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Full Name of Author — Nom complet de l'auteur

Borys Lev Brezden

Date of Birth — Date de naissance

July 17, 1949

Country of Birth — Lieu de naissance

Canada

Permanent Address — Résidence fixe

2120 Blossom Drive
Ottawa, Ontario
K1H 6G8

Title of Thesis — Titre de la thèse

The Physiological Basis of Frescon Action in Molluscan Muscles

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Carleton

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Name of Supervisor — Nom du directeur de thèse

Dr. D.R. Gardner

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Borys Lev Brezden

THE PHYSIOLOGICAL BASIS OF FRESCON
ACTION IN MOLLUSCAN MUSCLES

by

© Borys Lev Brezden, B.Sc., M.Sc.

A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Department of Biology
Carleton University
Ottawa, Ontario
August 5, 1982

The undersigned hereby recommend to the
Faculty of Graduate Studies and Research
acceptance of this thesis, submitted by
Borys Lev Brezden, B.Sc., M.Sc., in
partial fulfilment of the requirements
for the degree of Doctor of Philosophy.

.....
Chairman, Department of Biology

.....
Supervisor

.....
External Examiner

ABSTRACT

The molluscicide Frescon has been shown to cause irreversible contracture in smooth and cross-striated muscles of *Lymnaea stagnalis* and in a smooth muscle of *Helix aspersa*. The most likely mechanism for this action is an increase in the sarcolemmal permeability to calcium, where Frescon possibly induces existing calcium channels to remain in an open state. This conclusion was based on the following results:

- 1) The calcium entry blockers nickel, cobalt, manganese, lanthanum and a relatively high concentration of D-600, elevated magnesium and reduced calcium saline, and calcium-free saline prevented and, except for D-600, reversed the contracture induced by Frescon.
- 2) Frescon action is most unlikely to be at an intracellular site as this molluscicide does not exhibit some properties of agents which are considered to have an intracellular action. That is, caffeine, praziquantel and the calcium ionophore A23187 were all capable of promoting contraction in the absence of extracellular calcium, whereas Frescon-induced contractures were blocked under these conditions.
- 3) Frescon probably does not act on cell membrane-associated calcium extrusion mechanisms such as the Na-Ca exchange

process, since contractures induced by sodium-free saline (which probably inhibits the Na-Ca exchange) and Frescon action were not mutually exclusive. Furthermore, sodium-free saline, but not Frescon, caused a depolarization of the muscle cell membrane of *Lymnaea*.

- 4) Given the available evidence, it seems more likely that the action of Frescon is directed primarily against receptor-operated calcium channels as high potassium contractures (which probably utilize potential-dependent calcium channels) were superimposable over those of Frescon, and sodium pentobarbital and low concentrations of D-600, which reportedly block potential-dependent calcium channels, did not block the action of Frescon.

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To My Parents
and To Diane

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LIST OF ABBREVIATIONS

- Å: Angstrom (10^{-10} metres)
- ABRM: Anterior byssus retractor muscle
- ACh: Acetylcholine
- AP: Action potential
- ATP: Adenosine triphosphate
- ATPase: Adenosine triphosphatase
- BDAC: Bis(2-hydroxyethyl)dimethyl ammonium chloride
- cAMP: Cyclic adenosine 3',5'-monophosphate
- Ca_s: Sarcoplasmic reticular calcium
- CDCI: Calcium-dependent calcium channel inactivation
- cGMP: Cyclic adenosine 3',5'-guanosine monophosphate
- cm: Centimetre
- CNS: Central nervous system
- DMSO: Dimethyl sulphoxide
- EGTA: Ethyleneglycol-bis(β -aminoethylether)N,N'-tetraacetic acid
- E_x: Equilibrium potential (reversal potential) of an ion species 'x', or, where 'x' is not an ion, the equilibrium potential of the response evoked by 'x'.
- F: Faraday (23,060 calories/volt-equivalent)
- FX 2574 (blank): A dimethyl sulphoxide-based detergent formulation in which Frescon was dissolved
- g: Gram
- h: Hour
- HEPES: N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
- HV: Heart ventricle

HVMC: Heart ventricle muscle cell

I_x : Transmembrane current carried by ion species 'x'

kg: Kilogram

l: Litre

LC₉₀ (24h): Concentration of a toxic compound required to kill 90% of the treated organisms within 24 hours

LFS: *Lymnaea* foot strip

M: Molar

mg: Milligram

ml: Millilitre

μ l: Microlitre

mm: Millimeter

mM: Millimolar

msec: Millisecond

mV: Millivolt

n: Sample size or number of repetitions of a particular experiment

NAPB: Sodium pentobarbital

NE: Norepinephrine

PDC: Potential-dependent calcium (channel)

PDCI: Potential-dependent calcium channel inactivation

POPOP: 1,4-bis[2-(5-Phenyloxazolyl)] benzene

PPO: 2,5-Diphenyloxazole

PRM: Penis retractor muscle

PQ: Praziquantel

P_x : Membrane permeability of ion species 'x'

R: Gas constant (1.9872 calories/mole-degree)

ROC: Receptor-operated calcium (channel)

sec: second

S.D.: Standard deviation

SR: Sarcoplasmic reticulum

T: Absolute temperature

TTX: Tetrodotoxin

V: Volt

V_m : Membrane potential; the transmembrane potential difference (inside of cell negative) measured during alterations of the extracellular or intracellular ionic environment, or during exposure of the cell to any agent which alters the resting potential (V_r).

V_r : Resting potential; the transmembrane potential difference (inside of cell negative) under 'normal' intracellular and extracellular ionic conditions.

v/w: Volume per weight (expressed as % millilitre/gram)

v/v: Volume per volume

w/v: Weight per volume (expressed as % gram/millilitre)

$[X]_e$: Extracellular concentration of 'X'

$[X]_i$: Intracellular concentration of 'X'

X_e : Location of 'X' = extracellular

X_i : Location of 'X' = intracellular

For definitions of the abbreviations used to describe the experimental compounds D-600, A23187 and RMI 12330A, see Table 5, p. 85.

INTRODUCTION

1) THE PROBLEM OF SCHISTOSOMIASIS

The widespread tropical disease schistosomiasis (bilharzia) poses serious health and economic problems particularly in developing countries where its control is hindered by poor sanitation, poor social conditions and/or a weak economy. This disease, which affects at least 250 million people worldwide, is transmitted in humans by three main species of flatworm, *Schistosoma haematobium*, *Schistosoma mansoni*, and *Schistosoma japonicum*. At one stage in its life cycle, a schistosome parasite must enter an aquatic snail before it can develop into a form capable of infecting humans (Shell, 1974). Consequently, the transmission of schistosomiasis can be interrupted by reducing or eliminating the population of the secondary snail host.

2) THE MOLLUSCICIDE FRESCON: A HISTORICAL PERSPECTIVE

In the mid 1960's the governments of the countries worst affected by schistosomiasis, in collaboration with the World Health Organization, began a programme to retard the spread of this disease through the control of snail populations. To this end, the very effective molluscicide

Frescon (N-triphenylmethyl morpholine) was developed at the Shell Research Centre in England. This molluscicide is effective against host aquatic snails at very low concentrations (LC_{90} (24 h) = $0.023-0.1 \times 10^{-6}$ g/ml) and is highly specific. The concentrations which are required to kill snails have virtually no effect on plants and little effect on most other aquatic organisms. However, certain edible fish (e.g. *Tilapia* spp. and *Barbus* spp.) have been killed by the application of Frescon to schistosome-infested waters, although the reduction in the fish populations had no effect on the local fishing activities, and the fish populations quickly returned to normal (Boyce et al., 1966; Shell, 1974). Mammals have a very low susceptibility to Frescon, the percutaneous LD_{50} for rats, for example, being greater than 1000 mg/kg (Shell, 1974).

Concerted investigations into the mode of action of Frescon were first undertaken by Moreton & Gardner (1976) using the aquatic snail *Lymnaea stagnalis*, which is killed by 10^{-6} g/ml Frescon within 3 h. It was found that the exposure of the isolated brain of *Lymnaea* to 10^{-5} or 10^{-6} g/ml Frescon for 10-30 min results in the appearance of spontaneous, summing bursts of synaptic potentials which may depolarize the membrane by several tens of millivolts. These 'Frescon bursts' have a duration of 1-4 sec, and always appear in synchrony in

simultaneously monitored neurons (Moreton & Gardner, 1976). Similar findings were reported for the terrestrial snail *Archachatina marginata* (Plummer & Banna, 1979).

The electrical stimulation of intestinal and/or pallial nerve trunks of *Lymnaea* results in a neuronal response which is very similar to that induced by Frescon. Although the reversal potential of Frescon bursts (E_f) was found to vary from neuron to neuron, in the same cell E_f was found to be very close to the reversal potential of the response to nerve trunk stimulation (E_s). The similarity between E_f and E_s suggests that the synaptic pathways of Frescon bursts are very similar to, if not the same as, those utilized by nerve trunk stimulation (Gardner & Moreton, 1978).

Gardner & Moreton (1978) have suggested that the underlying mechanism of Frescon burst generation is a disinhibition resulting from the abolition of chloride-mediated inhibitory postsynaptic potentials. Such a disinhibition could result in a reverberatory sequence of synaptic activation which would lead to the synchronous generation of bursts throughout the snail's central nervous system (CNS) (Gardner & Moreton, 1978; Moreton & Gardner, 1981). Given these observations, it was conceivable that the molluscicidal activity of Frescon could be attributed to a large-scale disruption of the CNS with a consequent fatal disorganization of motor functions.

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In the examination of a range of Frescon analogues, Brezden & Gardner (1980a) found that those which were molluscicidal were also neurotoxic. Moreover, the molluscicidal effectiveness was closely correlated to the degree of neurotoxicity. These results suggested that the CNS was indeed a possible site for Frescon action. However, further investigation revealed that the primary site of action for Frescon in the whole animal is most likely not the CNS. This conclusion was based on the following observations.

- 1) The time course of Frescon poisoning in the whole animal was not correlated to the time course of appearance of bursts in the isolated CNS. The exposure of snails to 10^{-5} g/ml Frescon resulted in the appearance of symptoms of poisoning within 5-10 min. However, the same concentration of Frescon did not promote bursts in the isolated CNS neurons until after 20-60 min of exposure.
- 2) The neuronal activity of the CNS isolated from snails which had been exposed to Frescon for up to 2.5 h was found to be normal, although the snails were exhibiting symptoms of severe poisoning. The subsequent exposure of these 'normal' CNS preparations to Frescon, however, resulted in the eventual appearance of bursts, suggesting that electrophysiological abnormalities can only be elicited by direct exposure of the CNS to this molluscicide.
- 3) The injection of Frescon into the haemocoel of snails

promoted symptoms of severe poisoning within 2 min. However, only 3 out of 14 subsequently isolated brains of snails thus treated exhibited electrophysiological abnormalities (Brezden, 1977).

These results strongly argue against the hypothesis that the molluscicidal activity of Frescon can be attributed to its action on the snail CNS. However, the close correlation between the molluscicidal activity and the neurotoxicity of the Frescon analogues (Brezden & Gardner, 1980a) suggested that the principal mechanism (i.e. a nucleophilic attack at some target entity) for both neurotoxicity and molluscicidal activity of Frescon may be similar (Brezden & Gardner, 1980b). Since one of the first symptoms of poisoning in snails is the apparent loss of muscular coordination, and in later stages of poisoning, an irreversible rigour of the musculature, it was suggested that Frescon could act directly on molluscan muscles, i.e., without an intermediate involvement of the nervous system (Brezden & Gardner, 1980a).

