with the use of membrane-permeable 1,2-diacylglycerol receptor agonists, the phorbol esters (Ashendel *et al.*, 1983).

Phorbol esters are a class of turpenoids found in many members of the family Euphorbiacea, have been isolated from the oil of *Croton tiglium*, and are potent tumor promoters and inflammatory agents (Hecker, 1968, 1978). The parent structure, phorbol, has 5 hydroxyl groups that are important to activity (Figure 2). For activity, hydroxyls at carbons -12 and -13 should be esterified while hydroxyls at carbons -4, -9, and -20 should not. Carbon-4 is also a stereo center. The ß-isomers possess pharmacological and physiological activity while the α-isomers do not.

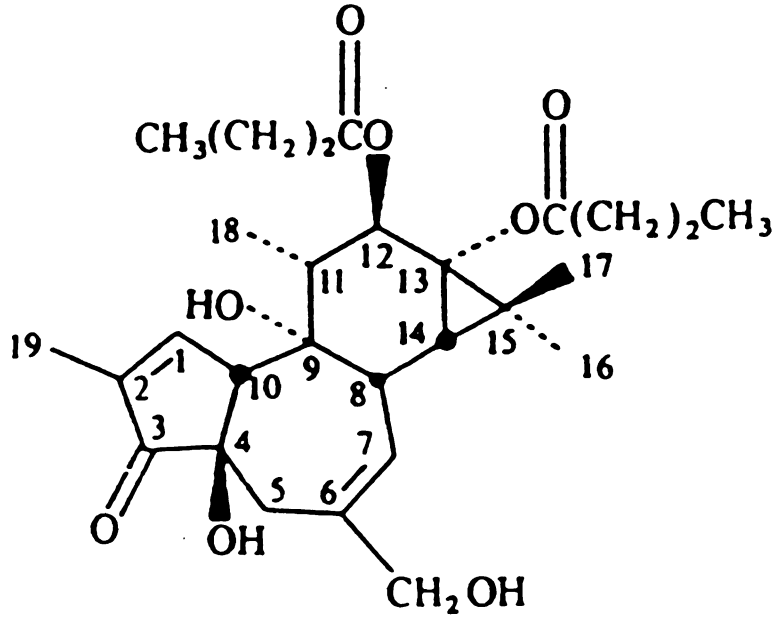

Figure 2. Structure of phorbol-12,13-dibutyrate.

Protein kinase-C was first identified in 1977 as a proteolytically activated protein kinase (Takai *et al.*, 1977; Inoue *et al.*, 1977) and was named protein kinase-M. Two years later, protein kinase activity of the protein kinase-M proenzyme was shown to be reversibly activated by Ca^{2+} and phospholipid and was named protein kinase-C (Takai *et al.*, 1979). Incomplete correlation between phorbol ester-induced tumor promotion and inflammation (Driedger and Blumberg, 1980) and curvilinear scatchard plots from [^{3}H]phorbol-12-deoxy, 13-isobutyrate binding studies (Dunn and Blumberg, 1983) indicated that protein kinase-C might not be a single, homogeneous enzyme. To date, at least seven isozymes of protein kinase-C have been identified biochemically and cloned (for reviews see Kikkawa *et al.*, 1989; Nishizuka, 1988).

Molecular analysis of brain protein kinase-C indicates that at least two sub-families of enzymes exist (Nishizuka, 1988). The first isozymes characterized (α, β_1, β_2 and γ) share four conserved peptide domains (C_1-C_4) and comprise one sub-family. The second sub-family (ϵ, δ and ζ) contain domains C_1, C_3, and C_4 only (Ono et al., 1988). Domains C_1 and C_2 contain the lipid and the Ca^{2+} binding sites while domains C_3 and C_4 contain the ATP binding site and the catalytic site. The number of members and the heterogeneity of the protein kinase-C family is expected to increase as more tissues are studied.

Various lipids and lipophilic compounds modulate protein kinase-C activity. Acidic phospholipids such as phosphatidylserine and phosphatidylinositol support protein kinase-C activity in the presence of 100 uM Ca^{2+} (Takai *et al.*, 1979), with an optimal stoichiometry of at least 4 moles of phosholipid per mole of protein kinase-C and mole of Ca^{2+}, presumably forming a complex that binds protein kinase-C to the membrane (Hannun *et al.*, 1985, 1986a,b). Basic phospholipids such as phosphatidylcholine and phosphatidylethanolamine do not support protein kinase-C activity, presumably because they do not form this complex. Neutral lipids such as 1,2-diacylglycerol (Kaibuchi *et al.*, 1981; Kishimoto *et al.*, 1980; Takai *et al.*, 1979) and tumor promoting phorbol esters (Castagna *et al.*, 1982) increase the affinity of protein kinase-C for Ca^{2+} such that it is active at physiological intracellular Ca^{2+} concentrations. 1,2-Diacylglycerol and phorbol esters bind to protein kinase-C at the same receptor site (Blumberg *et al.*, 1984; Ashendel, 1985; Niedel *et al.*, 1983).

The isozymes show differential sensitivity to activators. Arachidonic acid (McPhail *et al.*, 1984; Sekiguchi *et al.*, 1987; Tsukuda *et al.*, 1988) differentially stimulates protein kinase-C isozymes and they show varying sensitivity to Ca^{2+} (Tsukuda *et al.*, 1988). Ca^{2+} independent protein kinase-C activity has also been demonstrated (Ohne *et al.*, 1988; Schaap *et al.*, 1989). Because the isozymes are differentially

distributed (Huang *et al.*, 1987, 1988) and have differential sensitivity to activators, each isozyme may actually play a limited but well defined role in a receptor mediated response (Ono and Kikkawa, 1987; Kikkawa *et al.*, 1989).

Protein kinase-C is but one of several enzymes involved in the translation of plasmalemmal receptor-mediated cell stimulation into physiological responses. Generally, protein kinase-C dependent phosphorylation allows Ca^{2+}-dependent processes to proceed at reduced levels of intracellular Ca^{2+} (Alkon and Rasmussen, 1988; Kikkawa *et al.*, 1989). Specific physiological roles for any one of the protein kinase-C isozymes have not been demonstrated but they are expected to include modulation of secretion and exocytosis, modulation of ion channels and ion transporters, down regulation of receptors, tonic contraction in smooth muscle, gene expression and cell proliferation (for reviews see: Nishizuka, 1986, 1988; Kikkawa *et al.*, 1989). This is believed to be accomplished by several protein kinase-C dependent steps: down regulating receptor-mediated second messenger production, modulating Ca^{2+} influx through channels, and increasing the rate of Ca^{2+} efflux via the Ca^{2+} pump and Na^{+}/Ca^{2+} exchanger and shifting the Ca^{2+} sensitivity of the responding system so that it is active at lower Ca^{2+}_{i} concentrations.

OBJECTIVES AND RATIONAL

A. Objectives.

The overall objective of this study was to define further the nature of excitatory neuromuscular modulation in the schistosome. Previous studies were unable to identify adequately mechanisms coupling putative neurotransmitters to muscle contraction and relaxation. In vertebrate smooth muscle a recently identified excitatory pathway involves receptor mediated activation of phospholipid metabolism and the subsequent activation of protein kinase-C. Therefore, the first group of studies were designed to determine whether the protein kinase-C system was present in the schistosome. Once the presence of protein kinase-C was demonstrated, studies were designed to determine whether protein kinase-C-induced changes in muscle activity were neurogenic or myogenic and how activation of protein kinase-C led to this altered muscle activity.

B. Rationale.

The schistosome appears to be the best model organism with which to characterize parasitic flatworm physiology or the effects of anthelmintics. Since most of the available

information relevant to the interactions of drugs with schistosome neuromusculature has been generated using the adult male, I have confined my studies to it to maintain continuity with this data base.

There are several limitations inherent in studying this parasite. When I started these studies whole animal muscle contraction and surface electrical activity were the only techniques available for studying the effects of drugs and putative transmitters on the neuromusculature. The cells of this parasite are very small, making it impossible to maintain microelectrode penetrations unless the parasite is paralyzed, a procedure that suppresses active membrane responses. This makes difficult determination of whether the effects of the drug are direct on the muscle or somewhere in the nervous system. It is not possible to isolate specific tissues from the parasite for biochemical study. Biochemical studies, when performed, are limited to whole animal interpretations. Because of these limitations, the exact neuromuscular pharmacology has not been determined. Despite these limitations, it is my opinion that useful information can be obtained from whole animal muscle contraction and surface electrical activity. Alterations in dose-response curves must be interpreted in light of possible deviations from the law of mass action (Goldstein, *et al.*, 1974; MacKay, 1981). It is assumed from previous studies that the muscle is electrically excitable and sensitive to chemical transmission. The

specificity and selectivity of receptor agonists and antagonists are not necessarily similar to those described in mammals. The origin of electrical spikes is unknown but the origin of the slow wave electrical activity is believed to be the muscle. It is assumed that muscle contraction is due to elevated sarcoplasmic Ca^{2+}.

The results of the present study are divided into three sections. The first section provides physiological evidence for the presence of protein kinase-C in the parasite and a role in its neuromusculature. The second section provides biochemical evidence that protein kinase-C is present in the schistosome. The third section addresses the manner in which activation of protein kinase-C affects muscle activity and leads to increased muscle tone. It is hoped that this information will further the understanding of schistosome and flatworm neuromuscular modulation.

MATERIALS AND METHODS

A. Materials.

1. Parasites.

a. Intact parasites.

Adult male *Schistosoma mansoni* (St. Lucian strain), 45-55 days post infection were isolated from humanely sacrificed mice according to the method of Bennett and Seed (1977) and used throughout. These parasites have been in culture (Dr. Bennett, Department of Pharmacology/Toxicology, Michigan State University) for over 10 years. After removal from the host, the parasites were maintained in the commercial tissue culture medium, Roswell Park Memorial Institute (RPMI)-1640 (GIBCO, Grand Island, NY) to which had been added 20 mM 4-N(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (HEPES) buffer (pH 7.4) and 100 units ml^{-1} each of penicillin and streptomycin and will be referred to simply as RPMI-1640. Parasites were maintained at 37°C until used and all experiments were conducted at 37°C.

b. Detegumented Parasites.

Detegumented parasites were prepared according to the method of Oaks *et al.* (1981) with minor modifications as follows: parasites (10-20) were incubated in 3 ml ice cold

0.2% Triton X-100 in RPMI-1640 for 5 min and then vortexed at low speed (Vortex Genie) for 15 sec. The detergent solution was removed and the parasites washed 8-10 times with 3-4 ml RPMI-1640, plus 0.1% bovine serum albumin. To confirm that the tegument was removed, parasites were tested randomly for the loss of ability to exclude 0.1% trypan blue and for their lack of a tegumental membrane potential (Depenbusch et al., 1983). All of the parasites tested in this way failed to exclude trypan blue and lacked a tegumental membrane potential. Parasites treated in this manner retain muscle membrane potential (Bricker *et al.*, 1983) and are responsive to agents that affect the intact parasite, such as 60 mM K^+, ouabain, praziquantel, serotonin, and carbachol (Depenbusch, *et al.*, 1982, 1983). Exposing the parasites to higher concentrations of Triton X-100 or for a longer period of time (10-15 min) leads to a loss of responses to depolarization and to praziquantel. Thus the concentration of Triton X-100 used in this procedure does not appear to damage the muscle.

After the Triton X-100 treatment the parasites were allowed to recover for at least 1 hr in the RPMI-1640 plus 0.1% bovine serum albumin prior to testing. Once detegumented, worms were attached to the transducer system and tested to guarantee their physiological competency. Either carbachol (100 μM) or serotonin (100 μM) was added to the bath for 5 min, then the medium was replaced. Viable worms relaxed upon exposure to 100 μM carbachol or showed increased

contractions (rate and/or amplitude) upon exposure to 100 μM serotonin and returned to their original level of contraction within 15 min after the medium was replaced (Depenbusch *et al.*, 1983). Only worms that were sensitive to carbachol or serotonin in this reversible manner were tested further.

2. Salines.

a. General.

Whenever possible, studies were carried out in RPMI-1640. When a medium with an altered ionic composition was needed, the inorganic, ionic equivalent of RPMI-1640 (mM): NaCl, 102; KCl, 5.6; $Ca(NO_3)_2$, 0.4; $MgSO_4$, 0.4; Na_2HPO_4, 5.4; NaHEPES, 20 (pH 7.4) plus glucose, 11.1 and glutamine, 5 was used (Lane *et al.*, 1987) and will be called I-IRPMI. Lane *et al.* (1987) demonstrated that this saline was adequate for the short term (1-2 hr) maintenance of *S. mansoni* in culture. For the studies involving increased Mg^{2+}, Ca^{2+}, or K^+ concentrations, Na^+ was isotonically reduced. For the studies involving Cd^{2+}, Ba^{2+} or altered Ca^{2+}, the polyvalent anions were replaced with Cl^- to prevent precipitation. Previous studies (Fetterer *et al.*, 1978; Pax, unpublished observations) demonstrate that the parasite's muscle activity and membrane potential (tegument and muscle) are insensitive to these changes in Na^+ and Cl^- concentration.

b. Calcium studies and salines.

I-RPMI Ca^{2+} activities between 100 nM and 10 μM were prepared with a 10 mM ethyleneglycol-bis-(ß-amino-ethyl

ether)-N,N'-tetraacetic acid (EGTA)/Ca^{2+} buffer. A Ca^{2+} selective macro-electrode (Orion, Model 932000) was calibrated (Figure 3) with solutions of calculated Ca^{2+} activities from 10 nM to 100 μM (Tsien and Rink, 1980). The electrode had a voltage interval (slope) of 27 ± 2 mV per log Ca^{2+} activity from 100 nM to 100 μM. The voltage interval between 10 nM and 100 nM Ca^{2+} activity was 11 ± 2mV. The output of the Ca^{2+} selective electrode did not vary by more than 1 mV over a pH range of 6.6 to 9.6 demonstrating it to be insensitive to pH over the range (7.3 to 8.4) used in the calibration. Media of desired Ca^{2+} activities were prepared the day of the experiment. Mg^{2+} was omitted from these media to prevent the possibility of it blocking Ca^{2+} channels and thus the contractions. After the parasites had acclimated to the transducer system, the medium was exchanged twice with the solution of desired Ca^{2+} activity. After 15 min in the low Ca^{2+} medium, the response of the parasites were tested with various experimemtal protocols.

3. Drugs.

a. Preparation.

Drugs were prepared as stock solutions in dimethylsulfoxide (DMSO) or distilled water and diluted to the appropriate concentration for testing. The final concentration of DMSO never exceeded 1%, a concentration that had no significant effect on muscle activity. The drug concentrations reported are the final concentration of the

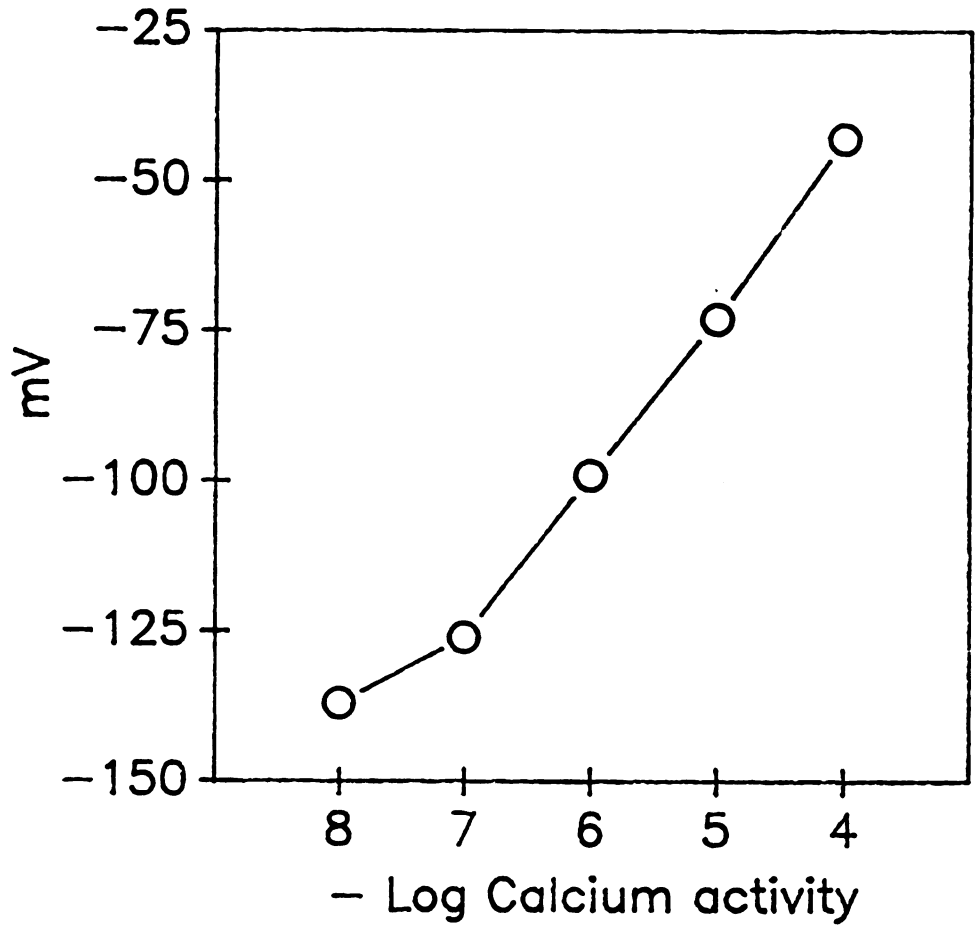

Figure 3. Sensitivity of the calcium selective macro electrode. The electrode was calibrated with solutions described in Tsien and Rink (1981) which were of calculated Ca^{2+} activities.

drug in the bath. The relationship between the concentration of a drug in the bath and the concentration of drug at the actual receptive site is not known.

b. Sources.

Praziquantel was a gift from Drs. P. Andrews and H. Thomas of Bayer AG. α-Phorbol-12,13-dibutyrate was purchased from LC Services Corp. (Woburn, MA, U.S.A.). Isotopic 5'-[γ-^{32}P]ATP (3000 Ci/mmole) and [^{3}H]PDB (10.2 Ci/mmole) were purchased from New England Nuclear. Tamoxifen•HCl was synthesized by precipitating with HCl_g tamoxifen dissolved in acetone (Tanaka, personal communication). The serotonin antagonists were obtained from Dr. Bennett (Department of Pharmacology and Toxicology, Michigan State Univerisity). All other drugs were purchased from Sigma Chemical Co., St. Louis, MO.