3) THE STRUCTURE AND FUNCTION OF MOLLUSCAN MUSCLE

A) *Gross structure and muscle types*

Molluscan musculature is represented by diverse structural and functional types, although the majority of the musculature is composed of small, non-striated

uninucleate cells. The body wall musculature consists of closely interwoven, antagonistic sheets working against a hydrostatic skeleton. Discrete muscle systems are difficult to recognize and are virtually inseparable. Only shell adductors (e.g. the adductor muscles of lamellibranch molluscs) and certain retractor and protractor muscles (e.g. byssus, radula, penis or antenna retractor and penis protractor muscles) are discrete units, but even these may be composed of different muscle types (e.g. the shell adductor of *Pecten* spp., Hanson & Lowy, 1957; Millman, 1964).

Those muscles which have been most extensively studied either form definable anatomical units (e.g. heart, buccal mass, penis retractor muscles), are functionally important (e.g. the foot) or are physiologically unique (e.g. the anterior byssus retractor muscle of *Mytilus edulis*). In addition to transient phasic contractions, the anterior byssus retractor muscle (ABRM) is capable of maintaining prolonged tonic contractions with very little expenditure of energy. For this reason, it is often referred to as a catch muscle, the catch state defining a physiological and/or structural mechanism which apparently locks the muscle in contracture.

Unlike vertebrate muscle, molluscan muscle cannot readily be classified into 'smooth' and 'striated' types. Many molluscan muscles exhibit properties (both physical

and physiological) which are intermediate between smooth and striated types, for which various names have been proposed. Heyer et al. (1973) suggested a comprehensive classification scheme for invertebrate muscle in general. However, the classification scheme of Hanson & Lowy (1960) and Hoyle (1964) are most often used in the literature. A synthesis of these two schemes defines all of the molluscan muscle types reported thus far.

Hanson & Lowy (1960) classified molluscan muscles into three distinct structural types, one of which is subdivided to give four categories. Hoyle (1964) further subdivided smooth muscle, defining a total of five categories. These are as follows (the nomenclature of Hoyle (1964) given in parentheses).

- 1) *Cross-striated* (ordinary striated) muscle fibres are identical in appearance under the light microscope to vertebrate and arthropod skeletal muscle fibres. These muscles are divided into sarcomeres by well-defined, regularly spaced 'Z-bands' and exhibit characteristic A and I bands. The molluscan 'Z-bands', however, are not ultrastructurally similar to vertebrate Z-bands (see later). The heart ventricle muscles of *Lymnaea stagnalis* (Plesch, 1977) and *Helix aspersa* (North, 1963) are cross-striated.
- 2) *Obliquely striated* muscle fibres appear banded under the light microscope, but the striations are at an

angle to the fibre axis. The banding is produced by an overlapping of thick and thin myofilaments as in the case of vertebrate striated muscles (Millman, 1967), but the striations are not the result of a unique division into sarcomeres (Hoyle, 1964). Z-lines are absent from obliquely striated muscles, but A and I bands can be discerned (Hanson & Lowy, 1961).

Obliquely striated muscle has been classified into

- a) *regular obliquely striated* (intermediate smooth) muscle fibres where the contractile filaments are arranged in a staggered, helical array on the outside of the muscle fibres around a central core of mitochondria, leading to a very regular pattern, and,
 - b) *irregular obliquely striated* (pseudo-striated) muscle fibres which contain contractile filaments in the centre as well as on the outside. There is no mitochondrial core, and the order of the contractile filaments breaks down in the central region. The overall pattern under the light microscope has been variously described as 'double-oblique', a 'diamond lattice' or 'herringbone'.
- 3) *Smooth* muscle fibres do not exhibit a band pattern. This type of muscle is found in the foot and penis retractor muscles (PRM) of *Lymnaea stagnalis* (Plesch, 1977) and the PRM of *Helix pomatia* (Schlote, 1963). Hoyle (1964) distinguishes between two types of smooth muscle,

viz.,

- a) ordinary smooth muscles showing very prolonged contraction and paramyosin banding (characterized by regular periodicities at 145 Å (Hanson & Lowy, 1969), since, in addition to myosin, the thick filaments of these muscles contain the protein paramyosin (see later), and,
- b) ordinary smooth muscles not showing paramyosin banding which are not capable of prolonged contraction. Electron microscopic examination of these fibres reveals only thin filaments, a situation analogous to vertebrate smooth muscle (Hoyle, 1964; Carlson & Wilkie, 1974; Kawaguti & Ikemoto, 1960).

B) Ultrastructure and mechanism of contraction

All molluscan muscles, except for the smooth muscles without paramyosin banding, have been shown to contain both thick and thin filaments (Hanson & Lowy, 1961; North, 1963; Nisbet & Plummer, 1968; Rogers, 1969; Sobieszek, 1973; Wabnitz, 1975; Plesch, 1977). The diameter of the thick filaments, which are usually tapered at both ends, varies from 120 Å to 1200 Å. Differences in diameter are generally attributed to the amount of paramyosin (tropomyosin A) present, which forms a core within peripherally arranged myosin molecules (Szent-Györgyi et al., 1971). The thin filaments are generally similar in diameter to those

of vertebrate skeletal muscles (ca. 50 Å) (Hoyle, 1964; Nisbet & Plummer, 1968; Rüegg, 1971), although they may vary from 40-100 Å, depending on the muscle and the method of fixation used (Heyer et al., 1973).

It is generally agreed that all molluscan muscles contract by the sliding filament mechanism analogous to that proposed by Huxley (1957) for vertebrate skeletal muscles. The structural evidence for this mechanism has been summarized by Lowy et al. (1964) and Hoyle (1964) and is as follows:

- 1) A double array of two types of filaments is present (except in certain smooth muscles; see above).
- 2) The thin filaments contain actin.
- 3) The thick filaments contain myosin.
- 4) Cross-linkages have been observed between thick and thin filaments.
- 5) The thick filaments are discontinuous along the contractile apparatus.
- 6) The filament lengths do not change during contraction.
- 7) The relative positions of the thick and thin filaments change as the muscle shortens.
- 8) The X-ray diffraction patterns are similar for different muscle lengths.

Two hypotheses have been proposed to account for contraction in those smooth muscles which apparently have only thin filaments. Hanson & Lowy (1961) suggested that

the filaments of vertebrate smooth muscles, which are all about 50 Å in diameter, might be of two kinds, viz., actin-containing filaments and myosin-containing filaments with little or no core protein. Contractions would therefore be effected by the usual actin-myosin interaction. A similar state of affairs may exist in molluscan muscle. However, X-ray diffraction analysis and birefringence studies of vertebrate smooth muscles failed to detect myosin (Elliot, 1964; Weber & Rüegg, 1966), although this protein can be extracted by salt solutions of low ionic strength, usually in the form of dimers or trimers. Consequently, it has been proposed that most or all of smooth muscle myosin exists in a soluble form and is dissociated from actin. During contraction, dimers of myosin may cross-link adjacent actin filaments which are in anti-polar alignment. With a large number of such interactions, actin filaments may be displaced in opposite directions with respect to each other, and the cells would contract (Rüegg, 1971).

The unique muscle protein paramyosin was first discovered in earthworm muscle by Hall et al. (1945) and subsequently in lamellibranch adductor muscles by Hanson & Lowy (1957). It is found in all molluscan muscles capable of prolonged contraction ('catch') (Millman, 1967), but not all muscles which contain paramyosin are capable of catch (Ishii & Takahashi, 1981). The role of this protein

with respect to catch has not yet been resolved. Lowy et al. (1964) proposed a structural role for paramyosin, since the thick filaments of the ABRM are approximately nineteen times longer than those of the frog sartorius muscle. Consequently, as a result of a greater number of cross-bridge formations, the tension generated along the length of the thick filament would be greater than that developed along a shorter filament. It was therefore proposed that the function of paramyosin is to strengthen the thick filaments, allowing them to bear the greater tensions produced during catch, and that the catch mechanism itself results from a slow decay of cross-bridge linkages (the 'linkage' hypothesis)* (Lowy et al., 1964; Szent-Györgyi et al., 1971).

Rüegg (1971) reviewed the alternate hypothesis that cross-bridge formation between molecules of paramyosin may be responsible for the catch state (the 'paramyosin' hypothesis). Wabnitz (1975) reported apparent paramyosin aggregates during catch in the PRM of *Helix pomatia*. However, Sobieszek (1973) concluded that such an aggregation is most likely a fixation artifact. Moreover, the thick filaments do seem to be discontinuous, as attachment sites to cell structures (e.g. the cell membrane) have not been observed (Lowy et al., 1964). Thus, it is difficult to see how thick filament interactions can be responsible for the maintenance of tension.

Currently, the experimental evidence favours the

linkage hypothesis (Twarog, 1976). If this hypothesis of prolonged contraction is to be substantiated, it must be shown that cross-bridge decay during the catch state is inhibited, since energy expenditure during catch is at a minimum. It has been proposed that the intracellular Ca (Ca_i) levels remain high during catch (Twarog, 1976; Twarog & Muneoka, 1972). However, prolonged contractions maintained by virtue of a high intracellular Ca concentration ($[Ca]_i$) must be supported by an additional process that shuts off the active state and stabilizes the bonds between actin and myosin, thereby reducing the energy expenditure. Szent-Györgyi et al. (1971) present evidence suggesting that paramyosin undergoes a structural transition during catch which specifically inhibits the ATPase responsible for cross-bridge turnover. Thus, a muscle in the catch state could maintain tension as a result of an inhibition of cross-bridge decay.

Evidence for a structural role for paramyosin is suggested by the report that the maximum length of the filaments of molluscan myosin formed *in vitro* does not exceed several μm (Szent-Györgyi et al., 1971), whereas the thick filaments may be as long as 30 μm *in vivo* (Lowy et al., 1964). Moreover, the paramyosin core of molluscan thick filaments is probably a bipolar structure (Szent-Györgyi et al., 1971). In view of these considerations, paramyosin may have a three-fold structural role,

i.e. to mechanically strengthen the thick filaments, to impose a polarity on the myosin surface array, and to increase the length of the myosin complex.

Z-lines are absent in most molluscan muscle fibres. Even in cross-striated muscles, the Z-lines are irregular and appear to consist of a dense mass of small particles (Z-particles) under high magnification (Kawaguti & Ikemoto, 1959, 1960; Hanson & Lowy, 1961; North, 1963; Sobieszek, 1973). The thin filaments appear to be attached to the Z-particles, some of which in turn are attached to the sarcolemma (Hanson & Lowy, 1961; Sobieszek, 1973). Their composition appears to be filamentous, resembling an aggregation of thin filaments (Rogers, 1969). Szent-Györgyi et al. (1971) have presented evidence to suggest that the filaments on the opposite sides of these particles are of opposite polarity. Accordingly, the Z-particles are believed to be responsible for the transmission of tension along the length of the muscle cell by allowing the formation of a continuous network of thick and thin filaments from one end of the cell to the other.

C) *Functional aspects of muscular contraction*

a) *Molluscan sarcotubular structures*

Sarcotubular systems, developed to a greater or lesser extent, have been reported in all molluscan muscle types examined, although in no case is the development as

extensive as in vertebrate striated muscles. However, the sarcoplasmic reticulum (SR) of most invertebrate smooth muscles is much more developed than that of its vertebrate counterpart (Rosenbluth, 1968).

Smooth muscle fibres from *Lymnaea stagnalis* (Plesch, 1977), *Helix aspersa* (Rogers, 1969), *Helix pomatia* (Schlote, 1963) and *Archachatina marginata* (Nisbet & Plummer, 1968) exhibit numerous sarcolemmal invaginations and subsurface cisternae, but the sarcotubular system is sparse. Structures analogous to the vertebrate T-tubule system do not exist. The SR of *Lymnaea stagnalis* smooth muscles occurs throughout the cells and is closely associated with the mitochondria, the sarcolemma (approaching to within 12-17 nm of the surface) and the subsurface tubules, forming diads (Plesch, 1977). A similar SR network is present in the PRM and foot muscles of *Helix* spp. (Rogers, 1969; Schlote, 1963).

The sarcotubular system is much more developed in the invertebrate heart, a cross-striated muscle. Plesch (1977) observed numerous cisternae in *Lymnaea stagnalis* ventricle muscle cells. These cisternae run in parallel rows between the contractile filaments and at the level of each line of Z-particles. A similar tubule organization has been observed in the heart muscles of *Helix aspersa* (North, 1963) and *Archachatina marginata* (Nisbet & Plummer, 1968).

The striated muscle fibres of the adductor of the oyster *Crassostrea angulata* contain numerous large, ovoid vesicles immediately under, and parallel to, the fibre axis, as well as smaller spherical vesicles separated from the ovoid vesicles by a 'dense zone'. No SR was observed inside the contractile apparatus in the fibre core. However, vesicles were associated with the peripheral Z-particles, forming diad-like structures (Hanson & Lowy, 1961).

The ABRM of *Mytilus edulis* has a relatively poorly developed sarcotubular system, exhibiting vesicular and tubular structures which are associated with the sarcolemma (Twarog, 1976). No tubules have been reported in the core of this muscle. The muscles of the buccal mass of *Ferrissia wautieri* show a well-developed longitudinal tubule system which appears to make close contact with the extracellular space through cisternae. The transverse tubule system is reduced to short sarcolemmal invaginations at the level of the Z-band (Richardot & Wautier, 1971).

In general, those molluscan muscles which have a higher degree of myofilament organization also possess a more extensive sarcotubular system. The muscles with an amorphous myofilament organization exhibit only a relatively small number of subsurface cisternae, which may open to the extracellular space (Rogers, 1969; Plesch, 1977). The possible functional role of these structures is discussed below.

b) *Calcium stores and the contraction-relaxation cycle*

The contraction-relaxation cycle can be resolved into the following components:

- 1) the translation of a chemical or electrical stimulus at the level of the sarcolemma (e.g. depolarization of the cell membrane) to the release of Ca ('activator Ca') from intracellular stores, and/or to an increase in the Ca permeability of the cell membrane to extracellular Ca (excitation-contraction coupling),
- 2) the interaction of Ca at the level of the contractile elements leading to the activation of myosin ATPase and cross-bridge cycling, and,
- 3) the resequestration of Ca by internal stores and/or the active extrusion of Ca to the extracellular space, leading to the inhibition of actin-myosin interaction with a consequent relaxation.

According to the current model of contraction of vertebrate skeletal muscle, Ca is released from sequestering sites into the sarcoplasm during excitation. The Ca ions then interact with the troponin component of the thin filaments. This interaction somehow allows the formation of actomyosin, and the myosin ATPase is activated by actin (myosin ATPase alone is not activated by Ca; Carlson & Wilkie, 1974). Cross-bridge cycling and contraction follow these events.

There is evidence that Ca regulates cross-bridge formation in molluscan muscle also (Kendrick-Jones et al., 1970; Szent-Györgyi et al., 1971). In these muscles, however, Ca triggers the contractile events by interacting with myosin which, in contrast to vertebrate skeletal muscle, contains a Ca-sensitive ATPase. Isolated molluscan thin filaments do not bind Ca (Kendrick-Jones et al., 1970) and contain only actin and tropomyosin. Components corresponding to vertebrate troponin are absent (Lehman et al., 1972). Moreover, molluscan thin filaments do not sensitize the ATPase of rabbit myosin. On the other hand, thin filaments of vertebrates, arthropods and annelids both bind Ca and confer Ca sensitivity to the ATPase activity of rabbit myosin (Lehman et al., 1972). Therefore, it appears that in molluscan muscles the Ca-binding component interacts directly with myosin, thereby inhibiting cross-bridge formation. Elevated Ca levels release the inhibitory component from myosin, allowing actin-myosin interaction (Kendrick-Jones et al., 1970). The Ca-sensitive regulatory component of molluscan myosin has been shown to be a particular light chain which can be reversibly removed by lowering the concentration of divalent cations with EDTA (Lehman et al., 1972).

In vertebrate skeletal muscle, the mechanism underlying the translation of cell membrane depolarization to an elevation of the intracellular Ca concentration ($[Ca]_i$) is

fairly well established (Endo, 1977). The spread of depolarization along the surface of the cell membrane is transmitted to the T-tubule system which is intimately associated with the SR. It is generally accepted that the spread of depolarization through the T-tubules leads to a release of Ca from the SR, although the mechanism of this process is not known (Franzini-Armstrong & Peachy, 1981).

The situation in most molluscan muscles, however, is probably more similar to that in vertebrate smooth muscles where both intracellular and extracellular Ca (Ca_e) have been implicated in the activation of contraction (Sulakhe & St. Louis, 1980). The source of activator Ca in molluscan muscle cells appears to depend on the type of stimulus (i.e. chemical or electrical) and on the type of muscle. For example, the ABRM of *Mytilus edulis* contracts in response to acetylcholine (ACh), caffeine, or a high extracellular K concentration ($[K]_e$). The application of ACh to the ABRM results in an increase of the sarcoplasmic Ca concentration. This increase has been attributed to both an increased influx of Ca_e and an increase in the Ca efflux from intracellular stores (Sugi & Tameyasu, 1975; Twarog & Muneoka, 1972). More recently, however, Sugi & Yamaguchi (1976) have suggested that the response to ACh is due primarily to the release of Ca from intracellular stores.

Caffeine can activate the contraction of the ABRM in the absence of Ca_e (Muneoka & Mizonishi, 1969), suggesting

that this agent releases Ca from intracellular stores. However, once these stores are depleted, Ca_e must be available to the resting muscle to restore Ca_i and the response to caffeine. The time required for the complete restoration of Ca_i in artificial seawater containing 10mM Ca is more than three hours. An elevation of $[K]_e$ greatly speeds up the rate of replenishment of Ca_i stores (Twarog & Muneoka, 1972). Hagiwara & Nagai (1970) reported an increased Ca influx in response to high $[K]_e$, which explains the increased rate of replenishment of Ca_i stores under this condition. Muneoka (1969) reported that procaine, which reportedly blocks the caffeine-induced release of Ca from the SR (Feinstein, 1963; Weber & Herz, 1968), reduces caffeine contracture in the ABRM.

Hagiwara & Nagai (1970) and Sugi & Tameyasu (1975) proposed that the contractile response of the ABRM to elevated $[K]_e$ depends on the presence of Ca_e . In contrast, Twarog & Muneoka (1972) reported that the contractile response of this muscle to DC current pulses depends on both Ca_e and Ca_i stores. Therefore, it would appear that the origin of activating Ca is extracellular during activation by high $[K]_e$, intracellular during activation by caffeine, both intracellular and extracellular during contraction induced by direct current pulses, and intracellular and/or extracellular during activation by ACh.

In contrast to the ABRM, Huddart et al. (1977)

proposed that the pharyngeal retractor and columellar muscles of *Buccinum undatum* and *Neptunia antiqua* respond to KCl-induced depolarization with a release of intracellular Ca, whereas Suzuki & Sugi (1982) reported that the contractile mechanism of the longitudinal retractor muscle of the sea cucumber *Stichopus japonicus* is activated by the release of Ca from subsarcolemmal cisternae as well as by an inward movement of Ca_e .

Hill et al. (1970) reported that the radula protractor muscle of *Busycon canaliculatum* (which has a poorly developed sarcotubular system) does not contract upon application of ACh in the absence of Ca_e . Restoration of Ca_e restores ACh-induced contraction. However, caffeine can induce contraction in a radula protractor muscle during depolarization by KCl and in the absence of Ca_e , although the response is weak and not sustained. These results suggest that, in this muscle at least, Ca_e is necessary for the ACh response, and that Ca_i stores are poor. A similar dependence on Ca_e has been reported for the contractile response to ACh in the radula protractor muscle of *Rapana thomasi* (Kobayashi, 1972a) and the longitudinal retractor muscle of the sea cucumber *Isostichopus badionotus* (Suzuki, 1982), which contains vesicles only near the plasma membrane and does not have a readily identifiable SR (Hill et al., 1978). Suzuki (1982) reported that ACh-induced contraction in this muscle was reduced by lowering $[Ca]_e$ as well as by Mn and La ions or

procaine, and was completely prevented by soaking the preparation in Ca-free saline for 30 min or more, suggesting that Ca_e stores are primarily responsible for the response to ACh. He also investigated the loci of Ca accumulations by means of pyroantimonate precipitates, which were found to be localized in the subsurface vesicles and along the inner surface of the plasma membrane in resting fibres. However, in fibres fixed during mechanical activity, the pyroantimonate precipitates were considerably decreased in the sub-surface vesicles and along the plasma membrane and were diffusely distributed throughout the sarcoplasm, suggesting that the contraction of this muscle depends not only on Ca influx, but also on the release of Ca from intracellular storage sites. Therefore, although Ca_i stores may play a role during ACh-induced contraction in this muscle, Ca_e stores probably represent the more significant fraction.

There may well be a relationship between the extent of sarcotubular or SR development, the capacity of Ca_i stores and the source of activator Ca in molluscan muscle, but there is not enough information about Ca fluxes in different muscle types for an accurate comparative analysis. Ultrastructural studies of various molluscan muscles *vis-à-vis* sarcotubular systems are more complete than studies related to Ca movements during contraction. Consequently, for most muscle types (including *Lymnaea stagnalis* and *Helix aspersa*, the subjects of the present investigations), the role of the sarcotubular

system in excitation-contraction coupling is largely speculative (cf. Plesch, 1977). The available evidence suggests that in those muscle types which have a poorly developed sarcotubular system, the primary source of activator Ca is extracellular, although intracellular structures may release their Ca contents in response to certain types of stimuli (e.g. caffeine) (Hill et al., 1970; Suzuki, 1982). However, these intracellular structures may play a more important role in the uptake of Ca during relaxation rather than serving as a source of activator Ca. There is some evidence that the vesicular structures of molluscan muscles are capable of accumulating Ca in an energy-dependent process. Microsomal fractions of the ABRM of *Mytilus edulis* have been shown to accumulate Ca in parallel with the hydrolysis of ATP (Sugi & Atsumi, 1973; Stössel & Zebe, 1968; Huddart et al., 1977), although the Ca binding activity of these microsomes is about one-ninth that of mouse skeletal muscle and about one-fourth to one-eleventh that of other invertebrate striated muscles (Stössel & Zebe, 1968). Heumann (1969) showed that Ca oxalate could be precipitated in the vesicles of the ABRM, suggesting that they can take up Ca. Atsumi & Sugi (1976) reported that the subsurface vesicles (also in the ABRM) can re-accumulate Ca following its release into the sarcoplasm. Huddart et al. (1977) showed that the protein profile of the smooth muscles of the welks *Buccinum undatum* and *Neptunia antiqua*, as determined by

polyacrylamide gel electrophoresis, is very similar to that of vertebrate and arthropod skeletal muscles. Therefore, it may be suggested that the sarcoplasmic reticulum of vertebrate skeletal muscle has its functional counterpart in at least some molluscan muscles. In vertebrates, there is a general relationship between the development of the sarco-tubular system and muscle contraction speed (Salviali et al., 1982) and dependence on extracellular or intracellular Ca stores during contraction (Devine et al., 1972). In view of the available, albeit limited, information, this relationship may extend to invertebrate muscles as well (see Plesch, 1977).