B. Methods.

1. Physiological Protocols.

a. Muscle physiology.

Muscle contraction and relaxation were recorded by the method of Fetterer et al., 1977). To measure muscle activity, parasites were attached to a transducer system as described in Fetterer *et al.* (1977). The posterior 1.5-2.5 mm of the worm was attached between two suction pipets constructed from polyethylene tubing (Intramedic type PE-50). One of the pipets was flexible and the other was rigid. The flexible

pipet coupled the movement of the portion of the worm between the pipets to a modified E&M type A myograph transducer. The tethered parasites were loaded with 4 mg and allowed to acclimate to the system for at least 10 min prior to the initiation of an experimental regime.

b. Membrane potential and intracellular ionic activity determinations.

Intracellular microelectrode recordings of the muscle layer were made using 20-40 megohm microelectrodes constructed from 1.2 mm o.d. borosilicate capillary tubing (World Precision Instruments, New Haven, CT, U.S.A.) filled with 3M KCl. A World Precision Instruments Model M-707A or Model FD223 electrometer was used and membrane potential was displayed on an oscilloscope screen (Tektronix 5113, Beaverton, OR) and a digital voltmeter. Several recordings were taken along the length of the parasite and averaged. A 3M KCl agar bridge and Ag/AgCl pellet served as the reference electrode. Parasites were immobilized with 100 μM carbachol to allow stable recordings and to increase the duration of the impalements.

Ion selective microelectrodes were prepared as in Pax *et al.* (1987) and Pax and Bennett (1989). Electrodes were pulled from acid (8M HNO_3) washed and ethanol (95%) washed 1.5 mm glass tubing as above. Electrodes were baked overnight at 200°C, then silanized by the addition of two drops of silane (hexamethyldisilane, Fluka Chemicals, Hauppauge, NY, U.S.A.)

to the 200°C storage chamber and baked further a minimum of 1 hr. The electrodes were allowed to cool and a 200-300 micron long column of sodium or proton cocktail (Fluka) was established at the electrode tip and the electrode was backfilled with 100 mM NaCl (sodium electrode) or 100 mM KCl and 10 mM imidazole, pH 8.4 (pH electrode). Voltage/reference electrodes were standard 3M KCl intracellular electrodes. Signals were recorded with a World Precision Instruments Model FD223 electrometer and displayed as above. Ionic activity was defined as the difference between the membrane potential in mV and the E.M.F. of the ion selective electrode (the sum of the electro-chemical gradients across the ion cocktail) in mV. Due to the small size of muscle cells, simultaneous impalement with voltage and ion-selective electrodes was not possible. Instead, several voltage and ion readings were taken along the length of the parasite and averaged to make the ionic activity calculation. This procedure should give a good approximation to the single cell response since the musculature is syncytial and isopotential (Thompson *et al.*, 1982). Parasites were immobilized with 100 μM carbachol to allow stable recordings.

c. Surface electrical activity.

Surface electrical activity was measured with a WPI DAM-60 amplifier. The non-inverting input was connected to the flexible pipet and the inverting input was grounded. The filters were set at 0.1 Hz and 1K Hz and the signal was recorded onto a chart recorder.

2. Biochemical protocols.

a. Receptor binding.

The procedure of Sando and Young (1983) was modified and used as follows: once isolated, parasites were thoroughly washed and then incubated for at least 3 hr to allow the digestion of ingested host blood. They were then homogenized in 200 vol of 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 10 μM leupeptin, and 1 mM diisopropylfluorophosphate with eight strokes of a glass/Teflon homogenizer rotating at 1000 rpm. To isolate the membranes and cytosol, the suspension was centrifuged at 1000g for 10 min and the pellet was discarded. Tissue was prepared fresh the day of the assay.

Generally, 25 μg of phosphatidylserine, 20 nM [^{3}H]phorbol -12,13-dibutyrate (10.2 Ci/mmole), and the test compound were added to silanized 12 X 75 mm test tubes. Binding was initiated by the addition of tissue (75 μg protein) to give a final volume of 250 μl and was carried out at 4 °C for 60 min. Binding was terminated by the addition of 4 ml cold 50 mM Tris-HCl (pH 7.4), and rapid filtration onto Whatman GF/C filters and washing twice with 4 ml cold 50 mM Tris-HCl. The protein was solubilized and radioactivity determined in a Beckman liquid scintillation counter at an efficiency of 30-40%. Specific binding was defined as the difference between the total radioactivity bound and that which remained bound in the presence of 10 μM phorbol-12,13-dibutyrate (non-specific binding).

b. Kinase activity.

The procedure of Martelly *et al.* (1987) was modified and used as follows. Worms were incubated as before prior to homogenization. To prepare a cytosolic fraction, parasites were homogenized in 100 vol of 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 5 mM $MgCl_2$, 5 mM EGTA, 10 μM leupeptin, and 1 mM diisopropylflurophosphate as before. The suspension was centrifuged at 1000g for 10 min and the pellet discarded. The supernatant was then centrifuged at 100,000g for 60 min and the resultant supernatant used as the kinase source. Tissue was prepared fresh the day of the assay.

Kinase activity was determined by the incorporation of ^{32}P from 5'-[γ-^{32}P]ATP into histone (Sigma type IIIs). Generally, 100 μg histone, 25 μg phosphatidylserine, 100 μM ATP (1 μCi), Ca^{2+}, and test compound were placed into silanized 12 X 75-mm test tubes. Free Ca^{2+} was determined with a Ca^{2+} selective electrode (Orion, Model 93000). Test compounds were dissolved in DMSO and diluted to the desired concentration in buffer. The experiment was initiated by the addition of cytosol (75 μg protein) to give a final volume of 250 μl. The mixtures were incubated at 35°C for 6 min. The assay was stopped by the addition of 200 μl of stop solution (50 mM Tris-HCl (pH 7,4), 20% sodium dodecyl sulfate, 20 mM p-nitrophenylphosphate and 20 mM ethylenediamine-N,N,N',N'-tetraacetic acid and boiled for 30 sec. The histone was co-precipitated by the addition of 200 μg of bovine serum albumin

and 2 ml of cold 25% trichloroacetic acid. The precipitate was pelleted in a benchtop centrifuge, the supernatant decanted, the pellet washed with 3 ml of 25% trichloroacetic acid, which was then decanted, and the pellet dissolved in 300 μl NaOH. The dissolved proteins were then precipitated and washed as before and re-dissolved in 300 μl of formic acid. The radioactivity was determined in a Beckman liquid scintillation counter. Protein kinase-C activity is defined as the difference between the precipitable radio-activity in the tubes containing phosphatidylserine only (unstimulated control) and those containing phosphatidylserine and 10 μM phorbol-12,13-dibutyrate (stimulated control) tubes.

3. Analysis of Physiological Data.

a. Data collection.

The schistosome is capable of a variety of phasic and tonic contractions and relaxations. Figure 4 shows some drug-induced changes in phasic and tonic contrations as an aid to explaining how these responses were analyzed. In each of these records, the test drug was added at the arrow, and t_0 is the level of tone at the time the drug was added to the bath. t' is the level of tone at the time the change in tone was measured, roughly 5-6 min for these examples. For all of the studies, the steady state muscle tone was taken as the average tone between the 14 and 15 min time points after the addition of the test drug unless stated otherwise. The peak contraction was determined for the experiments when

Figure 4. Determination of change in muscle tone. The response to a treatment is calculated as the change in tone from when the treatment was initiated (t_o) to the tone at a defined time point(s) after t_o, (t'). If a second treatment is added, the new t_o is that level of tone present when the second treatment was added. Several kinds of effects on tone and spontaneous contractions are possible as demonstrated in: A) 1% dimethyl sulfoxide; B) 100 μM dibutyryl cAMP; C) 100 μM carbachol. Calibration: Vertical bar, 2 mg; horizontal bar, 1 min.

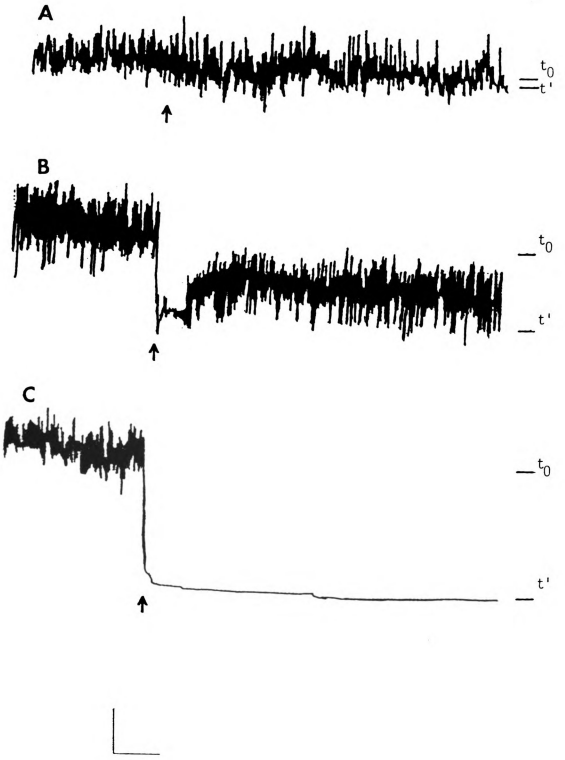

Figure 4

Figure 4 (continued). Responses to: D) 100 μM serotonin; E) 10μM phorbol-12,13-dibutyrate; F) 560 nM praziquantel. Calibration: Vertical bar, 2 mg; horizontal bar, 1 min.

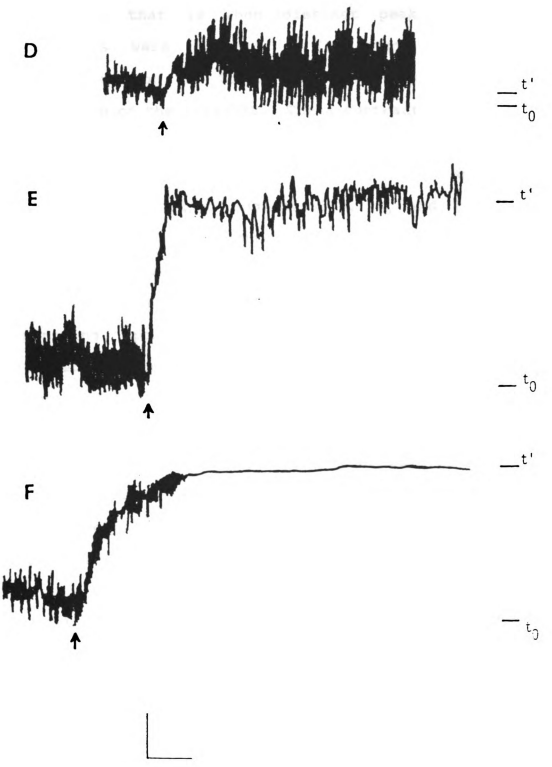

Figure 4 (cont'd.)

appropriate, that is when distinct peak and sustained contractions were evident. The vertical deflections superimposed upon the tonic level are spontaneous, phasic contractions of the parasite. These contractions and changes in their rate and/or amplitude were noted if there was an obvious change in them but they were not analyzed in detail. When drugs were added sequentially, t_0 and t' were re-established for each new drug added to the bath.

The schistosome is capable of generating a variety of responses to drugs. Tone can be reduced with or without eliminating spontaneous contractions (Figure 4B, 4C). Spontaneous contractions can be enhanced without an increase in muscle tone (Figure 4D) and tone may increase with out abolishing spontaneous contractions (Figure 4E). Since the schistosome can produce this variety of responses to drugs, tone and spontaneous contractile activity may be regulated independently. In this thesis, analysis of drug action has been concerned with changes in muscle tone only.

b. Statistics.

All data were analyzed by one-way analysis of variance. Comparisons for significance against a control were made using Dunnett's procedure. Testing for additivity and synergism was made using Scheffe's test. Synergism is defined as a response produced by the simultaneous treatment with more than one drug that is greater than the sum of each of the individual responses (Mackay, 1981). Multiple comparisons were made with

the Student Newman Keul procedure. The effects of drugs on surface electrical activity were analyzed with the signed rank test. Determination of drug EC_{50}'s and IC_{50}'s was done using the computer programs "Dose Effect Analysis with Micro-Computers" and "Ligand" (Elsevier publishing). Presented numerical data represent mean ± standard deviation and graphical data are mean ± standard error of the mean, n = 6 unless stated otherwise. Chart recordings were selected to demonstrate the most consistent or average responses observed in a given study.

RESULTS

A. Physiological evidence for the presence of protein kinase-C in the schistosome.

In preliminary visual observations of parasites *in vitro* (n=12) it was found that exposure to the protein kinase-C activator, phorbol-12,13-dibutyrate, at 1 μM in DMSO (0.1%) caused the worms to detach from the bottom of their container and to increase transiently their motor activity. After a time the worms became inactive, unpaired and partially contracted. By 5 min, none of them had reattached to the bottom of the container.

In contrast, control parasites in RPMI-1640 remained paired (male and female in copula) and the males readily attached to the bottom of the container by way of their oral and ventral suckers. They were elongate and exhibited a slow writhing motion with occasional stronger contractions. Addition of 0.1% DMSO (the solvent used in the phorbol-12,13-dibutyrate studies) resulted in a transient increase in the activity and a temporary loss of attachment of the parasites but within a minute they had reattached to the bottom of the container and appeared to return to their pretreated condition (n=8).

To obtain more quantitative data on the effects of protein kinase-C activators on the schistosme, a mechano-transducer system was used (Fetterer *et al.*, 1977). A variety of protein kinase-C activators were tested and, with this system it was obvious that changes in muscle tone were produced by low levels of a variety of these activators.

Phorbol-12-myristate-13-acetate induced a concentration-dependent increase in tone which was generally accompanied by a decrease in the spontaneous activity of the muscle (Figure 5A, Table 1). The figure illustrates a response to 1 μM phorbol-12-myristate-13-acetate but maximal tone was achieved with as little as 10 nM. The time to reach 1/2 maximal force ($t_{1/2}$) was also dose-dependent (Table 1). Once a maximal phorbol-12-myristate-acetate-induced tonic contraction was obtained and while phorbol-12-myristate-13-acetate was present, the muscle did not relax during the subsequent hour over which observations were made.

Phorbol esters exhibit a range in their abilities to stimulate protein kinase-C, efficacy depending on the sites of phorbol esterification and the specific fatty acids participating in the ester linkages (Ashendel, 1985; Jeffrey and Liskamp, 1986). If a protein kinase-C-like enzyme was involved in this phorbol ester-stimulated muscle contraction for the schistosome, a phorbol ester structure-activity relationship for the induced contractions should be similar to that observed for phorbol ester-activation of mammalian

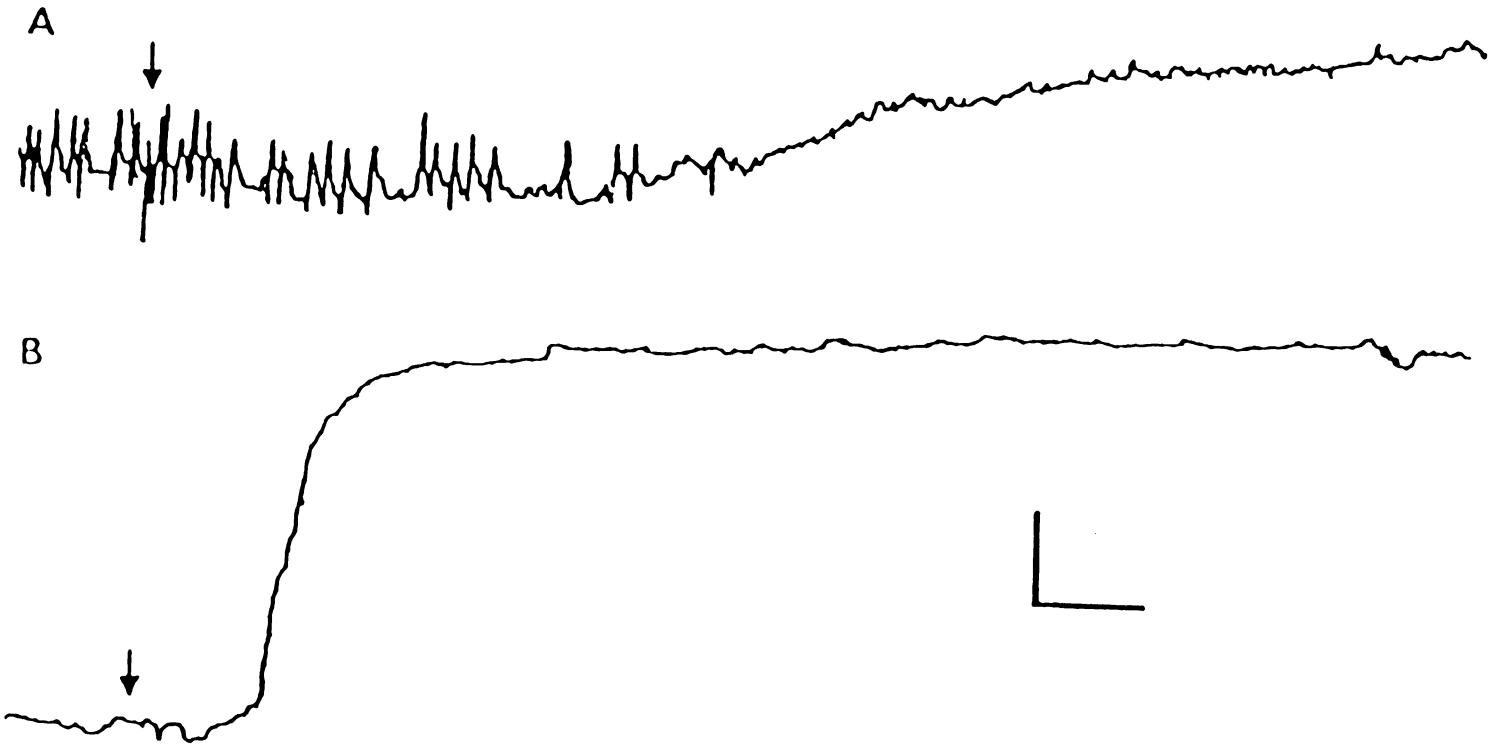

Figure 5. Representative responses of *Schistosoma mansoni* muscle to phorbol esters. Parasites were incubated in RPMI-1640 for 15 min and then 1 μM phorbol-12-myristate-13-acetate (A) or 1 μM phorbol-12,13-dibutyrate (B) was added (arrows) to the bath. Calibration: vertical, 2 mg; horizontal, 1 min.