It is not known whether the tubules of molluscs act like the transverse tubules of vertebrate striated muscles, i.e. to conduct electrical excitation to the cell interior. However, sarcolemmal invaginations and other specialized structures in molluscan muscle may play a parallel role. The smooth muscle cells of *Helix aspersa*, *Lymnaea stagnalis* and *Anodonta imbecilis* show areas of specialized contact between the sarcolemma and subadjacent vesicles. These structures may serve to couple membrane depolarization to Ca_i release in these muscles (Rogers, 1969; Plesch, 1977; Kidokoro et al. 1974; Heyer et al., 1973). The sarcolemmal invaginations which have been observed in many molluscan muscle types (Schlote, 1963; Rogers, 1969; Sanger & Hill, 1972; Plesch, 1977) have been considered to be permanent structures which

may also serve to increase the rate of conduction of membrane depolarization into the interior of the cell (Rogers, 1969; Sanger & Hill, 1972). However, Prescott & Brightman (1976) proposed that these invaginations may act as stretch receptors in the muscle, and that their deformation during stretch produces a membrane depolarization which leads to contraction.

c) *The regulation of intracellular calcium concentration*

A large transmembrane Ca concentration gradient exists across all muscle cells, $[Ca]_i$ being about four orders of magnitude lower than $[Ca]_e$. Apart from the SR (and possibly the vesicular structures observed in molluscan muscles) at least two additional processes are thought to be instrumental in maintaining low sarcoplasmic levels of free Ca. One of these processes is the Na-Ca exchange mechanism (Na-dependent Ca efflux) which has been investigated in vertebrate striated and smooth muscle (Chapman, 1979; Sulakhe & St. Louis, 1980), rat brain synaptosomes (Blaustein & Weismann, 1970) and the squid axon (Baker, 1972). This system is probably mediated by a carrier molecule or complex which exchanges 2 or more Na ions for each Ca ion (Chapman, 1979). Whether or not this process is energy-independent is not entirely clear. Since metabolic poisons or low temperatures appear to have little or no effect on Na-Ca exchange, it does not seem to be dependent on metabolic energy, at least

in the form of ATP (Baker, 1972; Blaustein, 1974; Brinley et al., 1975; Requena et al., 1977). However, it has been suggested that the residual levels of ATP in metabolically poisoned axons (Baker, 1972) may be sufficient to mediate $[Na]_e$ -dependent Ca efflux, which is influenced by micromolar levels of ATP in squid axons (DiPolo, 1973, 1977; Baker & McNaughton, 1976) and cardiac muscle (Jundt et al., 1975). However, the exact role of ATP in this mechanism is not yet resolved. Mullins (1977) suggested that the effect of ATP may be a catalytic one rather than that of a hydrolyzable substrate, although DiPolo (1977) and Baker & McNaughton (1978) have reported that non-hydrolyzable analogues of ATP do not influence Ca efflux activity, suggesting that the hydrolysis of ATP could play a role in modulating Ca efflux, and it has been shown that at least a part of the ATP-dependent Ca efflux is sensitive to $[Na]_e$ (Sulakhe & St. Louis, 1980).

The other mechanism for reducing $[Ca]_i$ is thought to involve the active transport of Ca via a specific membrane-bound ATPase which utilizes either Ca or Mg and which is regulated by $[Ca]_i$. This ATPase, however, can only assist the sarcoplasmic reticular Ca-ATPase during muscle relaxation, and is incapable of bringing about relaxation by itself (Sulakhe & St. Louis, 1980).

Virtually no information is available on the nature of cell membrane Ca fluxes in molluscs other than the squid.

The only other evidence for a passive Na-Ca exchange mechanism in molluscan tissue is for the ABRM of *Mytilus edulis*, where it was shown that Ca efflux was decreased by the exposure of this muscle to Na-free saline (Muneoka et al., 1978). Other molluscan muscle types have not been examined either for a Na-Ca exchange mechanism or an ATP-dependent Ca-ATPase.

In certain mammalian smooth muscles, mitochondria may be important in reducing the levels of Ca during relaxation. Batra (1973, 1975, 1977, 1982) reported that the Ca uptake rate and capacity of human myometrial mitochondria are sufficient to account for relaxation in this muscle. In contrast, Huddart & Syson (1975) reported that the Ca binding capacity of the rat ileal muscle mitochondria is insufficient to account for relaxation. It has been suggested that considerable species differences may exist in this context (Janis et al., 1976). There are no comparable studies for the mitochondria of molluscan muscles.

d) *The role of cyclic nucleotides in muscular contraction*

Cyclic 3',5'-adenosine monophosphate (cAMP), and to a lesser (or rather less well understood) extent, cyclic 3',5'-guanosine monophosphate (cGMP) have been implicated in the regulation of certain events during the contraction-relaxation cycle in smooth and striated vertebrate muscle (see reviews by Bär, 1974; Entman, 1974; Berridge, 1975).

The older concept of cyclic nucleotides as 'second messengers' (Robison et al., 1968) has been modified to include Ca ions since Rasmussen (1970) stressed the importance of the relationship between Ca, cyclic nucleotides and cell function. Changes in the levels of cyclic nucleotides are often secondary to, or occur in parallel with, an increase in $[Ca]_i$. In muscle, cAMP and cGMP may act as feedback vectors to modulate the levels of free Ca_i during the contraction-relaxation cycle. In vertebrate muscle, those agents which stimulate contraction act by raising $[Ca]_i$, whereas relaxing agents cause a reduction of $[Ca]_i$. The relaxation phase may be modulated by cAMP, which is thought to act by stimulating sarcoplasmic reticular and/or sarcolemmal Ca pumps (Shinebourne & White, 1970; Dietze & Hepp, 1972; Andersson & Nilsson, 1972; Berridge, 1975; Fabiato & Fabiato, 1978). Brooker (1973) reported that in the frog heart, the level of cAMP reaches its maximum just before or coincident with the beginning of the relaxation phase.

In most systems examined, elevated levels of cAMP result from the action of a hormone at the level of the cell membrane, presumably through the stimulation of adenyl cyclase (Robison et al., 1968; Rasmussen & Goodman, 1977). However, Brooker (1973) measured a transient increase in the level of cAMP during a single, electrically-induced contraction-relaxation cycle in the frog heart, and suggested that each action potential causes a transient stimulation of

adenyl cyclase. Epinephrine reportedly increased both the systolic and diastolic concentrations of cAMP. Thus, it would appear that either chemical or electrical stimulation of muscle contraction could result in elevated intracellular levels of this cyclic nucleotide.

Increased concentrations of Ca_i alone may have an indirect effect on the levels of cAMP. It has been reported that Ca_i in the presence of an 'activating factor' stimulates phosphodiesterase activity, thereby reducing the intracellular concentration of cAMP (Kakiuchi & Yamazaki, 1970; Kakiuchi et al., 1971; Kakiuchi et al., 1973; Teo & Wang, 1973). The activating factor reported by Kakiuchi & Yamazaki (1970) has since been identified as calmodulin and activates only one of several forms of phosphodiesterase (Klee et al., 1980). Calmodulin has now been recognized as a mediator of many Ca -dependent processes, having been implicated in the regulation of Ca transport by cardiac SR (Barany & Barany, 1977; Lehman, 1978), the stimulation of myosin phosphorylation in both skeletal and smooth muscle, and actomyosin ATPase activity (Wang & Waisman, 1979), as well as the regulation of cyclic nucleotide levels through the stimulation of adenylate cyclase activity (Ho et al., 1976; Donnelly, 1976). Although the exact nature of the interaction between Ca and cAMP is unclear at present, a dynamic balance between these two agents during the contraction-relaxation cycle is very likely to be closely controlled,

possibly through the mediation of calmodulin.

Elevated levels of cGMP appear to be associated with agents or treatments which induce muscle contraction. In the ductus deferens of the rat, cGMP levels were increased 2- to 3-fold by acetylcholine, carbachol and methylcholine or 125mM KCl. Atropine blocked the cholinergic effect on cGMP levels, suggesting the involvement of muscarinic receptors. No changes in cAMP levels were observed with these treatments. After incubation of the muscles in Ca-free saline, cGMP levels were reduced by about 85% and no longer responded to the cholinergic drugs or to high $[K]_e$ indicating that the elevation of cGMP levels was dependent on the influx of Ca (Schultz et al., 1973; Schultz, Hardman, Hurwitz & Sutherland, 1973). Similar results were obtained by Lee et al. (1972) in slices of the guinea pig ileum and heart. However, elevated cGMP levels are not always associated with contraction, since the treatment of the ductus deferens with a phosphodiesterase inhibitor elevated both cAMP and cGMP levels although the muscle relaxed (Schultz et al., 1973).

Elevated levels of cGMP are often associated with reduced levels of cAMP, and since in many cases the increased levels of these two nucleotides are associated with opposite responses, it has been proposed that they act as biological effectors with opposing actions (Goldberg et al., 1975; Rasmussen & Goodman, 1977). More recently, Fano et al. (1982) have observed opposite variations in the levels of cAMP and

cGMP during isometric tetanus of frog sartorius muscle, but could not ascribe a precise functional rôle to these variations. Also, it was not clear how the observed changes in Ca were related to changes in the cyclic nucleotide levels.

There is virtually no information on the relationship of cyclic nucleotides to the contraction-relaxation cycle in molluscan muscles, except for very preliminary work on the ABRM of *Mytilus edulis*. The application of 5-hydroxytryptamine (5-HT) to the ABRM results in an increase of Ca efflux (Nauss & Davies, 1966; Bloomquist & Curtis, 1972, 1975a, 1975b) which is accompanied by a relaxation of the muscle (Cole & Twarog, 1972). It has been suggested that 5-HT acts indirectly to alter Ca binding in the muscle cell, possibly through the mediation of cAMP. The application of dibutyryl cAMP produces relaxation of the ABRM as do the phosphodiesterase inhibitors theophylline and papaverine. Nicotinic acid, a stimulator of phosphodiesterase activity, decreases the extent of 5-HT-induced relaxation (Krishna et al., 1966; Cole & Twarog, 1972; Scratcherd, 1974). However, whether cAMP acts to increase the activity of the SR or sarcolemmal Ca pumps or whether it induces changes in Ca or ATP binding at the level of the contractile proteins (or both) is not clear at present. Also, direct measurements of cAMP levels have not been undertaken in molluscan muscle. Consequently, the results published to date are only suggestive insofar as they are consistent with the results obtained in vertebrate

systems, although even here the exact role of cyclic nucleotides in muscle contraction is not entirely clear.

e) *Calcium channels*

All cells which rely on extracellular Ca for the activation of specific cellular processes must possess a mechanism whereby the influx of Ca can be controlled. Although little is known about the coupling mechanism(s) between a stimulus (either chemical or electrical) and the activation (opening) of Ca channels, it is generally thought that Ca ions flow passively down their electrochemical concentration gradient through 'pores' or 'channels' which are more or less specific for this ion. Unlike the Na channel with its unique molecular structure which is highly conserved across many species, the Ca channel is characterized by a greater variety of types as evidenced by large differences in kinetics, pharmacological sensitivity and ion selectivity (Hagiwara, 1980). However, this is to be expected for a channel which must subserve such a wide range of physiological functions.

Current electrophysiological and pharmacological evidence favours the existence of at least two distinct types of Ca channels, viz., the 'potential-dependent' Ca channel (PDC channel) and the 'receptor-operated' Ca channel (ROC channel) (Bolton, 1979). As their names imply, the differences between these channels can be attributed to their modes of

activation. The PDC channel responds to changes in the membrane potential (V_m) where a depolarization of the membrane leads to an increase (or sometimes a decrease) in Ca permeability, whereas the ROC channel is activated by a specific chemical stimulant-receptor interaction which is independent of V_m .

The properties of ROC channels are not as well understood as those of the PDC channels. While it is generally agreed that ROC channel stimulants (e.g. norepinephrine (NE), 5-HT, histamine, etc.) produce their effects by first interacting with a specific receptor molecule, the evidence for this is inferential since no ROC channel receptor has yet been isolated or characterized. Moreover, there is some doubt as to the specificity of the ROC channel for Ca (cf. Rasmussen & Goodman, 1977, and Bolton, 1979). Most of the evidence for the existence of ROC channels rests on the differential actions of specific Ca channel 'agonists' and 'antagonists', the former mediating Ca_e -dependent activation of specific cellular functions which can be blocked by the latter (Bolton, 1979; Goodman, 1981).

i) Receptor-operated calcium channels

ROC channels have been implicated in the regulation of Ca -mediated functions in many cell types, some examples being blowfly salivary glands (Berridge et al., 1974, 1975), cockroach salivary glands (Ginsborg et al., 1980), rabbit lacrimal

gland cells (Botelho & Dartt, 1980), juxtaglomerular cells of the rat kidney (Fishman, 1976; Churchill, 1980), adrenocortical cells of the cat (Rubin, 1981) and canine vascular smooth muscle (Weiss, 1981). It is interesting to note that the granular juxtaglomerular cells of the kidney appear to be derived from vascular smooth muscle (Biava & West, 1966; Fishman, 1976), suggesting that ROC channels in secretory tissues and in smooth muscles may have similar properties.