Table 1. Changes in the Muscle Tone of Intact *Schistosoma mansoni* by Agents known to Modulate Protein Kinase-C

Agent	-log Conc	Contraction (mg)	$t_{1/2}$ (min)
CONTROL	1%	-0.1 ± 0.7	NA
PMA	5	4.5 ± 2.0**	4.9 ± 0.7
	6	3.7 ± 1.0**	5.4 ± 2.2
	7	3.7 ± 1.6**	6.1 ± 1.4
	8	3.5 ± 0.8**	10.5 ± 3.2
	9	0.7 ± 1.1	NA
PDB	5	7.0 ± 1.3**	0.8 ± 0.6
	6	7.1 ± 2.5**	1.5 ± 0.4
	7	4.4 ± 1.4**	1.3 ± 0.5
	8	2.8 ± 3.0*	16.4 ± 4.0
	9	-1.3 ± 1.0	NA
α-PDB	6	-0.5 ± 0.4	NA
12,13-PDA	5	3.5 ± 0.8**	1.5 ± 0.5
	6	0.8 ± 0.3	NA
13,20-PDA	5	0.1 ± 0.7	NA
12,13,20-PTA	5	0.2 ± 0.8	NA
20-PDB	6	0.2 ± 1.0	NA
Mezerin	5	3.1 ± 0.8**	1.8 ± 0.7

Numbers represent mean ± SD. All statistical comparisons are with the DMSO control group using a one-way analysis of variance and Dunnett's procedure.

* $p \leq 0.05$
** $p \leq 0.01$
NA: not applicable

protein kinase-C. For this reason a variety of other phorbol esters were also tested for their ability to produce muscle contraction in the schistosome.

Phorbol-12,13-dibutyrate produced contractions similar to those induced by phorbol-12-myristate-13-acetate (Figure 5B, Table 1) but a higher concentration was required. At the higher concentrations, however, the response was nearly twice that elicited by phorbol-12-myristate-13-acetate at equal concentrations. The response was also about four times faster than the response to phorbol-12-myristate-13-acetate.

Since the fatty acid chains in phorbol-12,13-dibutyrate are shorter than those in phorbol-12-myristate-13-acetate, phorbol-12,13-dibutyrate would be expected to have a higher solubility in the cytosol of cells. Thus, phorbol-12,13-dibutyrate may stimulate and translocate more protein kinase-C into the membrane, possibly explaining why it is more effective at higher concentrations. These short chain fatty acids in phorbol-12,13-dibutyrate also reduce its affinity for mammalian protein kinase-C (Ashendel, 1985) which would account for the requirement for higher concentrations of phorbol-12,13-dibutyrate to stimulate a maximal increase in tone. As with phorbol-12-myristate-13-acetate-treated worms, this increased muscle tone was maintained as long as phorbol-12,13-dibutyrate was present.

The diacetate ester, phorbol-12,13-diacetate, increased tone when applied at 10 μM but was inactive at 1 μM (Table 1).

Phorbol-12,13-diacetate also has in mammalian preparations, a lower receptor affinity than phorbol-12-myristate-13-acetate (Ashendel, 1985) and is consitent with the greater concentration of phrobol-12,13-diacetate needed to induce significant increases in the tone of the schistosome muscle. Though receptor affinity for phorbol-12,13-diacetate is much lower than that for phorbol-12,13-dibutyrate, both should have similar solubility in cytosol. This could account for the observation that the $t_{1/2}$ values of these two compounds are essentially identical in the schistosome. They probably penetrate to the target tissue(s) in the schistosome faster than phorbol-12-myristate-13-acetate.

Two other acetate esters, phorbol-13,20-diacetate and phorbol-12,13,20-triacetate, were inactive even at 10 μM (Table 1). The 20-oxo-20-deoxy derivative of phorbol-12,13-dibutyrate was inactive at 1 μM. These results are consistent with the reported phorbol ester structure activity relationship in mammals (Ashendel, 1985; Jeffrey and Liskamp, 1986).

Mammalian protein kinase-C is also stereo-selective, α-phorbol esters being inactive. This also appears to be the case for schistosmes since α-phorbol-12,13-dibutyrate at 1 μM was not active (Table 1). Though active in mammalian systems, phorbol-12,13-didecanoate produced erratic and inconsistent responses in the schistosome at 1 μM. In these experiments the end point being measured is contraction of muscle. Since

any compounds acting on the muscle must pass through the tegument to reach their site of action, a lack of response may reflect an inability of that compound to reach the muscle.

To increase the accessibility of the muscle to the applied compounds, a detegumented worm preparation was also tested. The detegumented worms exhibited no spontaneous activity and appeared to be slightly contracted. However phorbol-12,13-didecanoate, at 1 μM, still significantly increased tone (Table 2). The inactive isomer, α-phorbol-12,13-didecanoate, and the parent alcohol, α-phorbol, were still without effect. For purposes of comparison, phorbol-12,13-dibutyrate was tested in this preparation also. Phorbol-12,13-dibutyrate (1 μM) also increased tone but the increase was less than that in the intact animals, probably reflecting the partially contracted state of the detegumented parasites. From these results it is clear that the increased muscle tone produced by these protein kinase-C activators is not dependent on the integrity of the tegument or of surface sensory receptors.

Mezerin is a compound that does not contain the parent phorbol structure, but does interact with the diacylglycerol receptor (Ashendel, 1985). Mezerin, when applied to the schistosome, produced a response similar to that of the phorbol esters. That is, mezerin-treated parasites showed an increase in tone and a decrease in spontaneous activity (Table 1). However, mezerin was not as effective or as potent as

Table 2. Effect of Selected Phorbol Esters on the Muscle Tone of Detegumented *Schistosoma mansoni*

Agent	-log Conc	Contraction (mg)	$t_{1/2}$ (min)
DMSO	1%	0.1 ± 0.5	NA
PDB	6	4.4 ± 0.7**	1.7 ± 0.4
ß-PDD	6	3.5 ± 1.1**	10.9 ± 5.6
α-PDD	6	0.9 ± 0.6	NA
α-phorbol	6	0.6 ± 0.8	NA

Numbers represent mean ± SD. All statistical comparisons are with the DMSO control group using a one-way analysis of variance and Dunnett's procedure.

** $p \leq 0.01$
NA: not applicable

phorbol-12-myristate-13-acetate or phorbol-12,13-dibutyrate since a concentration of 10 μM was required to produce a significant contraction.

A series of 1,2-diacylglycerols (hexanoyl (C6:0), dioctanoyl (C8:0), didecanoyl (C10:0), and 1-octanoyl-2-acetyl glycerol) were also tested on intact and detegumented parasites but none increased tone. These compounds are metabolized to phosphatidic acid by diacylglycerol kinase (Severson and Hee-Cheong, 1989) and this may be a reason for their lack of action. To test this possibility, the diacylglycerol kinase inhibitor R 59 022 (de Chaffoy de Courcelles *et al.*, 1985) was tested on the parasites. Intact parasites contracted when exposed to 1 μM R 59 022 but their responses were biphasic when exposed to 3 μM R 59 022. That is, the parasites contracted initially but relaxed after 5 min. The reported active range of this drug as a diacylglycerol kinase inhibitor is 1 to 30 μM. The product information sheet accompanying this drug indicates that R 59 022 is also an antagonist of serotonin, dopamine, and histamine receptors at these concentrations. Thus the responses that I observed may not be due specifically to diacylglycerol kinase inhibition. This line of investigation was discontinued due to this lack of specificity.

There are several drugs that inhibit protein kinase-C activity *in vitro*. Tamoxifen, one such inhibitor (Su *et al.*, 1985), is not soluble in RPMI-1640 above a final concentration

of 1 μM. To circumvent this problem, the water soluble salt, tamoxifen•HCl, was synthesized as described in the methods section. Tamoxifen•HCl at a concentration of 10 μM produced a relaxation of 2.9 ± 1.4 mg ($p \leq 0.05$) while 100 μM relaxed the muscle by 5.1 ± 2.8 ($p \leq 0.01$). If tamoxifen•HCl relaxes the parasites by inhibiting protein kinase-C activity, the response to phorbol esters would be expected to be blocked since tamoxifen is a non-competitive inhibitor of phorbol ester-stimulated protein kinase activity (Su *et al.*, 1985). Though tamoxifen•HCl relaxed the parasites, it did not prevent the response to phorbol-12,13-dibutyrate. Phorbol-12,13-dibutyrate at 1 μM, a concentration that produced 7.1 ± 2.5 mg previously, still increased tone 5.0 ± 0.9 mg in the presence of 100 μM tamoxifen•HCl. Since tamoxifen is an estrogen antagonist, the estrogen agonist, 17-ß-estradiol (100 nM) was also tested but no significant changes in muscle tone were observed within 15 min.

Compound H-7, another protein kianse-C inhibitor (Hidaka *et al.*, 1984), had no apparent effect on parasite muscle activity nor did it block phorbol-12,13-dibutyrate-stimulated increases in tone. However this could be do to a lack of permeability of H-7. Sphingosine, a lipid metabolite released from the hydrolysis of sphingolipid, can inhibit protein kinase-C (Hannun and Bell, 1987). Sphingosine had no apparent effects on the schistosome at concentrations from 1 μM to 100 μM.

When intracellular Ca^{2+} is increased in mammalian cells, protein kinase-C translocates from the cytosol to the plasma membrane, enhancing the effectiveness of protein kinase-C activators (Nishihira *et al.*, 1986; Pontremoli *et al.*, 1986; Zatz *et al.*, 1987). The calcium ionophore A-23187 binds Ca^{2+} and translocates it across lipid membranes (Reed and Lardy, 1972). This makes it a useful compound for increasing intracellular calcium levels *in vitro*. For this reason A-23187 is commonly used in protein kinase-C/Ca^{2+} synergism studies (Park and Rasmussen, 1985; Nishihira *et al.*, 1986; Pontremoli *et al.*, 1986; Zatz *et al.*, 1987).

The schistosome muscle has a sensitivity to A-23187 which is similar to that of mammalian smooth muscle (Park and Rasmussen, 1985). In schistosomes, concentrations af A-23187 below 1 μM do not alter muscle tone or the rhythmic contractions. At 1 μM, A-23187 produces an increase in muscle tone (3.2 ± 1.3 mg) with a $t_{1/2}$ of 4.5 ± 2.3 min while at 10 μM the response is much greater (7.8 ± 1.5 mg) and much quicker ($t_{1/2}$ = 0.5 ± 2.3 min).

When phorbol-12-myristate-13-acetate at 100 nM, which by itself produced an increase in tone of 3.7 ± 1.6 mg, and A-23187 at 10 μM were applied simultaneously, muscle tone increased by 5.0 ± 1.3 mg. This level of tone is significantly less than the 7.8 mg produced by this level of A-23187 alone ($P \leq 0.01$) but it is not different from that for phorbol-12-myristate-13-acetate alone. The $t_{1/2}$ for the

response to this combined treatement was 1.9 ± 1.3 min and is significantly faster than that for phorbol-12-myristate-13-acetate alone ($t_{1/2}$=6.1 ± 1.4, $p \leq 0.01$) but not statistically different from that for A-23187 alone. Lower concentrations of phorbol-12-myristate-13-acetate (1, 3, and 10 nM) and of A-23187 (0.1 and 1 μM) also failed to synergize each other to increase muscle tone. This was not expected in light of the mammalian studies (Park and Rasmussen, 1985) and may indicate a difference between the protein kinase-C's of mammals and shistosomes. One possibility is that the schistosome protein kinase-C is not Ca^{2+} dependent. This possibility is explored further in the following biochemical studies.

B. Biochemical evidence for the presence of protein kinase-C activity in the schistosome.

1. Phorbol ester receptor binding.

[^{3}H]Phorbol-12,13-dibutyrate-specific binding to the schistosome membrane/cytosol preparation was very low (27%) but addition of phosphatidylserine (25 μg) and/or Ca^{2+} (100 μM) to the tissue preparation increased specific binding. Addition of phosphatidylserine increased specific binding to a level twice that obtained by the addition of Ca^{2+}. The combined addition of Ca^{2+} and phosphatidylserine to the tissue preparation did not increase specific binding beyond that with phosphatidylserine alone. Therefore, only phosphatidylserine was added to the tissue, giving a specific binding of 65-80%.

Greater than 90% of the nonspecific binding was to the filter. Specific binding was linear from 25 to 100 μg of tissue protein. Assays were conducted with 75 μg of tissue protein as a compromise between the signal to noise ratio and the linearity of the assay. No specific binding could be demonstrated in the absence of tissue.

[^{3}H]Phorbol-12,13-dibutyrate was displaced in a concentration-dependent manner by nonlabeled phorbol-12,13-dibutyrate with an approximate IC_{50} of 20 nM and displacement saturated by 1 μM (Figure 6). Phorbol-12-myristate-13-acetate displacement of [^{3}H]phorbol-12,13-dibutyrate binding was also concentration-dependent with an approximate IC_{50} value of 3 nM and saturated by 100 nM phorbol-12-myristate-13-acetate. Specific binding was also stereo-specific since 1 μM phorbol-12,13-didecanoate reduced binding while 1 μM α-phorbol-12,13-didecanoate did not. α-Phorbol-12,13-dibutyrate was not tested in this assay since it was not available at the time these experiments were performed. The putative protein kinase-C antagonists were not tested as their actions are not competitive with phorbol esters and would not be expected to interact with the diacylglycerol receptor. These binding results provide a link to the phorbol ester-induced contractions of the parasites and are consistent with receptor affinities reported for mammalian tissues (Ashendel, 1985).

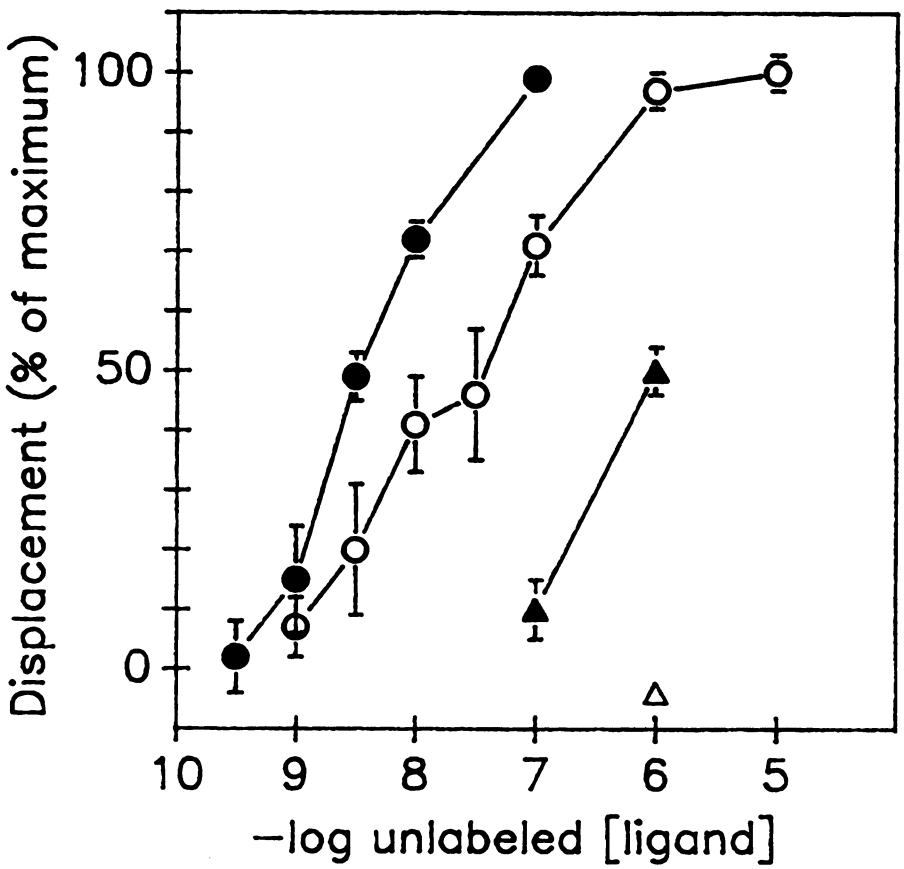

Figure 6. Displacement of [^{3}H]phorbol-12,13-dibutyrate from *Schistosoma mansoni* membranes and cytosol. The ligands tested are: phorbol-12-myristate-13-acetate (●); phorbol-12,13-dibutyrate (o); phorbol-12,13-didecanoate (▲); and α-phorbol-12,13-didecanoate (△). The assay was initiated by the addition of 75 μg tissue protein to silanized 12 X 75 mm glass culture tubes containing 100 μg phosphatidylserine, 20 nM [^{3}H]phorbol-12,13-dibutyrate, the competing unlabeled ligand, and 50 mM tris HCl, pH 7.4 to give a final volume of 250 μl. Competitive binding was carried out in an ice bath and terminated after one hour by the addition of 3 ml ice cold 50 mM Tris HCl, pH 7.4 and rapid filtration onto glass fiber filters. The maximal displacement was defined as that produced by 10 μM phorbol-12,13-dibutyrate. Data are presented as the mean ± SEM of experiments performed in triplicate.

2. Phorbol ester and diacylglycerol stimulated protein kinase activity.

The appearance of phorbol ester-stimulated protein kinase activity of the cytosol was time-dependent. No significant kinase activity was detected for incubations of less than four min; a time-dependent increase in activity was seen between four and six min, but after six min activity decreased. Phosphatase activity in this crude preparation could be one reason for this decline in activity.