The activation of ROC channels usually leads to a change in V_m which can be either a hyperpolarization (Fishman, 1976; Botelho & Dartt, 1980; Ginsborg et al., 1980), a biphasic response (Berridge et al., 1974) or a depolarization (Botelho & Dartt, 1980). However, these potential changes are a secondary response to a potential-independent influx of Ca and can be explained by changes in membrane permeability to ions other than Ca (Berridge et al., 1974; Bülbring & Tomita, 1977; Marier et al., 1978; Iwatsuki & Petersen, 1978).

Virtually nothing is known about the kinetics of ROC channels: their properties are very difficult to analyze because of a number of constraints (see review by Bolton, 1979). Moreover, it is not known if these channels inactivate in a Ca_i -dependent manner similar to PDC channels (see later), although, in some cases at least, it appears as though the ROC channels remain open as long as the 'activator' is bound

to the receptor site (Case, 1974; Berridge et al., 1974).

ii) *Potential-dependent calcium channels*

The PDC channels respond to changes in V_m whereby a depolarization of the membrane usually leads directly to an increase in Ca permeability. Generally, the activation of PDC channels leads to a transient inward Ca current where the influx of Ca is in sufficient quantity to be measurable with standard electrophysiological techniques. Virtually all excitable cells contain some form of PDC channels in the cell membrane. Because of practical considerations, the most extensively studied PDC channels are those of the giant axon of the squid and giant neurons of gastropod molluscs. The large size of these cells permits the use of techniques which would be difficult, if not impossible, to apply to other, smaller cells. However, even with amenable preparations and intensive investigation, the properties of PDC channels are largely unknown, though a considerable amount of information has accumulated regarding the kinetics, mode of activation and selectivity of these channels.

In the squid axon, depolarization-induced Ca entry appears to be made up of two components, the so-called 'early' and 'late' Ca currents. The early component is blocked by tetrodotoxin (TTX) and is believed to represent Ca entry through Na channels. The late component of Ca entry is insensitive to TTX or tetraethylammonium ions, but is

blocked by Mn, Co, Ni, La and the organic Ca entry blockers D-600 and iproveratril (Baker et al., 1971; Baker et al., 1973a). In contrast to the early Ca entry, which may be an influx through the Na channel, the late Ca entry appears to occur through channels which are pharmacologically distinct from either Na or K channels, i.e., they are probably specific Ca channels ('late' Ca channels) (Baker, 1972; Baker et al., 1973a).

One of the properties of the late Ca channel is that it appears to be capable of inactivation, although the rate of onset of inactivation is relatively slow in the squid axon (of the order of several seconds). The exposure of the squid axon to high $[K]_e$ ($>50\text{mM}$) results in an elevated $[Ca]_i$ which falls within about 5 min to a new steady level which is always greater than the control level. This fall in $[Ca]_i$ is probably due to inactivation of the late Ca channel (Baker et al., 1971; Baker et al., 1973b) and presumably to the activation of some process(es) to reduce the level of Ca_i . This inactivation was reported to be potential-dependent as prior depolarization of the squid axon reduced the Ca entry associated with subsequent depolarizations (Baker et al., 1973b). Potential-dependent Ca inactivation (PDCI) has also been reported for *Helix aspersa* and *Aplysia* spp. giant neurons (Standen, 1975; Kostyuk et al., 1977; Akaike et al., 1978; Adams & Gage, 1979).

Although earlier observations suggested that Ca channel

inactivation is a potential-dependent process, there is mounting evidence that this inactivation results from elevated levels of Ca_i and is independent of the transmembrane potential difference. Kostyuk & Krishtal (1977) found that the inward Ca current in *Helix pomatia* neurons is extremely sensitive to elevated levels of Ca_i . It was later proposed that this sensitivity represents inactivation of the Ca channel from the inner face of the cell membrane by Ca ions alone, i.e., that Ca channel inactivation results from an increase in $[Ca]_i$ during PDC channel activation (Eckert & Tillotson, 1978). Using the 'two pulse' technique of Hodgkin & Huxley (1952), Tillotson (1979, 1980) and Eckert & Tillotson (1978) found that Ca channel inactivation did not occur at very positive voltage steps (+160mV) where the Ca current (I_{Ca}) was small, but did occur at less positive voltages where a substantial I_{Ca} would be expected, suggesting that it is the entry of Ca, and not a potential change, which is responsible for Ca channel inactivation. Similar results were obtained for paramecium (Brehm & Eckert, 1978; Brehm et al., 1980), the skeletal muscle fibres of the stick insect (Ashcroft et al., 1979; Ashcroft & Stanfield, 1980) and the neurons of *Helix aspersa* (Plant & Standen, 1981). In support of the above hypothesis, Mullins & Requena (1981) found that squid axons depolarized with high K_e in the absence of Ca_e were still capable of producing a large phasic entry of Ca upon replacement of this ion, again

suggesting that Ca entry itself, rather than membrane depolarization, produces inactivation.

Although the evidence for Ca-dependent Ca inactivation (CDCI) is favourable, PDCI cannot be ruled out. Brown et al. (1981) examined the differences between Ba-induced and Ca-induced inactivation and concluded that both PDCI and CDCI occur in *Helix aspersa* neurons. This conclusion was largely based on the observation that Ba, which produces inactivation, inhibits CDCI, suggesting that Ba cannot replace Ca in CDCI, although it can replace Ca as a charge carrier during membrane depolarization. Consequently, the inactivation seen with Ba is likely to be voltage-dependent. Since Ba most likely utilizes the Ca channel, these results suggest that this channel is probably also capable of potential-dependent inactivation. However, there is also evidence that certain PDC channels appear to have no, or very slowly developed, inactivation. Blight (1980) reported that the stretch receptor/motoneuron synapse of the crab was capable of prolonged transmission under electrical depolarization, and concluded that the Ca conductance at the presynaptic terminal is non-inactivating.

4) THE PHARMACOLOGY OF CALCIUM ENTRY AND REGULATION

A) *Calcium entry blockers*

Many agents, including di- and trivalent cations and a range of organic compounds, can inhibit Ca-dependent processes with varying degrees of potency and specificity. The agents which will be considered here are the heavy metals Co, Ni and Mn, the rare earth La, and the organic compounds D-600 and sodium pentobarbital (NAPB). Many so-called 'Ca-antagonists' are believed to exert their Ca-blocking actions by somehow reducing or preventing the entry of Ca into the cell (the mechanism of this 'blocking' action is not known for any of the agents). For this reason it has been suggested that the term 'calcium entry blockers' be adopted to define this particular class of agents (Vanhoutte, 1981a).

a) *The heavy metals nickel, cobalt and manganese*

The heavy metals Ni, Co and Mn have been shown to inhibit Ca_e -dependent responses mediated by both PDC and ROC channels in many different tissues, including rat parotid glands (Marier et al., 1978; Putney et al., 1978; Putney, 1981), rat mast cells (Sullivan & Brown, 1981), squid axon (Baker et al., 1973a), barnacle muscle fibres (Hagiwara et al., 1969), mammalian heart (Sabatini-Smith & Holland, 1969; Kohlhardt et al., 1973), *Helix aspersa* neurons (Meech & Standen, 1975; Akaike et al., 1978), the radula protractor

of *Rapana thomasi* (Kobayashi, 1972a), *Mytilus edulis* ABRM (Twarog & Muneoka, 1972; Hagiwara & Nagai, 1970; Sugi & Yamaguchi, 1976), the musculature of *Schistosoma mansoni* (Fetterer et al., 1980) and the smooth muscle of the sea cucumber *Isostichopus badionotus* (Suzuki, 1982). It is generally agreed that the action of the heavy metals is to antagonize Ca entry at the level of the cell membrane. For example, Putney et al. (1978) reported that ^{45}Ca uptake by rat parotid glands was inhibited by Co, suggesting a cell membrane site for Co action. However, there is no direct evidence that the heavy metals do not penetrate into the cell interior, although there is one report suggesting that Mn, but not Ni or Co, can cross the cell membrane since it can replace the Ca current during action potentials in the muscle cells of the beetle *Xylotripes dichotomus* (Fukuda & Kawa, 1977).

The inhibition of Ca entry by the heavy metals appears to be competitive. Kohlhardt et al. (1973) found that while Ni, Co and Mn were able to block Ca entry in feline myocardial fibres, the transmembrane Ca conductivity could be partly restored by elevating $[\text{Ca}]_e$. The Ca-blocking action was attributed to a cell membrane locus, since depolarization-induced I_{Ca} was greatly reduced or abolished by Ni, whereas the Na current was unaffected, suggesting a relatively specific inhibition of late Ca entry. Similar results were obtained by Baker et al. (1973a) for the squid giant axon. The heavy metals blocked the late Ca current but not the Na

or K channels, although the early current was reduced by about 50%. However, Baker's group used a much higher concentration of the heavy metals than did Kohlhardt's group (25mM vs. 2mM), possibly explaining the different effects of these agents on the early currents of the squid axon and mammalian heart muscle.

b) *The rare earth lanthanum*

Lanthanum appears to have the same mechanism of action as the heavy metals, i.e., it blocks Ca entry through both PDC and ROC channels in a variety of tissues, although it is generally more potent (Weiss, 1974). In addition to its Ca influx blocking action, La has been reported to attenuate Ca efflux from mammalian smooth muscle (Deth, 1978; Freeman & Daniel, 1973; van Breemen & de Weer, 1970).

There is some controversy as to whether La can penetrate the cell membrane. Laszlo et al. (1952), using autoradiographic techniques, found La accumulations only at the cell membrane of mouse liver cells. On the basis of electron microscopic observations of La-treated muscles of *Anodonta imbecilis*, Kidokoro et al. (1974) reported accumulations of La at or near the surface membrane. Intracellular accumulations were not observed, but a diffuse intracellular distribution could not be ruled out. Similar observations of La accumulations were made by Garant (1972), Brightman & Reese (1969), Lane & Treherne (1972) and Payton et al. (1969).

Intracellular accumulations of La were not reported. Conversely, Hodgson et al. (1972) reported the uptake of ^{140}La by intracellular structures, particularly the mitochondria of mammalian smooth muscle. However, the muscle tissue was fractionated prior to the determination of La content in the various fractions, thus it was possible that a redistribution of La occurred during disruption of the tissue. Freeman & Daniel (1973), investigating the effect of La on Ca fluxes in rabbit uterine smooth muscle, suggested that La may slowly penetrate the cell and release Ca from intracellular sites, although they were not able to provide direct evidence for this. At the present time, there is no conclusive evidence as to whether or not La penetrates into cells.

c) D-600

The organic compound D-600 is a more effective Ca entry blocker than either the heavy metals or La (Fleckenstein & Grün, 1969). Unlike the inorganic cations which inhibit a wide range of Ca_e -dependent processes, D-600 appears to be more selective in that its blocking action, at low concentrations (see below), is restricted to PDC channels. Although this compound is not uniformly selective against all PDC channels (Vanhoutte, 1981b; Triggle, 1981), it has been shown to block potential-dependent Ca influx in many types of excitable tissues, e.g. mammalian heart (Fleckenstein,

1971; Kohlhardt et al., 1972), squid axon (Baker et al., 1973a), mammalian smooth muscle (Mayer et al., 1972; Triggle et al., 1979; Rosenburger et al., 1979; Goodman, 1981), barnacle muscle (Rojas & Luxoro, 1974; Lakshminarayanaiah, 1981), pancreatic islet and insulinoma cells (Fleischer et al., 1981) and juxtaglomerular cells (Churchill, 1980).