Addition of Ca^{2+} and/or phosphatidylserine to the tissue preparation did not stimulate protein kinase activity (Figure 7). When both phosphatidylserine and phorbol-12,13-dibutyrate were added in the presence or absence of Ca^{2+} (10 μM), protein kinase activity was stimulated by 30-40%. Other concentrations of Ca^{2+} were tested and were without stimulatory effect. In the absence of phosphatidylserine, phorbol-12,13-dibutyrate did not significantly stimulate protein phosphorylation (Figure 7). These results suggest that this protein kinase-C activity is independent of Ca^{2+} and is consistent with the lack of synergism between phorbol-12-myristate-13-acetate and A-23187 described in the previous section. The phorbol ester-dependent kinase activity of the cytosol was roughly 25% that of cAMP (1 mM)-stimulated protein kinase activity. Praziquantel (10 μM) did not stimulate protein kinase activity in any of these assay conditions.

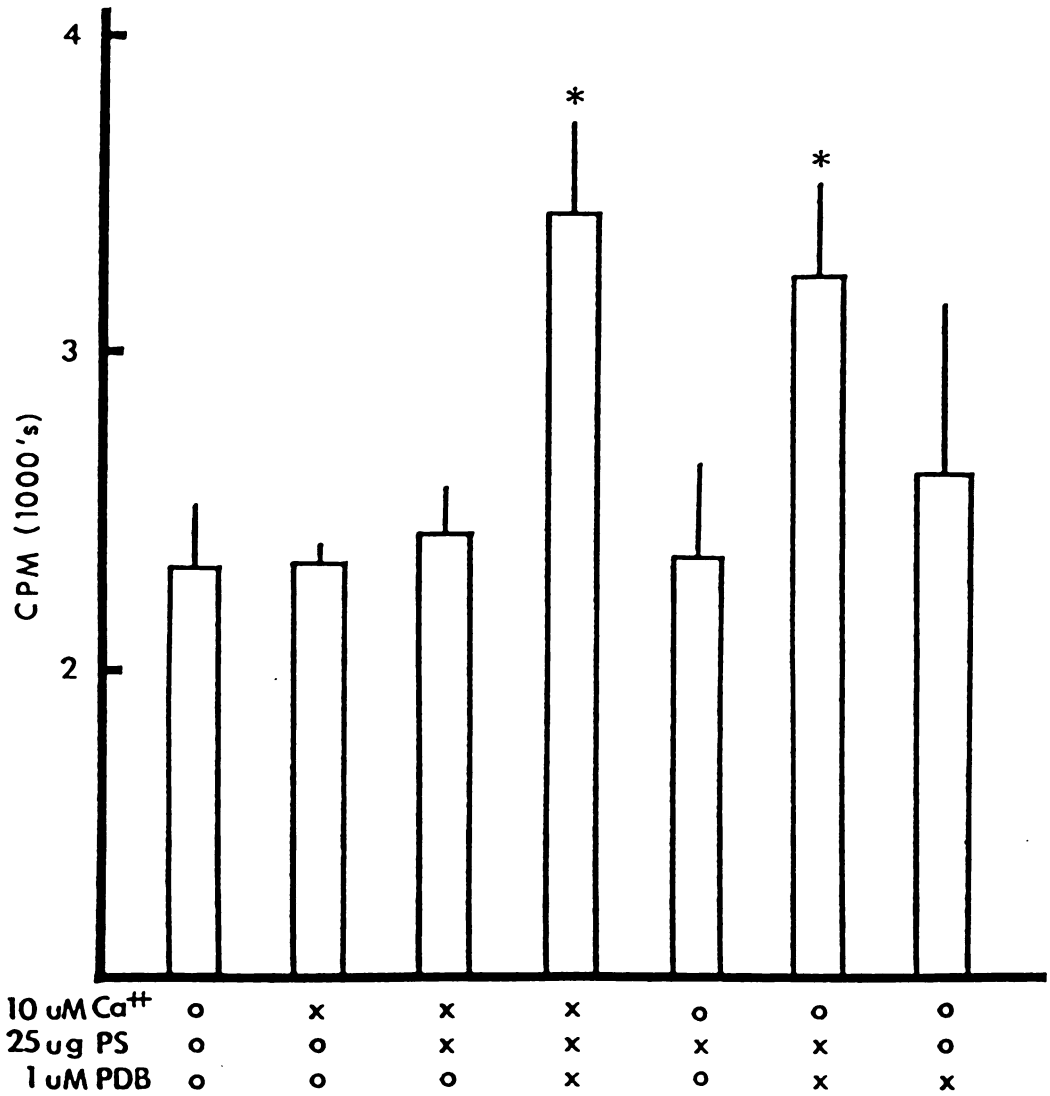

Figure 7. Phorbol ester-dependent protein kinase activity in *Schistosoma mansoni* cytosol. Protein kinase activity was determined by the incorporation of ^{32}P into histone. The assay was initiated by the addition of cytosol to silanized 12 X 75 mm culture tubes containing 100 μg histone, 100 μM ATP (1 μCi) and the test compounds: calcium, phosphatidylserine, and/or phorbol-12,13-dibutyrate. Test compounds were added to give the final designated concentration (X); or compound was not added (O). Values are presented as the means ± SEM of triplicates from a representative experiment. All statistical comparisons are with the tubes that received no test compounds using a one-way analysis of variance and Dunnett's procedure.

* $p \leq 0.01$.

The ability of phorbol esters to stimulate muscle contraction exhibited several structural requirements. These requirements were reflected in the structural specificity for the stimulation of protein phosphorylation (Table 3). The physiologically active phorbol esters phorbol-12-myristate-13-acetate, phorbol-12,13-dibutyrate, phorbol-12,13-didecanoate, and phorbol-12,13-diacetate stimulated protein kinase activity at the same concentrations at which they produced physiological responses. Figure 8 shows that the dose-dependency and saturation of phorbol-12,13-dibutyrate stimulated muscle contraction, receptor binding and kinase activition are similar. The kinase stimulation was stereo-specific with respect to the parent phorbol since α-phorbol-12,13-didecanoate and the parent α-alcohol were inactive (Table 3). Phorbol esters substituted at the 20-position did not stimulate protein kinase activity (Table 3). The non-phorbol ester, diacylglycerol receptor agonists, mezerin and 1,2-dioctanoyl glycerol also significantly stimulated protein kinase activity (Table 3). These results are consistent with the phorbol ester pharmacology and activator dependency of protein kinase-C activity (Nishizuka, 1988).

The putative protein kinase-C antagonists were not tested because they are not specific to protein kinase-C and it would not be possible to prove that decreased kinase activity was specific to an inhibition of protein kinase-C while other kinases and phosphatases were present in the preparation. A

Table 3. Protein Kinase C Activity and [^{3}H]phorbol-12,13-dibutyrate Receptor Displacement by Selected Compounds in Tissue Preparations of *Schistosoma mansoni*

Agent	-log Conc	PK-C Activity (% of control)	[^{3}H]PDB Displacement (% of control)
DMSO	1%	0	0
PDB	5	100 *	100 *
PMA	7	42 ± 19*	99 ± 20*
12,13-PDA	5	38 ± 23*	ND
Mezerin	5	130 ± 11*	ND
1,2-DAG (C8:0)	5	75 ± 35*	ND
ß-PDD	6	88 ± 17*	50 ± 16*
α-PDD	6	1 ± 30	-4 ± 17
α-phorbol	5	-16 ± 12	ND
13,20-PDA	5	-10 ± 11	ND
12,13,20-PTA	5	-7 ± 13	ND
20-PDB	6	-7 ± 12	ND

Numbers represent mean ± SD. All statistical comparisons are with the DMSO control group using a one-way analysis of variance and Dunnett's procedure.

* $p \leq 0.05$
ND: not determined

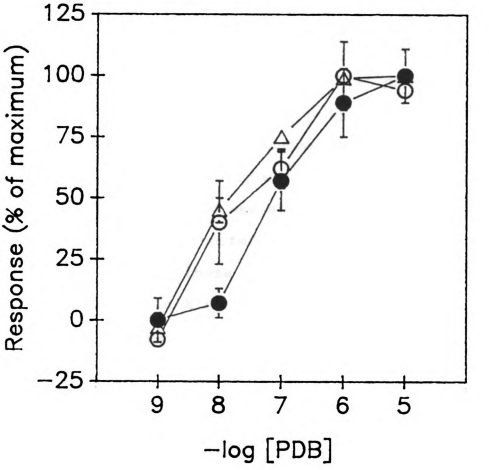

Figure 8. Dose-response relationship of phorbol-12,13-dibutyrate-induced responses of the *Schistosoma mansoni* preparations. The responses compared are muscle contraction (O), specific binding (Δ), and protein kinase activity (●). Curves are not significantly different by comparison of regression.

purified enzyme preparation will be necessary before a detailed kinetic and biochemical analysis of this protein kinase-C activity will be possible.

To compare the possible relationship between the protein kinase-C in the schistosome with mammalian protein kinase-C, schistosome extracts were sent to the UpJohn Co. to be tested with antibodies raised against mammalian (rat brain) protein kinase-C. If immunoreactivity was demonstrated, the presence of a protein kinase-C that was simmilar to rat brain protein kinase-C would be supported. No immunoreactivity was detected. At the time these experiments were conducted, only a few of the protein kinase-C isozymes were known and the available antibodies generally did not recognize multiple isozymes. Thus, these antibodies may not have recognized the specific protein kinase-C present in this primitive invertebrate.

In summary, phorbol esters are the most potent stimulants of schistosome muscle contraction tested to date. Phorbol esters stimulate muscle contraction with the structural requirements and stereo-specificity as observed for receptor binding and stimulated protein phosphorylation, indicating the involvement of a common mechanism. The dose-dependency of phorbol-12,13-dibutyrate-induced muscle contraction, receptor binding, and protein kinase activity were not statistically different indicating that all three of these events could be regulated by the same phorbol-12,13-dibutyrate-dependent

mechanism. This similarity also indicates that there is a close coupling between phorbol ester binding, kinase activation and increased muscle tone. These results are consistent with the presence of a protein kinase-C enzyme in the parasite that binds phorbol esters and when artificially activated by phorbol esters, leads to increased muscle tone.

C. Studies on the site and manner in which protein kinase-C affects motor activity in the schistosome.

While the previous studies indicate that protein kinase-C is present in schistosomes and affects the muscles of the parasite, they give no indication as to the site or sites at which the protein kinase-C might be acting. Protein kinase-C is present in most tissues and it can modulate several physiological and biochemical systems (Nishizuka, 1984, 1986, 1988). Two possible ways in which protein kinase-C activation leads to muscle contraction are that it changes neural input to the muscle or that it acts directly on the muscle itself. The following studies were undertaken to determine if either of these mechanisms are involved in the muscle contractions produced by protein kinase-C activation in schistosomes.

1. Changes in neural input to the muscle.

Phorbol esters could be acting presynaptically to enhance serotonin release or decrease acetylcholine release, or acting to alter postsynaptic sensitivities to these putative

neurotransmitters. A consequence of any of these actions would be expected to be an overall excitation of the muscle.

a. Serotonin.

Serotonin, when applied to whole schistosomes, causes marked stimulation of spontaneous motor activity. The phorbol esters might be acting as serotonin agonists, enhancing serotonin release, or enhancing postsynaptic sensitivity to serotonin. If any of these mechanisms are involved, then one would expect serotonin antagonists to interfere with phorbol ester activation of the muscle.

Flatworm serotonin-receptor pharmacology is different from that of known vertebrate serotonin receptors (Mansour, 1984). The following serotonin antagonists were tested for their ability to relax the parasite and to block 100 μM serotonin-induced muscle activity: metergoline, ketanserin, methylsergide, mianserin, cinanserin, lergotrile, quipazine, harmane, harmine, harmaline, and harmalol. These drugs were selected for their reported ability to inhibit serotonin-dependent adenylate cyclase activity or reduce muscle activity in trematodes (Estey and Mansour, 1988; Northrup and Mansour, 1978). Of these drugs, only metergoline and ketanserin relaxed the parasites consistently and blocked serotonin-induced contractile activity. They were active only at or above concentrations of 30 μM and 100 μM respectively. These values are much greater than the reported K_i's of these drugs for the serotonin-dependent adenylate cyclase activity in

S. mansoni tissues: 170 nM and 7.4 μM respectively (Estey and Mansour, 1987).

The ability of metergoline and ketanserin to interfere with phorbol ester activation of the muscle was tested in two experimental protocols: In the first, parasites were relaxed with either of these two serotonin antagonists for 15 min and then challenged with 1 μM phorbol-12,13-dibutyrate while still in the presence of that serotonin antagonist. In the second, parasites were contracted with 1 μM phorbol-12,13-dibutyrate for 15 min and then challenged with the serotonin antagonist while still in the presence of phorbol-12,13-dibutyrate.

Metergoline (30 μM) blocked spontaneous muscle activity and relaxed muscle tone by 3.9 ± 0.9 mg ($p \leq 0.01$) while ketanserin (100 μM) relaxed parasites by 1.8 ± 2.2 ($p \leq 0.05$) mg. Parasites that were pre-relaxed for 15 min by either 30 μM metergoline or 100 μM ketanserin were still responsive to 1 μM phorbol-12,13-dibutyrate. In the presence of metergoline, phorbol-12,13-dibutyrate produced a contraction of 4.4 ± 1.9 mg compared to control parasites in which phorbol-12,13-dibutyrate caused a contraction of 3.5 ± 1.3 mg. In ketanserin (100 μM), the response to phorbol-12,13-dibutyrate was 4.7 ± 1.9 mg compared to 4.5 ± 2.8 mg in control parasites. Likewise, there was no difference in the $t_{1/2}$ to maximal contraction between the antagonist and solvent treated parasites.

The serotonin antagonists were unable to relax parasites that had been pretreated with 1 μM phorbol-12,13-dibutyrate. When metergoline was added to parasites pretreated for 15 min with 1 μM phorbol-12,13-dibutyrate, there was a relaxation of only 0.7 ± 1.5 mg. Parasites pre-contracted with phorbol-12,13-dibutyrate and then treated with ketanserin actually contracted a further 2.9 ± 1.2 mg ($p \leq 0.01$) instead of relaxing.

Since the phorbol-12,13-dibutyrate-induced contraction was not reduced by doses of serotonin antagonists that completely blocked the effects of exogenous serotonin, it appears unlikely that the phorbol-12,13-dibutyrate-induced contraction is the result of increased serotonin release or sensitivity. The fact that ketanserin actually increased tone in animals already contracted by phorbol-12,13-dibutyrate is difficult to explain unless ketanserin has actions other than just serotonin antagonism in the schistosome.

b. Acetylcholine.

Cholinergic agonists cause relaxation and inhibition of schistosome muscle and it is generally assumed that there is an inhibitory cholinergic input to these muscles. One example is carbachol which produces a dose-dependent relaxation of the muscle with an EC_{50} of approximately 25 μM (Semeyn, 1987). Acetylcholinesterase inhibitors such as eserine also produce relaxation which one would expect if there were a tonic release of acetylcholine onto the muscle. If phorbol esters

acted to interfere with release of acetylcholine or the ability of acetylcholine to act on the muscle, then the end result of phorbol ester-stimulation might be the increase in muscle tone that is observed.

In parasites which were contracted with 100 nM phorbol-12-myristate-13-acetate, the cholinergic agonist carbachol, at 100 μM was still able to relax the muscle by 4.0 ± 0.6 mg ($p \leq 0.01$). This is to be compared to a relaxation of 3.9 ± 1.6 mg produced by this concentration of carbachol in the control animals. Parasites relaxed by 100 μM carbachol were still sensitive to phorbol esters since 1 μM phorbol-12,13-dibutyrate still contracted the muscle 6.6 ± 1.4 mg in the presence of carbachol compared to 5.8 ± 1.4 mg for control animals.

Eserine, an acetylcholine esterase inhibitor, caused a dose-dependent relaxation of the parasite. Pre-contracting the worms with 100 nM phorbol-12-myristate-13-acetate for 15 min did not reduce the worms' responses to eserine (Figure 9). If PMA reduced but did not block acetylcholine release, the time required to produce the same extent of relaxation would be increased since synaptic acetylcholine would build up at a slower rate. The $t_{1/2}$ for eserine-induced relaxation was not significantly different between the phorbol-12-myristate-13-acetate-treated and untreated groups at the various eserine concentrations (Figure 9b). From these results it seems unlikely that the muscle contraction produced by phorbol ester

Figure 9. Response of untreated and phorbol-12-myristate-13-acetate-contracted *Schistosoma mansoni* to eserine. Parasites were incubated in RPMI-1640 then treated with 100 nM phorbol-12-myristate-13-acetate (●) or dimethylsulfoxide (O). After 15 min, the parasites were challenged with various concentrations of eserine. Data are presented as the mean ± SEM (n=6). All statistical comparisons were made using a one-way analysis of variance and Dunnett's procedure.

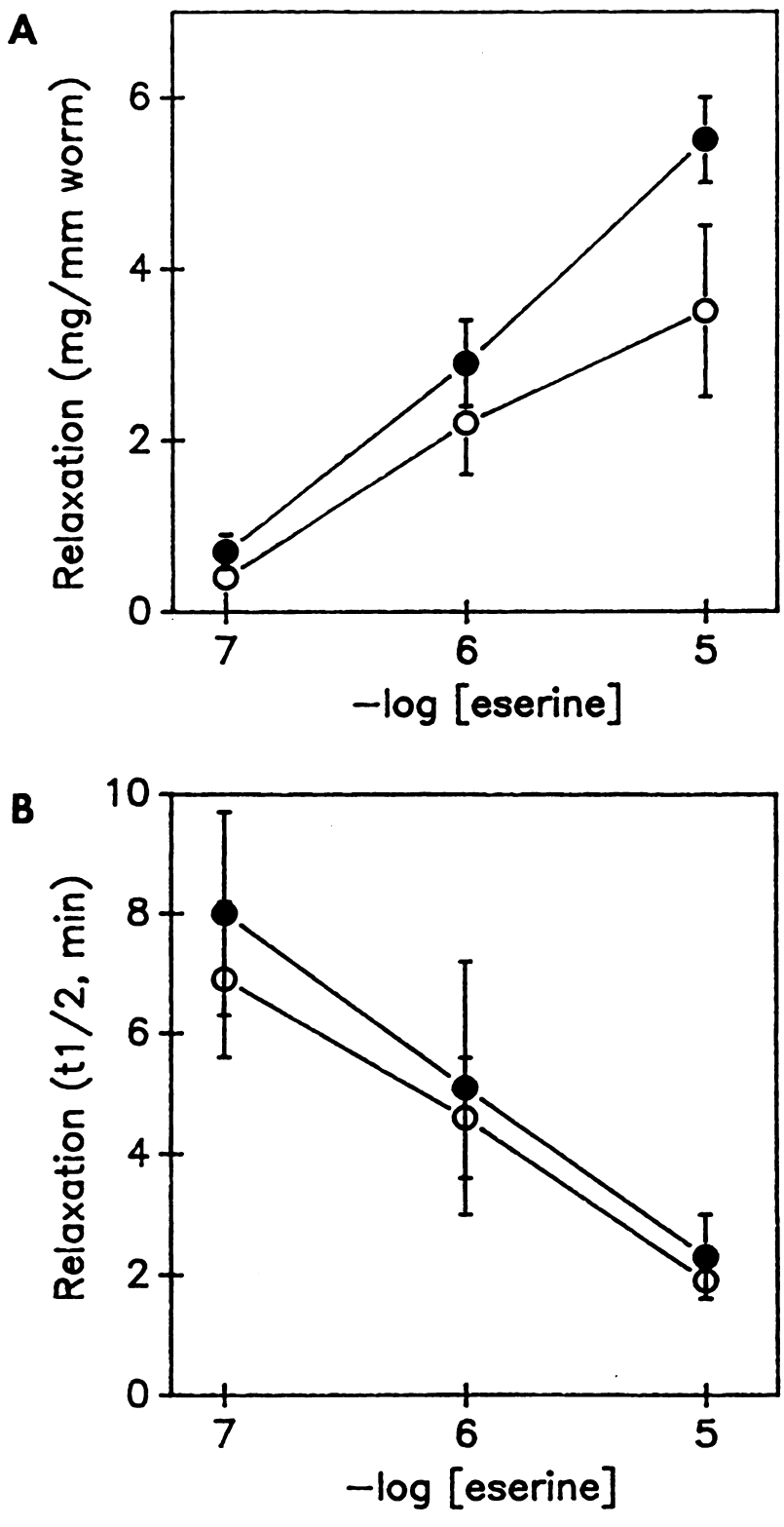
A
Relaxation (mg/mm worm)
0
2
4
6
7
6
5
−log [eserine]
B
Relaxation (t1/2, min)
0
2
4
6
8
10
7
6
5
−log [eserine]

Figure 9

application is the result of interfering with the action of cholinergic agents on the muscle.

c. Electrical activity.

An alternative approach in trying to determine how protein kinase-C activation is producing its effects on the muscle is to examine its effects on spontaneous electrical activity which can be recorded from these parasites. The procedure used (see Methods Section) allows for recording of electrical activity from the parasites for extended times.

The exact source of the electrical activity is unknown. The electrical and mechanical signals recorded from active parasites are asynchronous as described previously (Fetterer *et al.*, 1977; Semeyn *et al.*, 1982). This lack of association between the mechanical activity and electrical activity demonstrates that the electrical events are not artifacts of the pipet flexing or temporary loss of the seal between the parasite and the pipet.

It has been suggested that the recorded activity originates in the neuromusculature (Semyen *et al.*, 1982) but there is no direct proof of this. To eliminate the tegument as the sole source for the activity, electrical recordings were taken from parasites whose tegument had been removed. Initially such parasites exhibited very little mechanical activity or surface electrical activity (<20 μV). Stimulation with 100 nM serotonin and/or 100 μM 3,4-diaminopyridine, a putative K+ channel blocker (Rogawski, 1988), however, did

evoke electrical activity in these detegumented animals. These concentrations are near threshold for each of the drugs. From this it is clear that a significant portion of the electrical activity recorded originates in tissues below the tegument, probably nerve and/or muscle.

When whole parasites were exposed to 1 μM phorbol-12,13-dibutyrate, surface electrical activity decreased as muscle tone increased (Figure 10A). If the phorbol-12,13-dibutyrate-induced contractions were due to increased activity in serotonergic neurons or serotonin stimulation of the muscle, an increase in the surface electrical activity would have been expected (Semeyn *et al.*, 1982). There is a possibility that the electrical activity that remained was masking a phorbol ester-dependent component of electrical activity. Parasites were paralyzed with 100 μM carbachol to suppress all endogenous electrical activity before being treated with 1 μM phorbol-12,13-dibutyrate (Figure 10B). The parasite's muscle tone increased in response to the 1 μM phorbol-12,13-dibutyrate even in the presence of carbachol but no surface electrical activity was observed. On the other hand, exogenous serotonin (100 μM, Figure 10C) elicited electrical activity from 100 μM carbachol-paralyzed parasites ($p \leq 0.05$) without stimulating mechanical activity. This demonstrates that if activation of protein kinase-C leads to enhanced excitatory neurotransmitter release, it is via a system that does not stimulate electrical activity. These results also

Figure 10. Simultaneous muscle contractions and surface electrical activity of *Schistosoma mansoni*. For each pair, the upper trace is muscle activity and the lower trace is surface electrical activity. Chart recordings are representative of the responses elicited by: A) 1 μM Phorbol-12,13-dibutyrate was added at the arrows. B) Parasite was relaxed with 100 μM carbachol for 15 min then 1 μM phorbol-12,13-dibutyrate was added at the arrows. C) Parasite was relaxed with 100 μM carbachol for 15 min then 100 μM serotonin was added at the arrows. Calibrations: upper vertical, 2 mg; horizontal, 1 min; lower vertical, 200 μV.

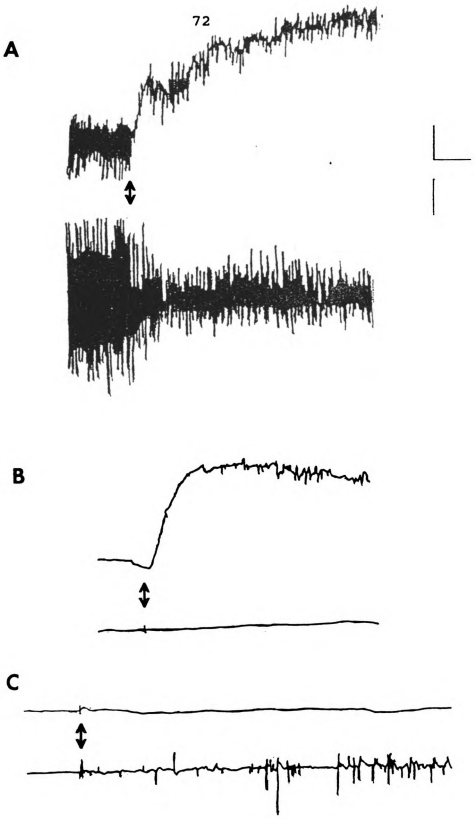

Figure 10

demonstrate that the propagation of electrical activity is not needed for the phorbol-12,13-dibutyrate-induced contraction and that no electrical activity is generated by this stimulus.

d. Involvement of neurotransmitters other than serotonin and acetylcholine.

From the results described thus far it appears unlikely that alteration of either serotonergic or cholinergic input to the muscle is the mechanism by which phorbol esters causes contraction of the schistosome muscles. However, since so little is known about neurotransmission in these animals, other unidentified transmitters might be involved. Possible excitatory amino acids and peptides were tested to search for unidentified neuromuscular transmitters that might be present in these animals. The amino acids glutamate, kainic acid, and N-methyl-d-aspartate were without effect on muscle tone when tested on intact and detegumented parasites in the concentration range of 10 μM to 1 mM. Since FMRF-amide and substance-P immunoreactivity are localized to the muscle (Gustaffson, 1988), these two peptides were also tested for their ability to stimulate contraction of intact or detegumented parasites. Neither peptide exhibited any effect on these parasites in a concentration range of 10 nM to 1 μM.

2. Direct actions on the muscle

Another mechanism by which phorbol esters might bring about contraction of the schistosome muscle could be via a direct action on the muscle itself. There are several

mechanisms by which these esters could be acting directly on the muscle to bring about contraction.

a. Membrane depolarization, alteration of sarcoplasmic Na^+ or pH.

One possibility is that activation of protein kinase-C causes contraction of the muscle by depolarizing its surface membrane, altering Na^+ activity or pH. To test this possibility, the membrane potential, sarcoplasmic Na^+ activity and pH were studied in parasites paralysed with 100 μM carbachol and treated with 1 μM phorbol-12,13-dibutyrate.

In these experiments the parasites were pre-treated with 100 μM carbachol for 15 min to reduce spontaneous movements to a level that allowed microelectrode penetration. In control animals the sarcolemmal potential was 25.9 ± 2.6 mV (n=12), sarcoplasmic pH was 7.01 ± 0.2, and sarcoplasmic Na^+ activity was 38.9 ± 11.7 mM. At 10 min after addition of 1 μM phorbol-12,13-dibutyrate, the sarcolemmal potential was 25.2 ± 5.6 mV, the sarcoplasmic pH was 7.01 ± 0.2, and Na^+ activity was 41.3 ± 12.9 mM. Measurements taken at earlier (when possible) and later time points were not different. This lack of an effect was not due to the presence of carbachol since, as described above, parasites still contracted in the presence of 100 μM carbachol. Also carbachol does not inhibit K^+ depolarization (Semeyn, 1987).

From these results it seems clear that the contractions induced by phorbol esters are not due to depolarization of the

parasite's muscles or to an alteration in the regulation of sarcoplasmic Na^+ or pH.

b. Altered Ca^{2+} permeability of the muscle membrane.

One possibility is that activation of protein kinase-C increases the Ca^{2+} sensitivity of the contractile system in schistosome muscle in a manner similar to phorbol ester-induced contractions in ferret aorta (Jiang and Morgan, 1987) and rat thoracic aorta (Itoh and Lederis, 1987). A second possibility is that there is an increase in the intramuscular free Ca^{2+}. Muscle contraction in schistosomes is normally dependent on the presence of Ca^{2+} in the incubation medium. If phorbol esters increase the Ca^{2+} sensitivity of the contractile system, then one would expect muscle contraction to be less sensitive to decreased levels of Ca^{2+} in the incubation medium in phorbol ester-treated worms compared to animals treated with other agents which act directly on the muscle but do not alter sensitivity of the contractile system to Ca^{2+}. One such agent is the Ca^{2+} ionophore A-23187.

Untreated parasites maintained muscle tone and spontaneous contractions in media with Ca^{2+} activities as low as 100 nM. Upon addition of A-23187 however, muscle tone was lost (-1.3 ± 0.5 mg) after a brief contraction and spontaneous activity was eliminated (Figure 11A, 12). When 1 μM phorbol-12,13-dibutyrate was tested in a similar experiment, similar results were observed (Figure 11B, 12). A phasic contraction

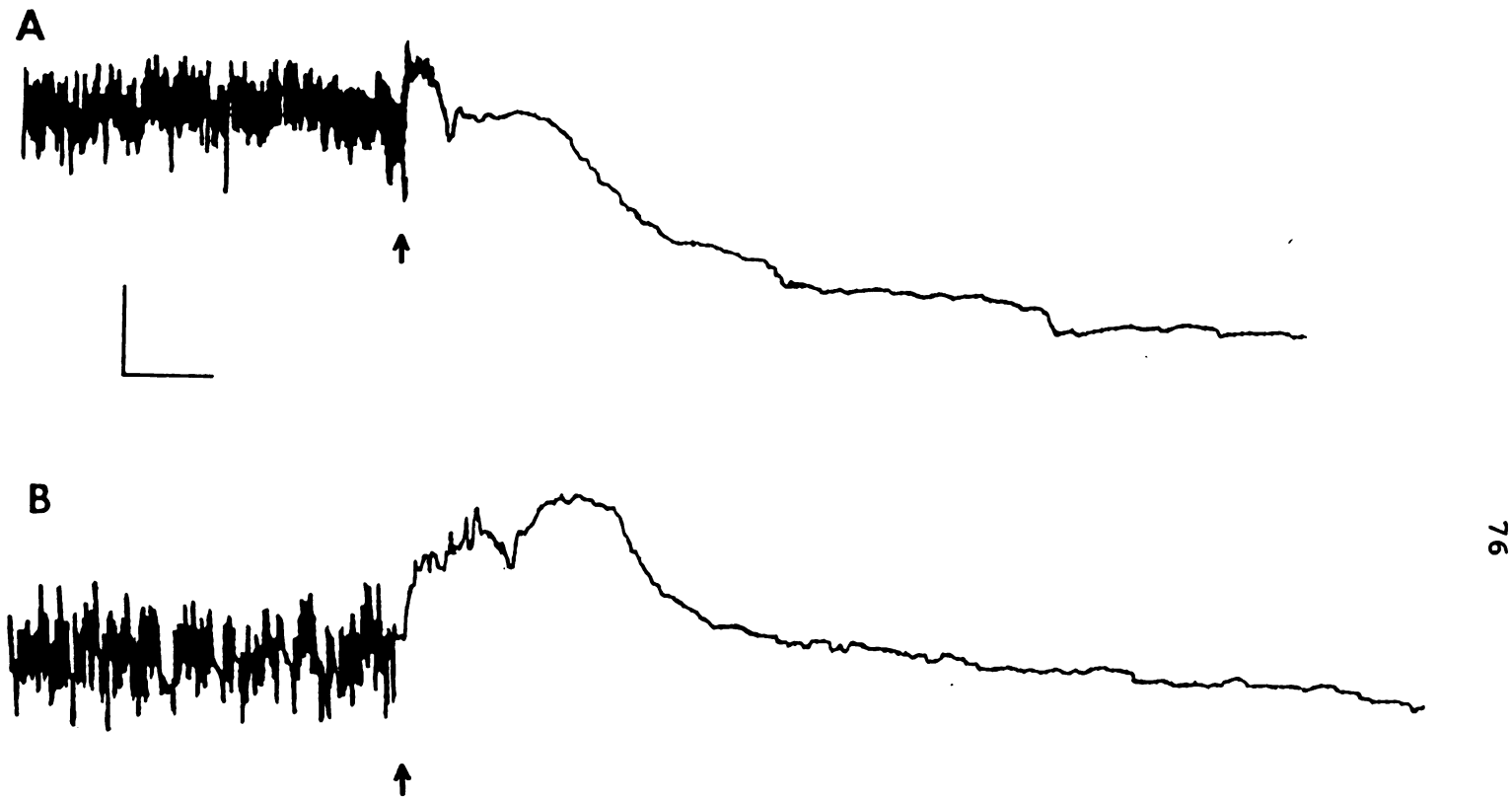

Figure 11. Response of *Schistosoma mansoni* in medium of 100 nM Ca^{2+} activity to 10 μM A-23187 or 1 μM phorbol-12,13-dibutyrate. Parasites were incubated in the 100 nM Ca^{2+} medium for 15 min and then the drug was added at the arrow. Calibrations: vertical, 2 mg; horizontal, 1 min.

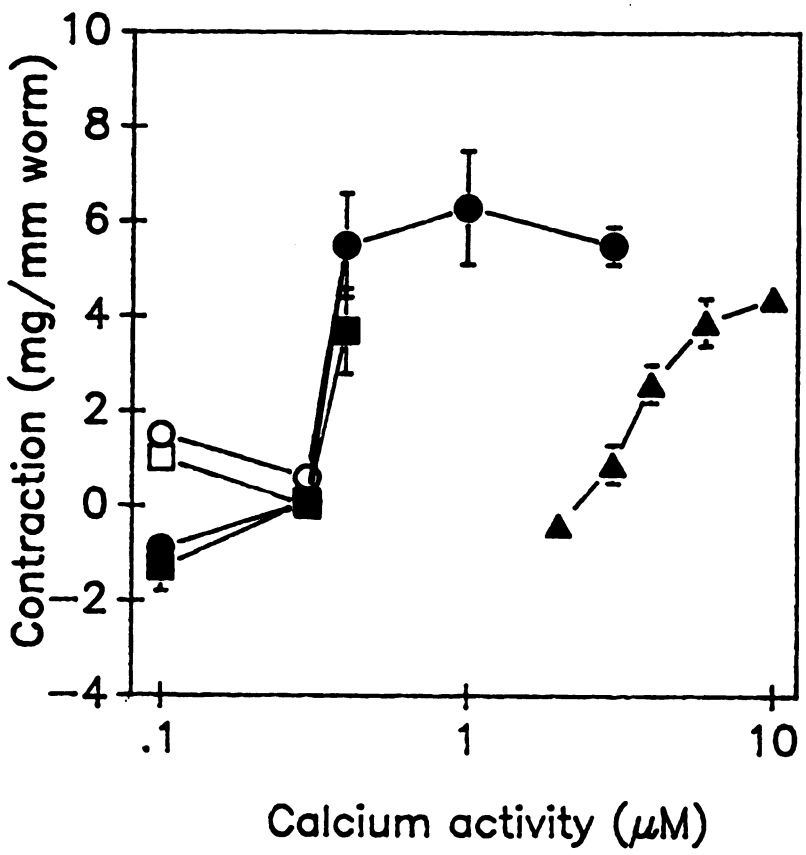

Figure 12. Ca^{2+} dependency of stimulated contractions. Parasites were incubated in the respective Ca^{2+}/10 mM EGTA-buffered I-RPMI for 15 min, then drug (1 μM phorbol-12,13-dibutyrate, circles and triangles or 10 μM A-23187, squares) was added to the bath. Open symbols represent the peak tone generated and closed symbols represent the tone at 15 min after the addition of the drug. Parasites were either intact (circles and squares) or detegumented (triangles). Data are presented as the mean ± SEM.

of 1.4 ± 1.0 mg was caused by 1 μM phorbol-12,13-dibutyrate but by 15 min muscle tone was 1.0 ± 0.3 mg below that when the phorbol-12,13-dibutyrate was added (Figure 11B, 12). Neither A-23187 nor phorbol-12,13-dibutyrate were able to produce tonic contractions until the Ca^{2+} activity was raised to 400 nM (Figure 12). These results do not support an increased sensitivity of the contractile system to Ca^{2+} but they are consistent with an increase in the membrane's permeability to Ca^{2+}.

One explanation for the responses described above might be that there is a Ca^{2+} pool within the parasite that can be maintained even though the external concentration of Ca^{2+} has been significantly reduced. As long as that pool is intact, the muscle is able to contract. The Ca^{2+} ionophore A-23187, when added to a medium with a Ca^{2+} activity below that of this pool, would be expected to allow Ca^{2+} to escape from this pool down its concentration gradient and as a result, there is a loss of muscle activity. The similar results that were observed with phorbol-12,13-dibutyrate may indicate it has a similar action, i.e. it results in loss of this internal pool. If protein kinase-C were acting to decrease Ca^{2+} efflux and/or to shift the Ca^{2+} sensitivity of the contractile system only, one would have expected to observe an increase in tone.