The limited available evidence supports the contention that the primary action of D-600 can be attributed to a selective blockage of PDC channels. Mayer et al. (1972) compared the actions of D-600 and La on Ca entry in K-depolarized mammalian smooth muscle. Both La and D-600 blocked K-induced contractures and reduced ⁴⁵Ca entry into the cells. However, La was found to be much more effective than D-600 in reducing Ca influx. It was proposed that while La blocks all Ca entry sites, D-600 blocks only those responsible for Ca entry during depolarization, i.e., PDC channels. Churchill (1980) reported that while D-600 blocked depolarization-induced inhibition of renin release by rat renal cortical slices, the Ca_e-dependent, potential-independent action of angiotensin II was unaffected. Schumann et al. (1975) reported that the concentration of D-600 required to inhibit the Ca_e-dependent K-induced contractile response in vascular smooth muscle was several orders of magnitude lower than that required to inhibit the NE-induced contractile response. The response to NE is presumably mediated by Ca_e-dependent ROC channels whereas the K-induced response utilizes PDC channels.

Although the above evidence favours a PDC channel-selective action for D-600, it is by no means conclusive. Apart from its effect on Ca channels, this compound has been shown to affect other types of ionic channels, especially at relatively high concentrations ($>0.1\text{mM}$). Whereas Fleckenstein (1971) and Kohlhardt et al. (1972) reported that D-600, at a concentration of about 10^{-6}M , inhibited the Ca current in mammalian cardiac tissue but had no effect on the early Na current, Baker et al. (1973a) reported that in addition to a blockage of late I_{Ca} , $2 \times 10^{-4}\text{M}$ D-600 also substantially attenuated the early current. Similar results were obtained by Van der Kloot & Kita (1975) who found that verapamil, a demethylated analogue of D-600, blocked both inward Na and outward K currents in frog muscle. Bregestovski & Iljin (1979) reported that 10^{-4}M to 10^{-5}M D-600 blocks the ACh-induced Cl current in the neurons of *Lymnaea stagnalis* as well as the inward Ca and Na currents. It should, however, be noted that the optical isomers of D-600 have different actions. The (-) isomer appears to be more specific in its action against PDC channels, whereas the (+) isomer blocks primarily the Na channel (Bayer et al., 1975; Ehara & Kaufmann, 1978). Since a specific isomer was not specified, most of the above results were probably obtained with a racemic mixture of D-600. This may partly explain the observed lack of specificity of this compound.

Neither the site nor the mechanism of action of D-600

is known, although the current evidence is consistent with a cell membrane locus of action. D-600 is ineffective in skinned smooth and myocardial muscle cell preparations (Weder & Grün, 1973; Fleckenstein, 1977; Fleckenstein et al., 1976) and blocks Ca movements in mitochondria and sarcoplasmic reticulum only at very high concentrations (Entman et al., 1972; Frey & Janke, 1975; Crankshaw et al., 1977; Thorens & Haeusler, 1979). Furthermore, in contrast to external application, intracellular D-600 does not inhibit Ca currents in barnacle muscle fibres (Rojas & Luxoro, 1974).

Payet et al. (1980) compared the time courses of action of D-600 and Mn on ~~Ca~~ blockage in rat myocardium and found that the time constant for D-600 was larger than that for Mn. Partly on this basis, it was suggested that the site of action for Mn is at the surface of the membrane whereas D-600 must pass into or through the membrane to exert its effect, probably at a site inside the membrane itself or at its inner face. This view is supported by the pH-dependence of D-600 action whereby the non-ionized form (at pH 10) is more effective than the ionized form (at pH 7.2) in blocking Ca entry, probably due to a facilitated penetration of the non-ionized form of this compound into the membrane (Payet et al., 1980).

It is, therefore, not likely that the action of D-600 is similar to that of the inorganic cations which function as non-selective Ca channel blockers and affect a greater variety of Ca-dependent processes. This non-selectivity is probably

due to the ability of inorganic cations to substitute for Ca at Ca binding sites with greater affinity than Ca, thereby blocking channel function. The relative selectivity of D-600 argues against a similar mechanism of action. Moreover, there is evidence that the blocking action of D-600 is potentiated by membrane depolarization and frequency of stimulation (Ehara & Kaufmann, 1978; McDonald et al., 1980). Also, Kohlhardt & Mnich (1978) found that D-600 can inhibit Ca channel recovery from inactivation. These results suggest that D-600 may act by modifying the voltage-dependent activation and/or inactivation parameters of the Ca channel (Weiss, 1981).

In contrast, Church & Zsotér (1980) proposed that verapamil acts intracellularly, i.e., at the inner surface of the membrane and possibly at deeper structures as well. This contention was based on the observed increase in Ca efflux from myocardial and smooth muscles by verapamil treatment. Although the origin of this Ca was not unequivocally established, it was reported that verapamil decreased the contractile response to NE, an agent whose action is probably mediated by Ca_i .

d) *Sodium pentobarbital*

The local anaesthetic sodium pentobarbital (NAPB) and related compounds have been reported to inhibit Ca currents in rat brain synaptosomes (Blaustein & Ector, 1975), lobster axon

(Blaustein & Goldman, 1966), vertebrate smooth and striated muscle (Feinstein, 1963, 1966; Nayler & Szeto, 1972; Altura & Altura, 1981) and feline adrenal glands (Rubin et al., 1967). Blaustein & Ector (1975) suggested that NAPB (4×10^{-4} M) acts primarily to inhibit PDC channels in synaptosomes, as the Ca entry induced by elevating intracellular and reducing extracellular Na was only slightly reduced (by about 18%), whereas the K-depolarization-induced Ca entry was reduced by about 50%. At higher concentrations (6×10^{-3} M), NAPB blocked both Na and K conductance in lobster axons (Blaustein, 1968).

The local anaesthetic procaine has been shown to block ROC channels in addition to PDC channels and K and Na currents (Fleisch & Titus, 1973; Adams, 1977; Ito et al., 1977; Bolton, 1979) and probably has an intracellular action as well (Feinstein, 1963; Seeman, 1972). Volatile anaesthetics, which readily penetrate the cell, interfere with mitochondrial Ca uptake (Lee et al., 1979a) and depress rat heart sarcolemmal Ca binding (Lee et al., 1979b). Whether or not NAPB has similar actions to procaine and the volatile anaesthetics has not been investigated in every case, although NAPB has been shown to inhibit Ca uptake by the SR of cardiac muscle (Lain et al., 1968; Nayler & Szeto, 1972) and inhibit mitochondrial respiration (Bunker & Vandam, 1965). In contrast, Dransfeld et al. (1969), reported that the Ca uptake by microsomes prepared from vertebrate skeletal and cardiac muscle

was not affected by NAPB, but Nayler & Szeto (1972) suggested that this negative result may have been due to the presence of oxalate in the incubation medium.

These studies were done on broken cell preparations, and relatively high concentrations of NAPB were required to produce the observed effects. Consequently, it is not known whether or not NAPB has similar actions in intact cells, nor is it known whether this anaesthetic can penetrate the cell membrane. Accordingly, it has been suggested that low concentrations of NAPB (<1mM) are relatively specific in blocking Ca currents, at least in some preparations (Blaustein & Ector, 1975).

B) *Other agents which affect cellular calcium distribution:*
A23187, caffeine and praziquantel

a) A23187

The divalent cation ionophore A23187 can activate a variety of Ca_e -dependent processes. This ionophore has been shown to stimulate amylase release by mouse pancreatic acinar cells (Ponnappa & Williams, 1980), mitogenesis in human lymphocytes (Jensen et al., 1977), K release from the rat parotid gland and enzyme secretion from the rat pancreas (Selinger et al., 1974), an increase in Ca permeability in human red blood cell membranes (Lew & Simonsen, 1980), contraction in vertebrate and invertebrate muscle (Entman et al., 1972; Swamy et al., 1975; Murray et al., 1975; Triggle

et al., 1975; Desmedt & Hainaut, 1976; Rosenberger & Triggle, 1979; Hill, 1980); Ca release from isolated SR (Scarpa et al., 1972) and H-Ca exchange in isolated mitochondria (Reed & Lardy, 1972). It is thought that A23187 acts to bypass normal physiological processes, which regulate Ca permeability, by translocating Ca_e into the cell interior via a carrier-type mechanism (Szabo, 1981) that is an electroneutral exchange process (Rosenberger & Triggle, 1978).

However, the action of this ionophore does not appear to be confined to a cell membrane locus. Certain events which are independent of Ca_e (i.e., dependent on Ca_i) can be induced by A23187 (Ponnappa & Williams, 1980; Karl et al., 1975; Ashby & Speake, 1975; Schroeder & Strickland, 1974), or Ca_e -dependent events can be triggered by this ionophore in the absence of Ca_e (Murray et al., 1975; Desmedt & Hainaut, 1976; Feinstein & Fraser, 1975), suggesting that A23187 can penetrate into the cell and release Ca_i stores.

b) Caffeine

The methylxanthine caffeine has been reported to induce contraction in many vertebrate and invertebrate muscle types, including barnacle muscle fibres (Ashley et al., 1972), the longitudinal retractor muscle of the sea cucumber (Sugi et al., 1982), the ABRM of *Mytilus edulis* (Muneoka & Mizonishi, 1969), mammalian smooth and striated muscle (Feinstein, 1963; Thorpe, 1973; Jundt et al., 1975; Itoh et al., 1981, 1982;

Niedergerke & Page, 1981; Coraboeuf et al., 1981) and insect skeletal muscle (Huddart, 1969; Huddart & Syson, 1975). In contrast, caffeine has also been reported to induce relaxation in some mammalian smooth muscle types (McFarland & Pfaffman, 1972; Sakai & Iizuka, 1972; Huddart & Syson, 1975), an effect attributed to an enhancement of intracellular Ca binding (Huddart & Syson, 1975).

The action of caffeine is largely independent of Ca_e (Frank, 1960; Itoh et al., 1981, 1982; Jundt et al., 1975) and can be evoked by intracellular injection (Caldwell, 1964). Caffeine contracture can also be produced in some muscle types during prolonged depolarization with high $[K]_e$ (Axelsson & Thesleff, 1958; Constantin, 1975). The effect of caffeine on the membrane potential appears to depend on the preparation. For example, Coraboeuf et al. (1981) reported that caffeine induces strong contractions in canine Perkinje fibres without altering V_m . However, Kuba (1980) found that caffeine induces a hyperpolarization of the membrane of a bullfrog sympathetic neuron, an effect which was attributed to a Ca-induced increase in K conductance (cf. Meech & Standen, 1975), whereas Itoh et al. (1982) showed that caffeine induces a slight (about 5mV) depolarization of the porcine smooth muscle membrane at high concentrations ($>10mM$) and a slight hyperpolarization (about 3mV) at concentrations below $1mM$. Both of these changes were associated with a decrease in the membrane resistance, and it was suggested that

the hyperpolarization is due to an increase in K conductance whereas the depolarization at higher concentrations of caffeine may be due to an increase in Na conductance.

Weber (1968) and Weber & Herz (1968) reported that caffeine stimulates the release of sufficient quantities of Ca from the SR to account for the force of contracture in intact frog muscle. These results have since been duplicated for several muscle types (Thorpe, 1973; Batra, 1974; Huddart & Syson, 1975). Furthermore, caffeine can induce contractions in 'skinned' muscle preparations (Fabiato & Fabiato, 1972; Miller & Thieleczek, 1977; Itoh et al., 1981, 1982) and has been shown to inhibit the uptake of Ca by the isolated SR of insect skeletal muscle (Thorpe, 1973; Huddart & Syson, 1975). The fraction of Ca released from the SR reportedly ranges from 27% (Thorpe, 1973, 20mM caffeine) to almost 100% (Endo, 1977, 25mM caffeine).