Assuming there is an "intraworm" pool of protected Ca^{2+}, one possible site of regulation of this pool could be the tegument. To determine whether in fact the tegument does

regulate this pool, the Ca^{2+} dependency of phorbol-12,13-dibutyrate-induced contractions on detegumented parasites was determined. Detegumented parasites were inactive and produced little if any tone in Ca^{2+} media below 1 μM. The Ca^{2+} dependency of the phorbol-12,13-dibutyrate-induced contraction in the detegumented parasites, compared to intact parasites, shifted to the right (Figure 12). From this it appears that the tegument may be important in maintaining an "intraworm" Ca^{2+} pool.

While these results indicate that Ca^{2+} permeability is altered by the activation of protein kinase-C, they do little to prove or disprove that its activation alters the Ca^{2+} sensitivity of the contractile system. This question could be addressed more appropriately with a skinned muscle preparation, in which the sarcolemma is removed, and one has sufficient control over intracellular Ca^{2+} activity to study directly the Ca^{2+} sensitivity of contraction.

c. Modulation of contraction by carbachol.

The previous studies indicate that activation of protein kinase-C might lead to increased Ca^{2+} permeability of the muscle. If this is the case, then phorbol-12,13-dibutyrate-induced contractions might behave in a manner similar to contractions elicited by other agents that are believed to enhance Ca^{2+} influx.

It has been proposed by Semeyn (1987) that cholinergic agonists cause muscle relaxation by stimulating Ca^{2+} efflux.

If this were the case then one would expect that when a cholinergic agent such as carbachol is present, a shift to the right in the dose-response curves of agents that increase Ca^{2+} influx would occur. To examine this relationship, dose-response curves for A-23187 were generated in the presence and absence of 100 μM carbachol. The carbachol was added 15 min prior to the A-23187 stimulus and remained throughout the test. Carbachol shifted the dose-response curve for A-23187 to the right in a parallel manner as expected for a competitive-like interaction (MacKay, 1981) (Figure 13A).

High K^+ depolarization would also be expected to increase Ca^{2+} influx into the muscle. When this treatment was given in the presence of carbachol, the dose-response curve did not shift in a simple competitive-like manner as expected (Figure 13B). The curve became more sigmoidal, lower concentrations of K^+ no longer induced as marked a contraction but maximal contractions were reached at the same concentrations as in non-carbachol treated parasites. Similar results were obtained with praziquantel (Figure 13C) and phorbol-12,13-dibutyrate (Figure 13D). Concentrations of phorbol-12,13-dibutyrate below 30 nM or of praziquantel below 420 nM were no longer able to stimulate contractions but maximal contractions were reached at the same concentrations as in non-relaxed parasites. From these data it appears that carbachol increases the minimally effective dose of these three stimuli without altering the maximally effective dose. This effect of

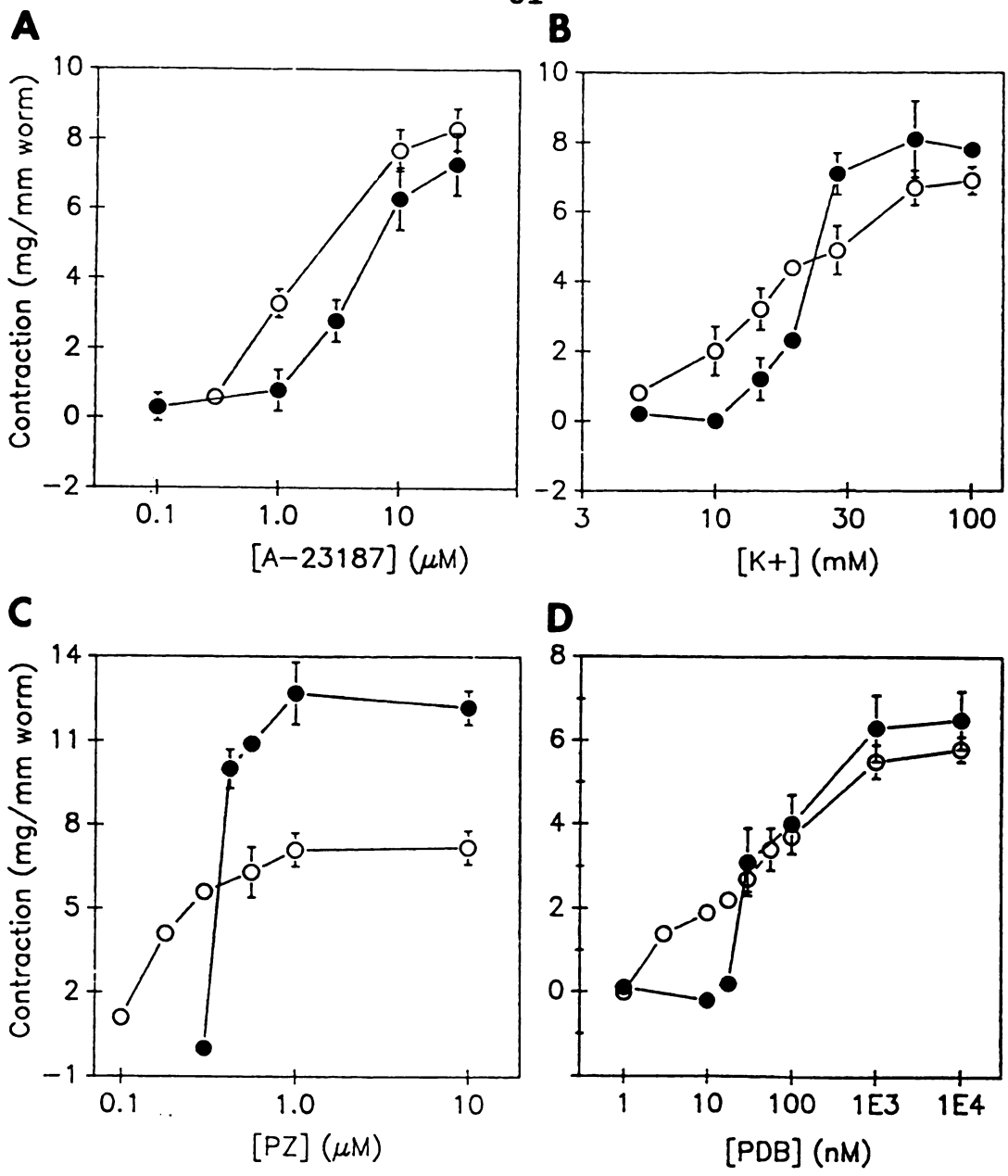

Figure 13. Effect of carbachol on stimulus dose-response curves. Parasites were treated with 25 μl water (open symbols) or 100 μM carbachol (closed symbols) for 15 min then treated with a test drug. The symbols represent muscle tone at 15 min after the test drug treatment. Data are presented as the mean ± SEM.

carbachol is not due simply to the relaxed state of the parasite since parasites relaxed 4.1 ± 2.1 mg with 100 μM dopamine contracted 2.8 ± 1.1 mg ($p \leq 0.05$) in response to 10 nM phorbol-12,13-dibutyrate and 9.1 ± 1.4 mg in response to 300 nM praziquantel.

While the magnitude of the maximal muscle contractions produced by A-23187, depolarization, praziquantel and phorbol-12,13-dibutyrate in the absence of carbachol are not significantly different from each other, contractions evoked in the presence of 100 μM carbachol are not all the same (Table 4). The maximal contractions produced by 1 μM phorbol-12,13-dibutyrate, 10 μM A-23187, and 60 mM K^+ were significantly less than that produced by 1 μM praziquantel. However if both 1μM phorbol-12,13-dibutyrate and 60 mM K^+ were administered to carbachol pre-relaxed worms, a complete contracture of the parasites was produced. Thus praziquantel is a full contraction agonist in the absence or presence of carbachol while the other agents are only partial contraction agonists in the presence of carbachol.

If A-23187, high K^+, praziquantel and phorbol-12,13-dibutyrate all increase internal Ca^{2+} they could be modulating a common or different sites. If they modulate a common site, then one might expect that at low doses their effects should be synergistic, that is the effects of the two combined should be greater that the sum of the two applied separately. If they elevate sarcoplasmic Ca^{2+} via independent mechanisms then

Table 4. Effect of Carbachol on stimulated contractions of *Schistosoma mansoni*.

Stimulus	Dose	Contraction (mg) Untreated	Carbachol
A-23187	(10 μM)	8.3 ± 1.5	7.3 ± 2.3
K^+	(60 mM)	6.9 ± 0.9	8.1 ± 2.6
Praziquantel	(1 μM)	7.1 ± 1.5	12.7 ± 2.1*
PDB	(1 μM)	5.8 ± 1.4	6.6 ± 1.4
PDB + K^+	(1 μM, 60 mM)	6.9 ± 2.1	12.2 ± 3.1*

Parasites were treated with either solvent (25 μl water) or 100 μM carbachol for 15 min then stimulated to contract. The single stimulatory drug data are from Figure 13. Numbers represent mean ± SD. All statistical comparisons are made using a one-way analysis of variance and the Student Neuman Keul procedure.

* $p \leq 0.01$

their combined responses should be only additive (MacKay, 1981; Segel, 1986).

To test for synergism, parasites were relaxed with 100 μM carbachol, and after 15 min, tested with various combinations of two of the test agents as well as serotonin. Synergistic interactions were observed with 10 nM phorbol-12,13-dibutyrate plus 15 mM K^+ (Figure 14) and 300 nM praziquantel plus 18 nM phorbol-12,13-dibutyrate, 15 mM K^+, or 100 μM serotonin (Figure 15) but not with 1 μM A-23187 plus 10 nM phorbol-12,13-dibutyrate (Figure 14) or 300 nM praziquantel (Figure 15). If the initial assumption about how these agents cause contraction is correct then one would conclude that phorbol-12,13-dibutyrate, praziquantel, high K^+ and serotonin are modulating a common mechanistic pathway or site to elevate Ca^{2+} while A-23187 is elevating intracellular Ca^{2+} independently. One site that might be common to these agents are Ca^{2+} channels in the muscle membranes. This could explain the lack of synergism with A-23187 since this agent is a Ca^{2+} ionophore and directly translocates Ca^{2+} across the membrane, bypassing the Ca^{2+} channel.

There are two common ways in which Ca^{2+} channel activity could be modulated to increase contraction: 1)a shift in the voltage sensitivity of channel opening to less depolarized potentials to increase channel probability of opening or 2)to simply increase the number of channels available for opening. A less common way is that one or more of these treatments

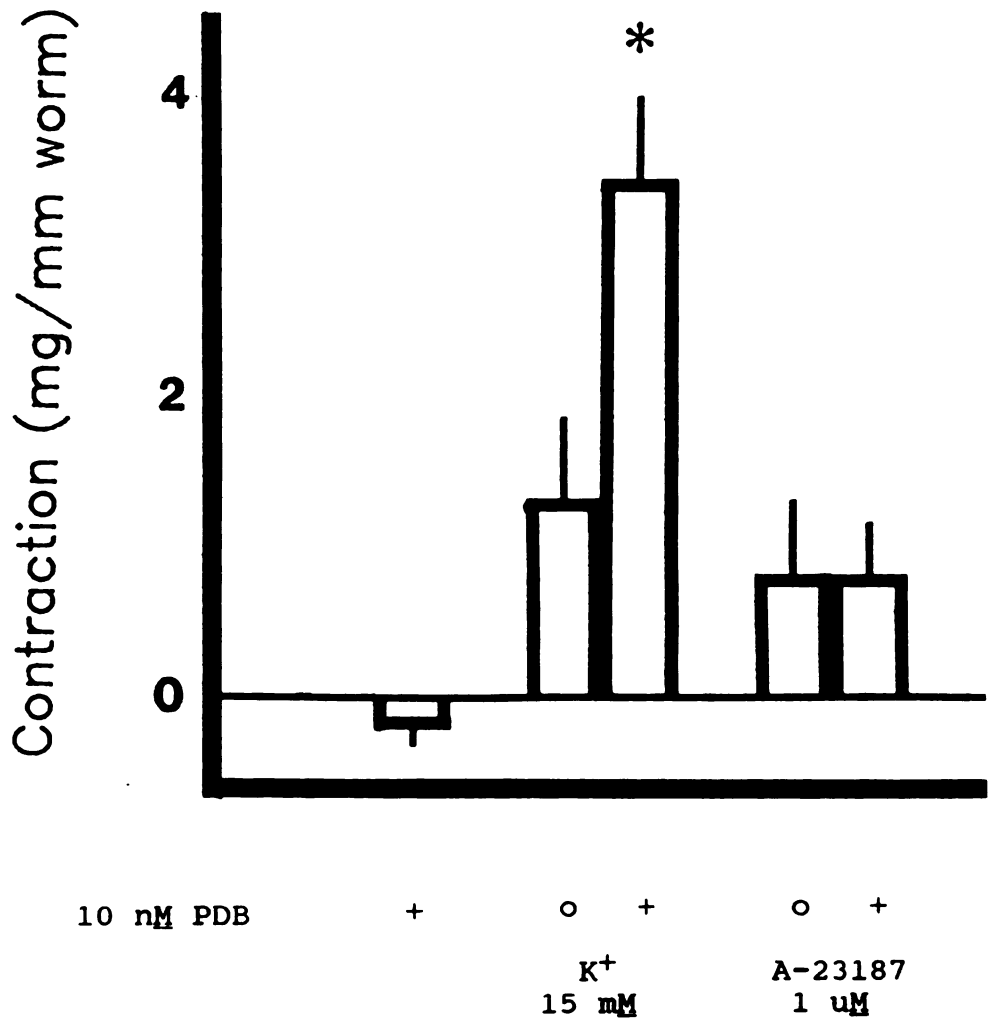

Figure 14. Synergism of phorbol-12,13-dibutyrate-induced contractions in *Schistosoma mansoni*. Parasites were incubated in the presence of 100 μM carbachol for 15 min then stimulated to contract in the presence (+) or absence (O) of 10 nM phorbol-12,13-dibutyrate. The concentration of carbachol remained constant. The change in muscle tone was determined 15 min after the drug treatment. Data are presented as the mean ± SEM.

* $p \leq 0.05$, analysis of variance and Scheffe's procedure

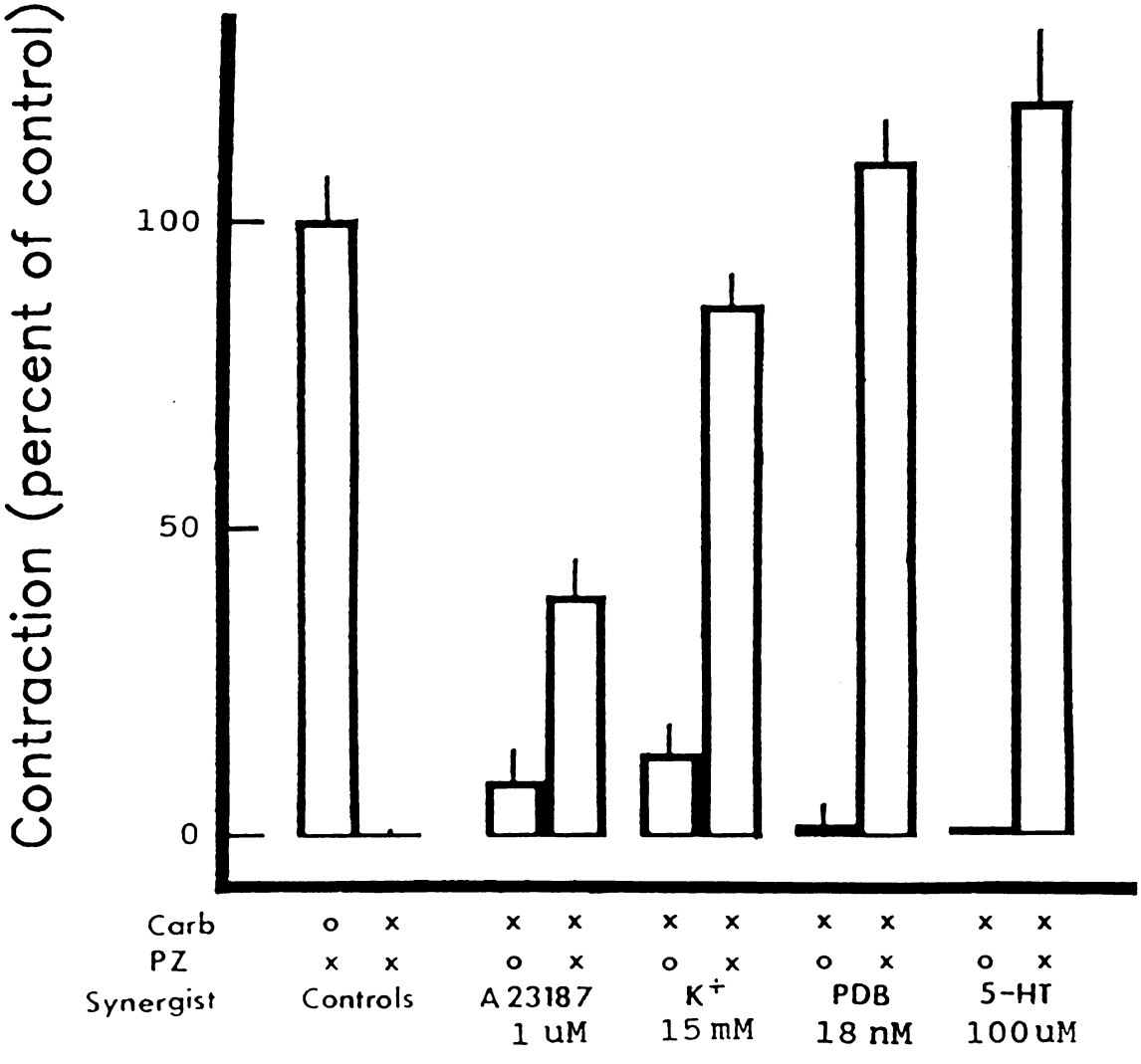

Figure 15. Synergism of praziquantel-induced contractions in *Schistosoma mansoni*. Parasites were incubated in the presence (x) or absence (O) of 100 μM carbachol for 15 min and then stimulated to contract in the presence (x) or absence (O) of 300 nM praziquantel. The concentration of carbachol remained constant. The change in muscle tone was determined 15 min after the test drug treatment. Data are presented as mean ± SEM

[a] Significant synergism, $p \leq 0.01$, analysis of variance and Scheffe's procedure.
[b] No significant difference from control praziquantel response, analysis of variance and Dunnett's procedure.

could be altering the basic biophysical properties of the channel itself. This has been reported for dihydropyridine modified Ca^{2+} channels in vascular smooth muscle (Lacerda and Brown, 1989) and ryanodine modified Ca^{2+} channels from skeletal muscle sarcoplasmic reticulum (Smith *et al.*, 1988). If protein kinase-C activity increased the number or sensitivity of Ca^{2+} channels in the muscle, the phorbol-12,13-dibutyrate-induced contraction might be inhibited by agents that block Ca^{2+} channel activity.

d. Sensitivity of the contractions to Ca^{2+} channel blockers.