Whether or not caffeine releases mitochondrial Ca stores is not entirely clear, and may depend on the type of tissue. Huddart & Syson (1975) found that although caffeine did not affect Ca binding in rat smooth muscle mitochondria, it blocked Ca uptake by cockroach skeletal muscle mitochondria. Weber (1968) showed that the amount of Ca released by caffeine in frog muscle was not reduced by prior depletion of mitochondrial Ca stores with Dicumarol, suggesting that the mitochondria are not a locus of caffeine action. Whether or not mitochondrial Ca is involved to some degree in the

action of this agent, there is little doubt that: a) caffeine releases bound Ca from the SR, and, b) caffeine has an intracellular action in intact preparations, i.e., it readily penetrates the cell membrane.

However, other notable actions have been proposed for this methylxanthine. Niedergerke & Page (1981) suggested that caffeine also increases the Ca permeability of the sarcolemma, as the magnitude of caffeine-induced contractures in the atrial muscle of the frog heart was proportional to $[Ca]_e$. In the same preparation, Kimoto et al. (1974) measured a caffeine-induced increase in the inward Ca current. Conversely, Eisner et al. (1979) reported a caffeine-induced reduction of inward I_{Ca} in sheep Perkinje fibres, and Huddart & Syson (1975) observed a caffeine-induced reduction of Ca efflux from rat ileal smooth muscle. Therefore, it would appear that the nature of caffeine action is dependent on the type of tissue. This suggestion is strengthened by the report that the atrium of the frog heart is considerably more sensitive to this methylxanthine than the ventricle, although there are no obvious differences in either the appearance or quantity of their SR (Niedergerke & Page, 1981).

In some mammalian smooth muscles, caffeine has been shown to relax K-depolarization-induced contractures. Theobald et al. (1978) reported a 200% increase in the levels of cAMP following caffeine treatment of bovine ileal smooth muscle and suggested that such an increase may be responsible for stimu-

lating the uptake of Ca by intracellular organelles. Although this interpretation is speculative, there is evidence that elevated levels of cAMP are involved in the relaxation of smooth muscle (see above). There is also evidence that the Na-Ca exchange mechanism may be stimulated directly or indirectly by caffeine. Jundt et al. (1975) reported that in the presence of extracellular Na (Na_e) caffeine increased the efflux of Ca from guinea pig auricles but did not produce a contracture. In the absence of Na_e , caffeine produced a contracture which was relaxed by the restoration of Na_e . Thus, there are potentially two mechanisms by which Ca_i can be reduced following treatment of mammalian smooth muscles with caffeine, i.e., a cAMP-mediated increase in SR Ca pump activity and an increase in the rate of the Na-Ca exchange process. In other muscle types the magnitude and duration of caffeine-induced contractures would presumably depend on the balance between a possible caffeine-induced influx of Ca through the sarcolemma and release from the SR, and the capacity of Ca sequestering or extrusion processes. The degree of efficiency of the latter (or the degree to which caffeine can stimulate these processes) may partly account for the different actions of caffeine in various tissue types.

c) *Praziquantel*

The relatively new anthelmintic compound praziquantel (PQ) is used for the treatment of schistosomiasis (Fetterer et al., 1980). This compound has been reported to induce contractures in adult schistosome body wall musculature (Pax et al., 1978) and guinea pig ileal muscle (Jim & Triggle, 1979), and to increase the contractile force of isolated rat atria (Chubb et al., 1978). The action of PQ on mammalian muscles is blocked by verapamil and D-600, is not blocked by adrenergic or cholinergic receptor antagonists, and appears to be dependent on Ca_e (Chubb et al., 1978; Jim & Triggle, 1979). PQ does not act as an ionophore since it does not translocate Ca from an aqueous into an organic phase (Chubb et al., 1978), and it probably doesn't affect Na channels as its action on the ileal smooth muscle is insensitive to TTX (Jim & Triggle, 1979). Accordingly, it has been proposed that at least one of the actions of PQ may be an alteration of Ca channel function.

However, the action of this compound on schistosome musculature differs from that on rat atrial muscle. In the schistosome muscle, D-600 does not block the response to PQ although La , and to a lesser extent Co , partially attenuate it. Moreover, the response of this muscle to PQ is a sustained contracture which is reportedly dependent on Ca_e (Fetterer et al., 1980), although more recent evidence suggests that Ca_i stores may also be involved during PQ action, albeit to a

lesser extent than Ca_e stores (Wolde Mussie et al., 1982). The muscle cell membrane is only slightly depolarized by PQ, and only after 6-8 min of exposure. On the basis of these results, it was suggested that PQ acts on schistosome musculature by altering Ca permeability of the sarcolemma by a mechanism other than the alteration of PDC channel function (Fetterer, Pax & Bennett, 1980). However, this action appears to be non-specific since PQ can also raise the intracellular level of Na and inhibit the uptake of K (Pax et al., 1978).

5) THE IONIC BASIS OF RESTING AND ACTION POTENTIALS IN MOLLUSCAN MUSCLES

A) The resting potential

The resting potential (V_r) of molluscan muscles studied thus far appears to be a function of $[K]_e$ to a greater or lesser extent. Hill et al. (1970), applying the sucrose gap technique to the radula protractor of *Busycon canaliculatum*, reported an average V_r of -55mV. An increase in $[K]_e$ resulted in a depolarization of the membrane, whereas a decrease promoted a hyperpolarization. However, the quantitative relationship of $[K]_e$ to V_m was not reported. Burnstock et al. (1967) reported a V_r of -60mV for *Poneroplax albida* rectal muscle and -38mV for the posterior jugalis muscle (sucrose gap), although they considered that the latter value

may have been artificially low. The superfusion of the rectal muscle with 'isotonic' KCl resulted in a 44mV depolarization of V_m . Potassium concentrations were not reported.

Kater et al. (1971) recorded the V_r 's of the posterior jugalis and columellar muscles of *Heliosoma trivolvis* intracellularly. These averaged -40mV and -27mV, respectively, although the V_r of the columellar muscles was occasionally found to be as high as -40mV, suggesting the possibility of membrane damage resulting from impalement with microelectrodes. Kidokoro et al. (1974), recording intracellularly, reported a V_r of -42mV for the adductor muscle of *Anodonta imbecilis*. Florey & Kriebel (1969) measured a V_r of -20mV to -50mV for the chromatophore muscles of *Loligo opalescens*, and in the radula protractor muscle of *Rapana thomasiana*, the V_r was found to be -45mV (Kobayashi & Shigenaka, 1978). The ionic basis of these V_r 's was not investigated.

Twarog (1967) reported an average V_r of -65mV for the ABRM of *Mytilus edulis*. The V_m was depolarized by 45mV for a 10-fold (decade) increase of $[K]_e$. Shigeto (1970), recording intracellularly from *Mytilus edulis* and *Crassostrea gigas* myocardia, reported a V_r of about -45mV for both species. The V_m was altered by about 30mV per decade change in $[K]_e$ when $[K]_e$ was between 30mM and 186mM. However, no change in V_r was observed when $[K]_e$ was between 1mM and 18mM. When Cl ions were replaced with sulphate ions, a gradual depolarization ensued. Whether this depolarization was due to a genuine

contribution of Cl ions to V_r or to some effect of sulphate on membrane properties was not ascertained. If Cl contributes to V_r in these muscles, it would be expected that alterations in the extracellular Cl concentration ($[Cl]_e$) would result in more rapid changes in V_m , although Cl trapped in interstitial spaces could mask this response. A similar response of V_m to $[K]_e$ has been reported for the obliquely striated muscles of *Ascaris lumbricoides* (Brading & Caldwell, 1964; del Castillo et al., 1964a, 1964b). In this muscle, a decade increase in $[K]_e$ over a range of 1-40mM results in only a few mV change in V_m . If $[K]_e$ is increased above 45mM, V_m begins to depolarize. If $[Na]_e$ is reduced or removed, V_m responds with a 42mV change per decade change in $[K]_e$ over a range of 3-35mM. If Cl_e is removed as well, V_m changes by 50mV per decade change in $[K]_e$, suggesting that Na and Cl permeabilities contribute significantly to V_r . Although there is no substantiating evidence, it is possible that a similar situation may exist in some molluscan muscles.

The reported V_r 's of molluscan muscles are low compared to vertebrate skeletal muscles (cf. -92mV for frog sartorius muscle; Adrian, 1956). This could either reflect damage of the sarcolemma during impalement, or a genuine phenomenon. In many cases V_r declined rapidly following impalement (e.g. Kidokoro et al., 1974), which would suggest membrane damage. The values for V_r 's determined by the sucrose gap technique, on the other hand, incorporate other errors. For

example, Sugi & Yamaguchi (1976), using an oil gap voltage clamp, reported a V_r of -12mV to -25mV for the ABRM of *Mytilus edulis*, whereas Twarog (1976), recording intracellularly, reported a V_r of -65mV for the same preparation. However, the V_r 's of different invertebrates, e.g. *Ascaris lumbricoides*, may also be relatively low (-30mV to -40mV, Toida et al., 1975). The apparently low V_r 's of some molluscan muscles, therefore, does not represent a unique situation. Moreover, the equilibrium potential of K (E_K) may be comparatively low in molluscan muscles. For example, the V_r of myocardial muscle fibres of *Crassostrea gigas* has been reported to be about -45mV, whereas E_K was calculated to be -68mV (Shigeto, 1970), which is considerably less negative than the E_K of about -100mV in vertebrate skeletal muscles (Adrian, 1956; Hodgkin & Horowicz, 1959).

B) The action potential

Some molluscan muscles exhibit action potentials (Burnstock et al., 1967; Twarog, 1967; Florey & Kriebel, 1969; Hill et al., 1970; Shigeto, 1970; Kater et al., 1971; Kobayashi, 1972; Kidokoro et al., 1974) while others do not (Cohen et al., 1978). In many cases, the action potentials have been reported to be of relatively long duration. Twarog (1967) observed action potentials (AP's) of about 100 msec in duration in the ABRM of *Mytilus edulis*. Florey & Kriebel (1969) recorded AP's of up to 200 msec in duration from the

chromatophore muscles of *Loligo opalescens*. However, they also recorded AP's of much shorter duration and larger amplitude, suggesting that the longer AP's may have been artifacts, possibly resulting from damage to the muscle cell membrane by the microelectrodes.

The AP's of molluscan muscles appear to be dependent on Ca and, in some cases, possibly Na as well. Shigeto (1970) found that the myocardia of *Crassostrea gigas* and *Mytilus edulis* are spontaneously active in Na-free solution, suggesting that some ion other than Na may be responsible for the inward current during an AP. Florey & Kriebel (1969) reported that TTX did not abolish the AP's of *Loligo opalescens* chromatophore muscles and concluded that the spike potentials are not accompanied by an appreciable Na current. Twarog (1967) found that TTX also has no effect on the AP of *Mytilus edulis* ABRM, again suggesting the lack of involvement of Na. However, Kobayashi (1972) reported that TTX does not affect the Na current in the radula protractor muscle of *Rapana thomasi*. Therefore, the lack of a TTX action on different molluscan muscles may not necessarily reflect a lack of involvement of Na during the AP. Furthermore, Sugi & Yamaguchi (1976) found that the ABRM of *Mytilus edulis* is capable of producing repetitive AP's in the complete absence of Ca_e , suggesting that Na may well be involved in the generation of AP's in this muscle, even though TTX has no effect in this preparation (Twarog, 1976).

Kobayashi (1972) reported that the complete removal of Na_e also did not abolish the generation of AP's by the radula protractor muscles of *Rapana thomasi*. However, in the absence of Ca_e and presence of Na_e , the membrane became depolarized and transient spontaneous AP activity ensued. After the spontaneous activity ceased, the muscle membrane responded to brief depolarizing currents with a prolonged depolarization (ca. 20 min in duration). The subsequent removal of Na resulted in the complete abolition of the prolonged depolarization, suggesting its dependence on Na_e . The rate of rise and the amplitude of the AP, however, were found to be dependent on Ca_e . Moreover, AP's produced in solutions containing both Ca and Na were abolished by 10mM Mn, but Mn did not inhibit the prolonged Na depolarization under Ca-free conditions. These data suggest that under normal ionic conditions, Ca is the major charge carrier, whereas in the absence of Ca_e , the Na becomes the major charge carrier.