The ability of maximally effective doses of phorbol-12,13-dibutyrate (1μM), K^+ (60 mM), or praziquantel (10 μM) to stimulate contractions was tested in parasites treated with either nicardipine, a member of the dihydropyridine family of Ca^{2+} channel blockers or Cd^{2+}, a Ca^{2+} antagonist. Nicardipine was selected because it was the most potent and effective of seven organic Ca^{2+} channel blockers tested by Semeyn (1987). Likewise, Cd^{2+} is reported to be among the most potent inorganic Ca^{2+} channel blockers (Nachschen, 1984; Narahashi *et al.*, 1987) and inhibits Ca^{2+} currents in muscles of other primitive animals such as the ctenophore, *Mnemiopsis* (Dubas *et al.*, 1987).

The blockers caused relaxation of the parasites (Figure 16). In the experiments described below the parasites were simultaneously relaxed with 100 μM carbachol and the desired

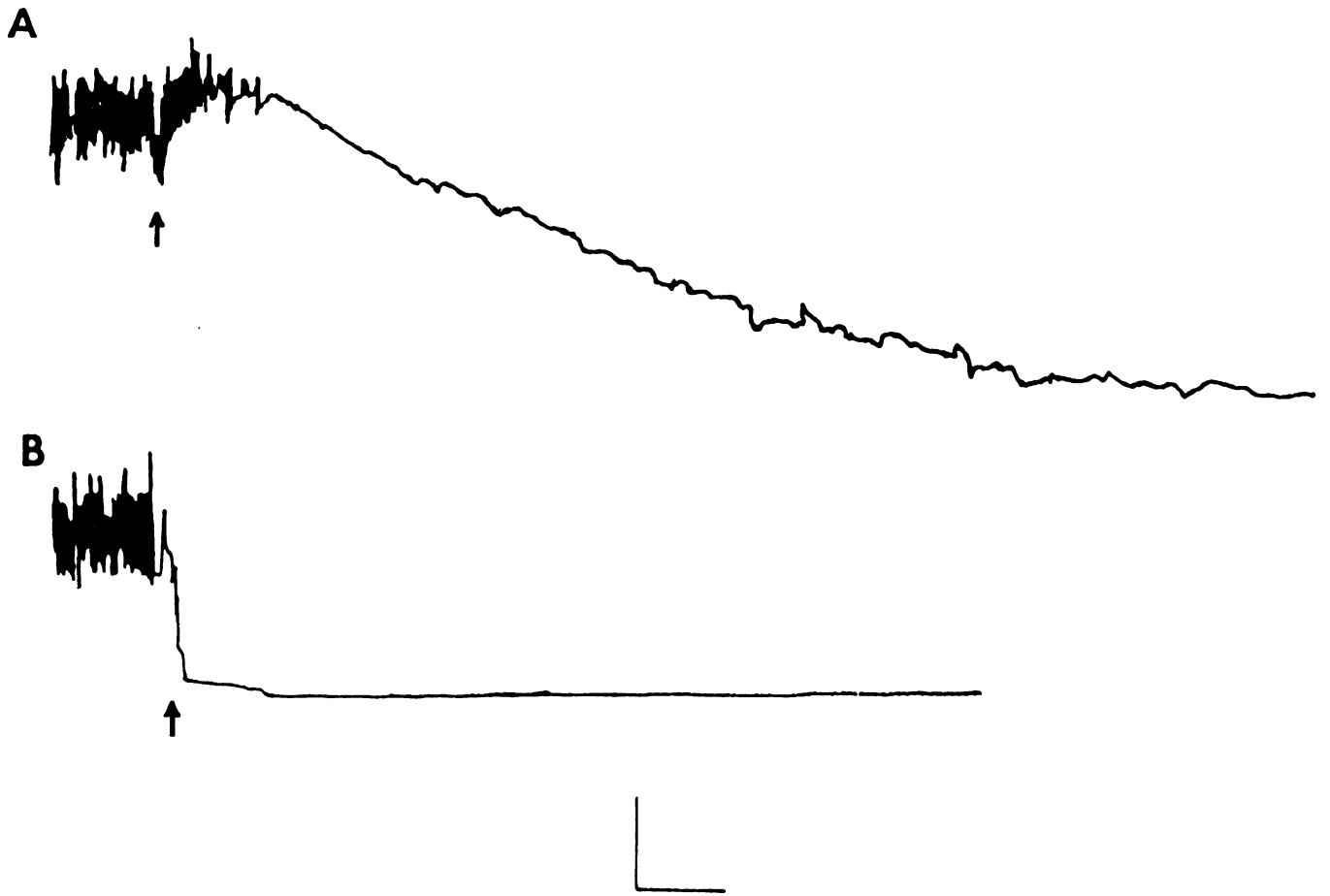

Figure 16. Representative responses of *Schistosoma mansoni* muscle to Ca^{2+} channel blockers. Parasites were incubated in I-RPMI for 15 min then treated with 10 μM nicardipine (A) or 1 mM Cd^{2+} (B). Calibrations: vertical, 2 mg; horizontal, 1 min.

Ca^{2+} channel blocker under study for 15 min. This was done to assure that all of the parasites were starting at the same level of tone and to simplify analysis of the data. The parasites were then challenged with 1 μM phorbol-12,13-dibutyrate, 60 mM K^+, both phorbol-12,13-dibutyrate and K^+, or 10 μM praziquantel.

Cd^{2+} blocked the tonic phorbol-12,13-dibutyrate and 60 mM K^+-stimulated contractions with IC_{50}'s of 32 μM and 20 μM respectively (Figure 17). This block was not due to parasite death as 10 μM praziquantel still stimulated contractions in 1 mM Cd^{2+} (Figure 17A).

Nicardipine blocked the contractions induced by 60 mM K^+ with an IC_{50} of 1.4 μM (Figure 18A) but the phorbol-12,13-dibutyrate-stimulated contracture was not completely blocked (Figure 18B). Nicardipine is believed to block in a voltage dependent manner, that is the degree of block increases as the cell membrane is depolarized further or longer (Nelson and Worley, 1989), and since the parasite is not depolarized by phorbol-12,13-dibutyrate, nicardipine may not be fully active. To test this, parasites were simultaneously treated with 60 mM K^+ to depolarize the muscle and 1 μM phorbol-12,13-dibutyrate but nicardipine was no more effective at blocking these contractions than it was at blocking the phorbol-12,13-dibutyrate-induced contraction (Figure 18B). Likewise, nicardipine did not block 10 μM praziquantel-stimulated contractions (Figure 18A). Similar results were obtained with

Figure 17. Dose-dependency of cadmium-block of stimulated contraction. Parasites were relaxed with 100 μM carbachol plus Cd^{2+} for 15 min then exposed to their respective stimulus: A) 60 mM K^{+}, (O, ●) or 10 μM praziquantel, (▲); B) 1 μM PDB. Open symbols represent the peak contraction and closed symbols represent the level of tone after 15 min. Data are presented as mean ± SEM.

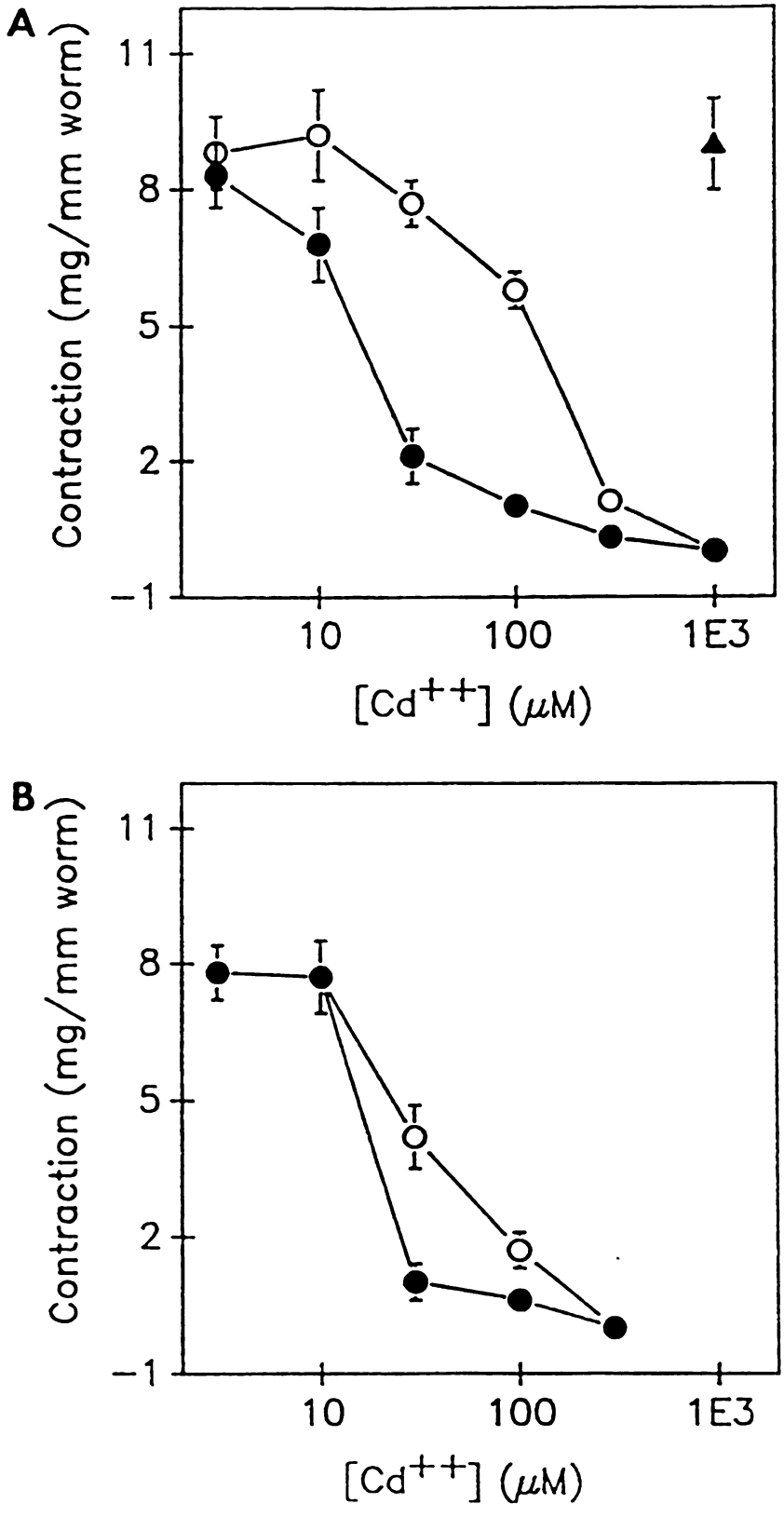
A
Contraction (mg/mm worm)
11
8
5
2
−1
10
100
1E3
[Cd++] (μM)
B
Contraction (mg/mm worm)
11
8
5
2
−1
10
100
1E3
[Cd++] (μM)

Figure 17

Figure 18. Dose-dependency of nicardipine-block of stimulated contraction. Parasites were relaxed with 100 μM carbachol plus nicardipine for 15 min then exposed to their respective stimulus: A) 60 mM K^+ (O, ●) or 10 μM praziquantel (▲); B) 1 μM PDB (●) or combined 1 μM PDB and 60 mM K^+ (■). Open symbols represent the peak contraction and closed symbols represent tone remaining at 15 min after the stimulus. Data are presented as mean ± SEM.

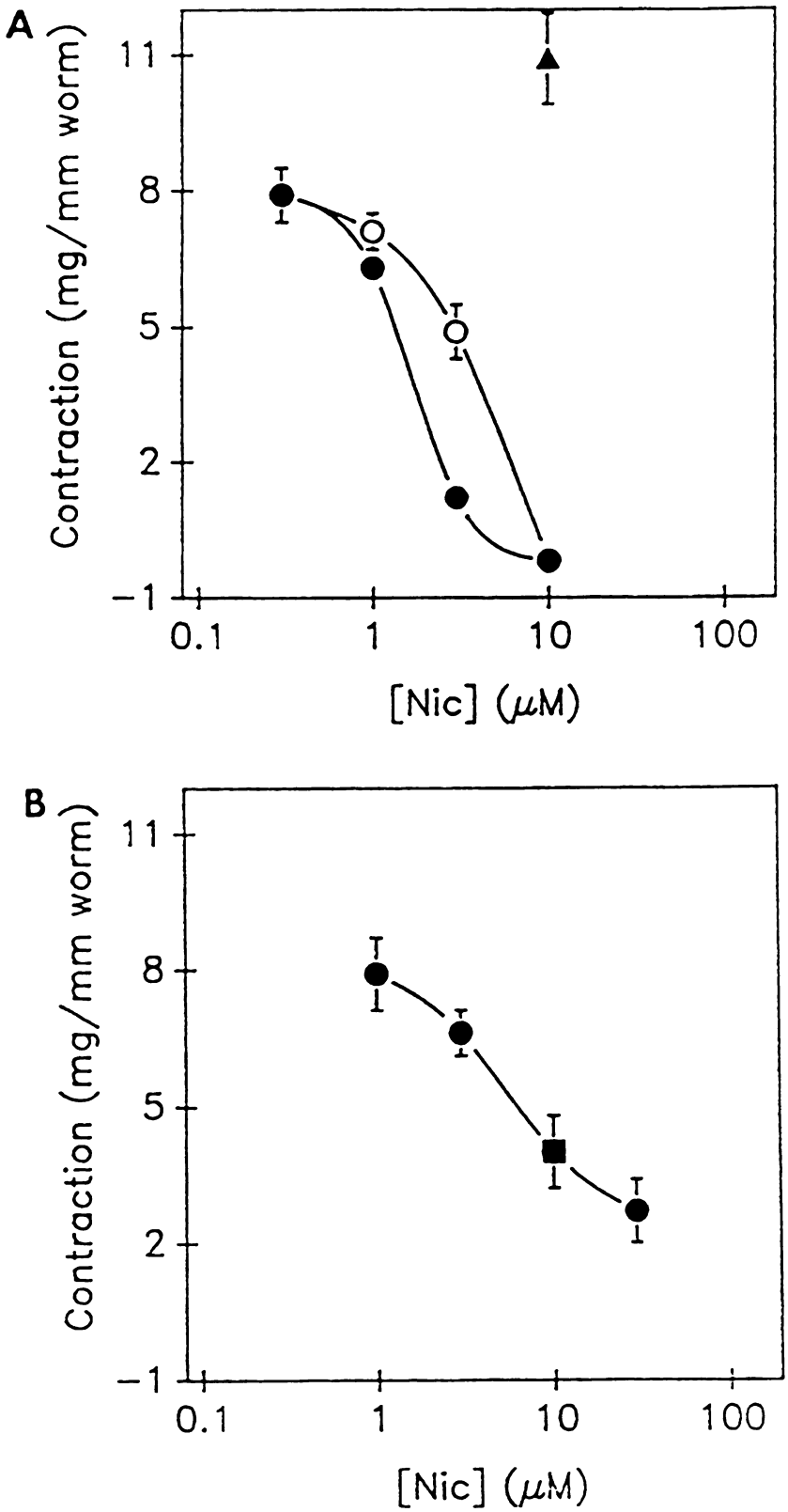
A
Contraction (mg/mm worm)
11
8
5
2
−1
0.1
1
10
100
[Nic] (μM)
B
Contraction (mg/mm worm)
11
8
5
2
−1
0.1
1
10
100
[Nic] (μM)

Figure 18

100 μM D-600, another organic Ca^{2+} channel blocker. These concentrations of blockers are high compared to doses needed to block vertebrate Ca^{2+} channels (Fox *et al.*, 1987) but 100 μM D-600 is required to block 90% of the Ca^{2+} current in ctenophore muscle (Dubas *et al.*,1987).

Assuming that these blockers are specific for Ca^{2+} channels in this primitive animal, the ability of Cd^{2+} and of nicardipine to block both high K^{+} and part of the phorbol-12,13-dibutyrate-induced contractions suggests that one end point of protein kinase-C activation is at the Ca^{2+} channels in the muscle membrane. The remaining phorbol-12,13-dibutyrate-induced tone in the presence of 10-30 μM nicardipine may represent a component of this induced tone that is independent of channel activity, i.e., altered Ca^{2+} flux by another mechanism or altered sensitivity of the contractile system. Alternatively, activation of protein kinase-C may lead to an alteration in the channel that makes it less sensitive to organic Ca^{2+} channel blockers. Why these channel blockers do not block praziquantel-induced contractions is not obvious. One possibility is that at 10 μM, praziquantel is not as specific for Ca^{2+} channels as it is at 300 nM, at which concentration synergism was observed. Another possibility is that at this higher dose the Ca^{2+} channel is modified in such a manner that these blockers become less effective.

In summary, these results demonstrate that activation of protein kinase-C with phorbol esters leads to a contraction that is dependent upon extracellular Ca^{2+} but does not depolarize the muscle as does high K^{+}. This contraction is sensitive to agents known to be Ca^{2+} channel blockers in smooth muscle from other organisms and is probably due to increased sarcoplasmic Ca^{2+}. No evidence was observed that would indicate that this contraction was due to an alteration of neuromuscular transmission, decreased Ca^{2+} efflux, or a shift in the sensitivity of the contractile system to Ca^{2+}. These results do not rule out possible other, more subtle effects of phorbol esters on the parasite that were not detectable by the methods employed in these studies.

DISCUSSION

A. Evidence that protein kinase-C is present in the schistosome and its activation causes muscle contraction.

For several years, protein kinase-C has been biochemically defined as a protein kinase that depends for maximal catalytic activity upon three cooperative activators: acidic phospholipid, Ca^{2+}, and a neutral lipid such as diacylglycerol (Takai *et al.*, 1979) or phorbol esters (Castagna *et al.*, 1982). This definition was relaxed when Ohne *et al.* (1988) demonstrated the presence of a Ca^{2+}-independent protein kinase-C isozyme. There are now at least three Ca^{2+}-independent protein kinase-C isozymes (Nishizuka, 1988).

The most common method for demonstrating the presence of protein kinase-C and/or the physiological effects of its activation has been to demonstrate a response to phorbol esters. Phorbol esters are preferred to diacylglycerol because they have greater membrane permeability and are not metabolized as readily as diacylglycerol (Ashendel, 1985). The presence of protein kinase-C is supported further if the response to phorbol esters is stereo-specific and shows a typical and consistent structure-activity relationship with respect to the sites of phorbol esterification. Phorbol

esters do exhibit non-specific effects at high (micromolar) concentrations (Hockberger *et al.*, 1989) but these effects are not stereo-specific. Indeed, when present in nanomolar concentrations, phorbol esters have no other demonstrated specific effects apart from binding to and activating protein kinase-C.

1. Biochemistry.

In the biochemical studies presented here it is shown that in the schistosome a phorbol ester receptor and a phorbol ester-stimulated protein kinase activity are present. The EC_{50}'s of phorbol-12-myristate-13-acetate (3 nM) and phorbol-12,13-dibutyrate (20 nM) for binding to the schistosome phorbol ester receptor are similar to values reported for mammalian preparations (Ashendel, 1985; Jeffrey and Liskamp, 1986) and binding to the schistosome receptor is stereo-specific.

Schistosome protein kinase-C activity like mammalian protein kinase-C, is dependent upon phospholipid and is activated by 1,2-diacylglycerol, phorbol esters or the non-phorbol, mezerin. Also, phorbol esters with substituted -20 hydroxyls as well as the α-phorbol ester are unable to stimulate the schistosome protein kinase-C. This structure-activity relationship is similar to that for phorbol ester activation of mammalian protein kinase-C (Ashendel, 1985; Jeffrey and Liskamp, 1986).

2. Physiology.

In the schistosome, one presumed physiological effect of protein kinase-C activation is muscle contraction. This physiological response has a phorbol ester structure-activity relationship similar to that of mammalian protein kinase-C (Ashendel, 1985; Jeffrey and Liskamp, 1986). In the schistosome phorbol-12-myristate-13-acetate and phorbol-12,13-dibutyrate are both active with EC_{50}'s in the nanomolar range while the acetate di-ester is less potent, requiring micromolar concentrations for activity. The non-phorbol, mezerin, also stimulated contraction but phorbol esters with substituted -20 hydroxyls as well as the α-phorbol esters are unable to stimulate contraction.

3. Assays compared.

The ranked potency of the phorbol esters in the schistosome assays with respect to binding, kinase activation and muscle contraction are similar. For the binding assay and muscle contraction the ranked potency of the phorbol esters is the same: phorbol-12-myristate-13-acetate > phorbol-12,13-dibutyrate > phorbol-12,13-didecanoate. Phorbol-12,13-diacetate is consistently the least potent of the phorbol esters tested. However, phorbol-12,13-dibutyrate is the most effective and potent of the phorbol esters with respect to protein kinase activation. Phorbol esters that induce contraction exhibit a similar potency for stimulating protein kinase activity. The α-phorbol esters as well as ß-phorbol

esters with substituted -20 hydroxyls are inactive in all three of the assays. Mezerin also stimulates muscle contraction but the 1,2-diacylglycerides are not effective. The inactivity of the 1,2-diacylglycerides may be due to an inability of these compounds to penetrate the parasite's tissues and reach the muscle. Alternatively rapid metabolism (Severson and Hee-Cheong, 1989) may inactivate them before they can become effective.

In these schistosome assays, the dose-dependency of the phorbol-12,13-dibutyrate responses are very similar indicating that they are interrelated or coupled e.g., phorbol ester binding initiates phorbol ester-stimulated protein kinase activity, which leads to the phorbol ester-induced contraction.

In one respect schistosome protein kinase-C appears to be different from the enzyme discussed in a majority of the studies on other tissues. The schistosome protein kinase-C as measured in the biochemical assay appears to be Ca^{2+}-independent in the presence of phorbol esters. Also, in the muscle contraction assay, there was no facilitation of the phorbol ester-induced contraction by threshold doses of the Ca^{2+} ionophore A-23187, which one would expect if schistosome protein kinase-C were Ca^{2+}-dependent. The binding of [^{3}H]phorbol-12,13-dibutyrate is increased by Ca^{2+}, but this enhancement is less than that produced by acidic phospholipid in the absence of Ca^{2+}. These data do not reveal whether the

kinase activity becomes sensitive to Ca^{2+} at low levels of phorbol esters or in their absence. Since the Ca^{2+} sensitivity of mammalian protein kinase-C is increased by phorbol esters (Castagna *et al.*, 1982), the schistosome enzyme may be sensitive to Ca^{2+} but is fully active in the presence of low concentrations of phorbol esters and EGTA, essentially making it Ca^{2+}-independent. Ca^{2+}-independent protein kinase-C has also been demonstrated in the triclad flatworm, *Dugesia gonocephala* (Martelly *et al.*, 1987: Moraczewski *et al.*, 1987). Perhaps the Ca^{2+}-independent isozymes of protein kinase-C are ancestral to the Ca^{2+}-dependent isozymes present in mammalian systems.

B. How activation of protein kinase-C causes muscle contraction in *Schistosoma mansoni*.

Stimulation of muscle contraction is one effect of phorbol ester commonly observed in a variety of smooth muscle systems including: vascular tissues of pig (Chatterjee and Tejada, 1986; Miller *et al.*, 1986), dog (Chiu *et al.*, 1988), ferret (Jiang and Morgan, 1987), rat (Cheung, 1988; Litten *et al.*, 1987; Wakabayashi *et al.*, 1988a,b) and rabbit (Gleason and Flaim, 1986; Laher and Bevan, 1987; Rasmussen *et al.*, 1984); rat myocyte (Dosemeci *et al.*, 1988), guinea pig ileum (Holzer and Lippe, 1989; Sasaguri and Watson, 1989), and bovine trachea (Park and Rasmussen, 1985). The contractions produced by these tissues vary with respect to their

sensitivity to phorbol esters and their dependence on extracellular Ca^{2+}, indicating that there may be some inter-species variability in the role or efficacy of protein kinase-C in stimulating contraction. In the schistosome, the phorbol ester-induced contraction is proportionally among the greatest and most rapid reported. Comparison with these vertebrate model systems may give some insight as to the manner in which schistosomes are stimulated to contract by the activation of protein kinase-C.

A physiological role for protein kinase-C in vertebrate smooth muscle contraction is well supported. Several transmitter and hormone substances that stimulate contraction in model muscle systems are associated with increased protein kinase-C activity including: acetylcholine (Vivaudou *et al.*, 1988), angiotensin II (Dosemeci *et al.*, 1988; Griendling *et al.*, 1986), substance P (Holzer and Lippe, 1989) and endothelin (Lee *et al.*, 1989; Sugiura *et al.*, 1989). One mechanism by which activation of protein kinase-C causes contraction is that it increases sarcoplasmic Ca^{2+} channel activity. This occurs in several model smooth muscle systems including: toad stomach (Clapp *et al.*, 1989; Vivaudou *et al.*, 1988), cerebral artery (Laher *et al.*, 1989), rat heart myocyte (Dosemeci *et al.*, 1988) and porcine coronary artery (Goto *et al.*, 1989; Silberberg *et al.*, 1989) and is the basis for the contraction's dependence on extracellular Ca^{2+}. A second mechanism by which protein kinase-C causes contraction could

be that it shifts the Ca^{2+} sensitivity of the contractile system (Jiang and Morgan, 1987; Laher *et al.*, 1989; Ruzycky and Morgan, 1989). As a result, the contraction becomes less dependent on extracellular Ca^{2+}. This shift may be one component in the control of tonic contraction (latch state) (Murphy, 1989).

For the schistosome, the best supported mechanism by which activation of protein kinase-C leads to muscle contraction appears to be one in which activation of protein kinase-C leads to elevated sarcoplasmic Ca^{2+}. This could be brought about by either increasing Ca^{2+} permeabilty of the muscle or by decreasing Ca^{2+} efflux.

Schistosome muscle is dependent upon extracellular Ca^{2+} to support contraction (Wolde Mussie *et al.*, 1982) indicating a role of Ca^{2+} influx sites. A role for voltage-gated Ca^{2+} channels in schistosome stimulus-contraction coupling is supported by several observations. First, acutely isolated schistosome muscle fibers contract immediately upon depolarization with KCl (25-100 mM, presure ejection). Second, this contraction is associated with an elevation of intracellular Ca^{2+} as determined by the change in fluoresence of a Ca^{2+} sensitive dye. Third, this contraction is prevented by the inclusion of 5 mM Co^{2+} or the exclusion of Ca^{2+} from the ejected saline (Lewis, personal communication). Praziquantel exhibits similar effects on isolated schistosome muscle (Lewis, personal communication) and it prolongs the Ca^{2+}-

dependent plateau phase of the cardiac action potential (Chubb *et al.*, 1978).

The phorbol ester-induced muscle tone in schistosomes also is dependent on extracellular Ca^{2+}. Muscle tone of parasites incubated in a 100 nM Ca^{2+} medium decreased when treated with phorbol ester, indicating a reduced ability of the muscle to retain Ca^{2+}, while parasites incubated in medium containing 400 nM Ca^{2+} or greater contracted. Since sarcoplasmic pH and Na^{+}_{i} are not alterd by treatment with phorbol esters, this increased permeability appears to be specific to Ca^{2+}.

Phorbol esters synergize with KCl-stimulated contraction of rat aorta (Wakabayashi *et al.*, 1988a,b), rat saphenous vein (Cheung, 1988), pig artery (Miller *et al.*, 1986), and rabbit facial vein (Laher and Bevan, 1987) and they faciltate contractions induced by the Ca^{2+} channel agonist, BAY k 8644 (Litten *et al.*, 1987). In the schistosome, phorbol-12,13-dibutyrate synergizes with depolarization and praziquantel-induced contractions. This synergism might be expected if activation of protein kinase-C enhanced Ca^{2+} channel activity. It is doubtful that activation of protein kinase-C or the drug, praziquantel leads to decreased Ca^{2+} efflux or an increased sensitivity of the contractile system to Ca^{2+}, since A-23187, an ionophore that should transport Ca^{2+} into cells independently of Ca^{2+} channel activity, did not synergize with contractions induced by threshold concentrations of either of

these drugs. A role for enhanced Ca^{2+} influx therefore is supported.

The phorbol ester-induced contractions of schistosomes are blocked completely by pre-incubation with Cd^{2+} and partially by members of the dihydropyridine and phenylalkylamine classes of organic Ca^{2+} channel blockers, compounds that block Ca^{2+} channels in more complex (Fox *et al.*, 1987; Tytgat *et al.*, 1988) and simpler (Dubas *et al.*, 1987) organsisms. These results are similar to the effects of Ca^{2+} channel blockers on protein kinase-C dependent muscle tone in vertebrates. Organic Ca^{2+} channel blockers do not completely block protein kinase-C dependent muscle tone in vertebrate vascular tissues. The dihydropyridine, nitrendipine (1 μM) partially inhibits (50%) the phorbol ester-induced contraction of rat aorta (Litten *et al.*, 1987). Contractions of human bladder by agents believed to lead to the activation of protein kinase-C (neurokinin A, carbachol, and endothelin) were partially inhibited (roughly 50%) by 1 μM nifedipine, a dihydropyridine, but were reduced by 80-90% by 1 mM La^{3+} (Maggi *et al.*, 1989). Contraction of human bladder, induced by depolarization with KCl, is reduced by roughly 90% by either 1 μM nifedipine or 1 mM La^{3+}. The contractions evoked by all four of these stimuli are reduced by roughly 90% in Ca^{2+} free medium demonstrating a strong dependence on extracellular Ca^{2+} for each stimulus. That activation of protein kinase-C synergizes voltage-gated Ca^{2+} channel-

dependent muscle contraction and that this contraction is partially insensitive to organic Ca^{2+} channel blockers indicates that either the activation of protein kinase-C leads to an alteration of the channels such that they become less sensitive to the blockers or that a second class of Ca^{2+} channels is activated. Activation of protein kinase-C in *Aplysia* bag cell neurons does "uncover" a voltage-gated Ca^{2+} channel that was not detectable prior to enzyme activation (Strong *et al.*, 1987; Conn *et al.*, 1989).

Whether a shift in the Ca^{2+} sensitivity of the contractile system occurs as in some latch systems (Jiang and Morgan, 1987; Laher *et al.*, 1989; Ruzycky and Morgan, 1989) could not be determined from these results. A skinned muscle preparation would be essential for these studies to be conducted correctly.

A variety of possible alternate mechanisms by which protein kinase-C activation might bring about the schistosome muscle contraction do not appear to be supported by the data presented here. If the phorbol ester-induced contraction were due to decreased Ca^{2+} efflux, one would expect to have observed synergism between the simultaneous addition of threshold concentrations of phorbol ester and A-23187, but none was observed. Likewise, if Ca^{2+} efflux was reduced, the parasites in 100 nM Ca^{2+} activity medium should have maintained their muscle tone or contracted further rather than relaxing. Furthermore, activation of protein kinase-C leads

to increased Na^+/Ca^{2+} exhanger rate in rat aorta (Vigne *et al.*, 1988).

No evidence was found to support an obligatory role of neurotransmission in the phorbol ester-induced contraction of schistosome muscle. This contraction was not mimicked by exogenous application of putative neurotransmitters or peptides, it was not inhibited by known inhibitors of the excitatory transmitter, serotonin, neither was it associated with a decrease in the release or efficacy of the inhibitory neurotransmitter, acetylcholine.

Likewise, a specific involvement of gap junctions could not be supported. The phorbol ester induced contraction did not generate or depend on alterations in membrane potential, thus conduction of electrical events from cell to cell would seem to be of little importance. Surface electrical activity of active parasites decreased during the contraction, indicating an alteration in the properties of the membranes from which these signals arise. This might be due to an increase in the activity of inhibitory ion channels such as the calcium-dependent potassium channels in the muscle (Blair *et al.*, 1989).

Also, the possibility that activation of protein kinase-C leads to a catch state in the schistosome does not appear likely. Catch muscle has several stereo-typed characteristics (Achazi, 1982; Ishii *et al.*, 1989 a,b). Catch muscle like the schistosome, is capable of both phasic and tonic contractions,

but unlike schistosome muscle, catch muscle possesses a well developed sarcoplasmic reticulum and T-tubule network. Also catch muscle is not dependent on extracellular Ca^{2+} for tonic contraction. The inhibitory transmitter of catch, serotonin, elevates cAMP and induces relaxation of tonic contraction. When catch is "relaxed" in this manner, subsequent contractions are reduced in amplitude and are phasic only. In trematodes, cholinergic (inhibitory) agonists do not appear to stimulate cAMP production (Mansour, 1984; Northup and Mansour, 1978) and actually inhibit serotonin-stimulated cAMP production in *F. hepatica* (Northup and Mansour, 1978). Praziquantel, or the combination of 60 mM K^+ depolarization and 1 μM phorbol-12,13-dibutyrate, produce full tonic contractions in carbachol-relaxed schistosomes. There is sufficient dissimilarity between the phorbol ester-induced contraction and the catch system of mollusks to conclude that a catch system is probably not involved in this contraction of longitudinal muscle in the schistosome.

From these results it appears that an isozyme of protein kianse-C that, in the presence of phorbol esters, is independent of Ca^{2+} for activity is present in the schistosome. Activation of this protein kinase-C with phorbol esters leads to muscle contraction, but the endogenous regulator of the physiological activation of protein kinase-C remains unknown. These results support the hypothesis that activation of protein kinase-C with phorbol esters leads to an

enhanced Ca^{2+} channel activity and subsequently increases sarcoplasmic Ca^{2+} concentrations to increase muscle tone. However, these experiments were carried out on whole organisms and did not examine channel activity directly. Therefore the interpretations cannot be as conclusive or as clear-cut as interpretations attainable from experiments conducted on single cells under voltage clamp conditons. Only once muscle cells can be isolated and studied directly will specific interactions and modulation of channel activity by the various drugs used in this study be unequivocably demonstrable and interpretable.

SUMMARY

1) The physiological effects of various phorbol esters are evaluated in adult male *Schistosoma mansoni*. Phorbol esters stimulate muscle contraction in a dose-dependent manner, with a structural specificity that is consistent with the activation of protein kinase-C.

2) A stereo-specific phorbol ester receptor is demonstrated.

3) A phospholipid and phorbol ester-dependent protein kinase activity, consistent with protein kinase-C, possessing the same requirements for phorbol ester stereo-specificity and sites of esterification as the physiological responses is demonstrated.

4) The phorbol ester-induced muscle contraction is independent of membrane potential, but surface electrical activity is reduced during the contraction.

5) The phorbol ester-induced muscle contraction is dependent on extracellular Ca^{2+}, appears to be associated with an increase in permeability of the parasite to Ca^{2+} but not Na^{+} or H^{+}, and is blocked by agents known to be Ca^{2+} channel blockers in smooth muscle of other organisms. Furthermore, threshold doses of phorbol ester synergize with the contraction induced by threshold depolarization or the

threshold dose of praziquantel, agents that are believed to alter voltage-gated Ca^{2+} channels.

6) The contraction could not be explained by altered release of or sensitivity to putative neuromuscular transmitters, decreased Ca^{2+} efflux, or an increase in the sensitivity of the contractile system to Ca^{2+}.

7) These results support the hypothesis that activation of protein kinase-C in the schistosome with phorbol esters leads to muscle contraction by enhancing Ca^{2+} channel activity.

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