Kidokoro et al. (1974) reported that the overshoot of the AP in the adductor muscle of *Anodonta imbecilis* is not altered by the complete removal of Na_e . The overshoot, however, is reduced by decreasing $[\text{Ca}]_e$ and is abolished by the addition of 1mM La. These results suggest that the AP of this muscle is wholly dependent on Ca. However, because of the difficulty of maintaining satisfactory impalements with microelectrodes, quantitative relationships between the overshoot potential and $[\text{Ca}]_e$ could not be obtained.

6) INTRODUCTION TO THE EXPERIMENTS

The purpose of the investigations reported herein was to examine the mechanism of the direct contraction-inducing effect of the molluscicide Frescon on snail musculature. The approach was three-fold, consisting of the following:

- 1) an investigation of the effect of Frescon on the transmembrane potential difference in *Lymnaea stagnalis* heart ventricle muscle cells, utilizing intracellular recording techniques,
- 2) an analysis of the actions of Ca entry blockers on Frescon-induced contractions, and,
- 3) a comparison of the action of Frescon with the actions of other agents which are thought to interfere with cellular processes or constituents associated with the regulation of cellular Ca.

MATERIALS AND METHODS

1) THE EXPERIMENTAL ANIMALSA) *Lymnaea stagnalis*

Two subspecies of the freshwater snail *Lymnaea stagnalis* were used to investigate the mode of action of the molluscicide Frescon on snail muscles. *L. stagnalis appressa* were collected locally while *L. stagnalis stagnalis* were imported from England (Griffin & George Ltd., Gerrard Biological Centre, East Preston, West Sussex). The animals were kept and bred in large aquaria containing standing, aerated water, and were fed on lettuce leaves *ad libitum*. During the course of the experiments no intersubspecific physiological differences were observed, therefore the two species were used interchangeably.

The choice of experimental animal was based on the following considerations:

- 1) *Lymnaea* is highly susceptible to low concentrations of Frescon, succumbing to a 10^{-5} g/ml suspension within 1 h (Brezden & Gardner, 1980a).
- 2) Although *Lymnaea* is not host to the species of parasite which causes human schistosomiasis, it is host to a variety of less pathogenic species. For instance, *Schistosomatium douthitti* and *Trichobilharzia ocellata* which cause dermatitis in humans (Swimmer's Itch), have been found in *L. stagnalis appressa*. Although human infection by these parasites

is generally not of serious consequence, *T. ocellata* causes the equivalent of schistosomiasis in ducks (Bourns, 1961; Bourns et al., 1973).

- 3) *L. stagnalis appressa*, being indigenous to Ontario, are easily collectable during the summer months and can be maintained in large numbers with a minimum of effort. During the winter they can usually be obtained from England, thus ensuring a constant supply.

B) *Helix aspersa*

The terrestrial snail *Helix aspersa* was used for comparative experiments related to the action of Frescon. In contrast to *Lymnaea*, *Helix* is not affected by direct exposure to Frescon (Gardner & Moreton, 1978). Therefore, it was of interest to examine the effect of this molluscicide on its isolated muscles.

H. aspersa were obtained from Griffin & George Ltd. and kept in a ventilated plexiglas box wherein they remained in a state of estivation. Before an experiment, estivation was terminated by placing a snail into a shallow pool of warm water for a few minutes.

2) THE PREPARATIONS

The experimental preparations for both *Helix* and *Lymnaea* were the penis retractor muscle (PRM) and the heart

ventricle (HV). In addition, several experiments were performed on strips of *Lymnaea* foot (LFS). These muscles represent two distinct morphological types. The HV muscles are cross-striated whereas the PRM and the muscles of the foot are smooth (Plesch, 1977).

Dissection procedures for both *Lymnaea* and *Helix* were similar (see below). Following isolation, the tissues were immediately transferred to their respective bath chambers (Section 3-C). All experiments were performed at room temperature.

A) *The penis retractor muscle*

Several PRM's originate from the columellar muscles of the body wall and attach to the posterior and lateral portions of the penial complex. The posteriormost muscle is the largest and for this reason it was used exclusively. The shell of an unanaesthetized snail was removed with a pair of scissors and the snail was pinned through the dorso-anterior part of the foot to a Sylgard substrate (Sylgard 184 Silicone Elastomer, Dow Corning Corp., Midland, Michigan). The mantle was pinned back and a midline incision along the length of the body wall exposed the internal organs. The two halves of the body wall were pinned aside and the ovotestis overlying the PRM's was cleared away (Fig. 1). At the posterior end, part of the body wall was removed with the PRM to facilitate handling and mounting in the bath. The other end of the muscle was cut at

the point of attachment to the penial complex.

B) *The heart ventricle*

a) *The whole heart preparation*

The heart of an unanaesthetized, de-shelled snail was exposed by an incision through the integument just behind the mantle on the left side of the body wall. The thin connective

Figure 1. A diagrammatic representation of the dissection procedure for the penis retractor muscle.

- A. The de-shelled snail. M, mantle
- B. The exposed internal organs. M, mouth; O, ovotestis; PC, penial complex
- C. Close-up of B. I, line of incision; PC, penial complex; PRM, penis retractor muscle
- D. The isolated PRM. BF, body wall fragment

tissue sheath covering the aorta was cleared away and the distal aorta was cut leaving as long a length as possible. The proximal aorta was cut close to its point of entry below the gut. The cut end of the distal aorta was grasped with a pair of forceps and, by cutting away the remaining connective tissue, the heart was lifted free of the snail (Fig. 2). A cut through the proximal portion of the atrium completely freed the preparation.

Figure 2. A diagrammatic representation of the dissection procedure for the isolated heart preparation.

- A. The snail pinned down with the left side facing up. I, line of incision
- B. The exposed internal organs. AT, atrium; DA, distal aorta; F, foot; G, gut; PA, proximal aorta; V, ventricle
- C. The isolated heart

b) *Other ventricle muscle preparations*

i) *The open ventricle preparation*

When the transmembrane potential difference (V_m) of *Lymnaea* ventricle-muscle cells was to be measured, an 'open ventricle' preparation was used. The atrium and the aorta of an isolated heart were pinned to a small Sylgard mounting block, thereby immobilizing the preparation. The ventricle was opened by cutting along its long axis with a pair of fine scissors. After cutting away the atrium and the aorta, the ventricle was spread apart and its edges were pinned to a small Sylgard mounting block with miniature tungsten staples, exposing the inner face (see Fig.. 6).

-ii) *The ventricle strip preparation*

When V_m and muscle tension were to be measured simultaneously, an open ventricle preparation was prepared as described above. Under a dissecting microscope, the part of the ventricle having the largest proportion of parallel-oriented trabeculae was selected and cut out with a razor blade fragment, yielding a strip about 1.5mm X 3.5mm.

c) *The foot muscle strip*

The foot was cut free from an unanaesthetized, de-shelled snail (*Lymnaea* only) and placed dorsal side up in a shallow pool of saline in a plastic petri dish. A thin (about

1.5mm) longitudinal strip was cut from the centre-most portion of the foot with a razor blade. This portion of the foot has the highest concentration of muscle cells (Plesch et al., 1975). The integument on the ventral side of the strip was cut away.

3) THE APPARATUS

A) *Intracellular microelectrode techniques*

The V_m of *Lymnaea* heart muscle cells was measured using intracellular glass microelectrodes. Micropipettes were pulled from Omega Dot capillary tubing (#1011, Glass Co. of America, Bargaaintown, New Jersey) on a Narashige (Narashige Scientific Instrument Laboratory, Tokyo, Japan) or Associated Scientific (model M1, Industrial Science Associates, Inc., Ridgewood, New Jersey) micropipette puller. The tips were filled with 3M KCl by capillary action and the shafts were backfilled by injecting 3M KCl through a 31 gauge syringe needle. The microelectrodes had a tip resistance of 10-40 M Ω , the lower resistances being characteristic of the micropipettes pulled on the Narashige instrument.

A microelectrode was secured in a holder (model EH-2R, WPI Instruments Co., New Haven, Connecticut) containing a 3M KCl salt bridge and coupled to the input probe of a WPI model KS 700 amplifier via a chlorided silver (Ag/AgCl) electrode,

the latter being used to minimize junction potentials and random electrical fluctuations at the saline/electrode interface (Geddes, 1972). Silver wire electrodes were chlorided by passing 50 μ A of current per electrode for 12 h through a 0.1M KCl solution with a silver wire coil at the cathode. The plated electrodes were stored dry.

The input probe of the KS 700 amplifier had an input resistance of $10^{11} \Omega$, and the leakage current could be adjusted to zero. The output of the amplifier was fed to a Linear Instruments model 395 chart recorder (Linear Instruments Corp., Irvine, California) and a Tektronix model 5113 oscilloscope (Tektronix, Inc., Beaverton, Oregon). A block diagram of the recording apparatus is presented in Fig. 3.

The reference electrode consisted of a 2% agar/3M KCl salt bridge (made from a pasteur pipette) fixed in a plexiglas holder containing 3M KCl in contact with an Ag/AgCl electrode, the latter being at ground potential. The two recording poles were symmetrical with respect to ionic composition and electrode/fluid interface, minimizing currents due to differences in junction potentials between the poles. Junction potentials in this system (including the microelectrodes) rarely exceeded 5mV and were usually of the order of 2mV.

Microelectrodes were positioned with a Brinkman model CP-V micromanipulator (Brinkman Instruments, Rexdale, Ontario). In addition, the preparation bath X-Y positions could be adjusted by means of a translation stage (model XYT-25, Jodon

Figure 3. A block diagram of the recording and associated apparatus.

B: Bath	OS: Offset circuit
C: Clamps	P: Preparation
CR: Chart recorder	RE: Reference electrode
CRO: Cathode ray oscilloscope	S: Stimulator
FA: Force transducer amplifier	SE: Stimulating electrode
FC: Faraday cage	SL: Superfusion line (from 9-way valve)
FT: Force transducer	ST: Superfusion line (for toxic compounds)
HP: High input impedance probe	SIU: Stimulus isolation unit
M: Microelectrode	SR: Stimulating electrode (reference pole)
MA: Microelectrode amplifier	TMG: Time mark generator

This arrangement was used when both muscle tension and membrane potential were measured simultaneously (HV strip). When only muscle tension was measured (e.g. PRM), the components M, HP, MA and RE were not used.

Engineering Associates, Inc., Ann Arbor, Michigan). The micromanipulator, input probe and preparation bath were placed in a grounded Faraday cage to minimize ambient electrical interference. The bottom of the cage consisted of two 1.3cm thick plates (60cm X 90cm and 47cm X 75cm) set on five tennis balls (Tretorn pressureless) to reduce the transmission of mechanical vibrations to the preparations. The Faraday cage-enclosed components of the recording apparatus are illustrated in Fig. 4.

B) Muscle tension measurements

Muscle tension was measured with a Gould Statham UC-2 semi-isometric force transducer (Gould, Inc., Measurement Systems Division, Oxnard, California). The mechanical coupling of the preparations to the transducer is described in the next section. An offset circuit and a high-gain DC amplifier were designed and built to condition the signal from the force transducer (see Appendix). The output of the amplifier was fed to the chart recorder and oscilloscope. A time mark generator was designed to provide pulses at 1 or 10 sec to one of the chart recorder channels. The amplifier gain was adjusted to provide a 3mV output per mg of muscle tension. Calibration of the transducer/amplifier arrangement revealed a very linear response within the range of tension measurements encountered in these experiments (see Appendix).

2

C) The baths and preparation mounting procedures

a) The perfusion bath: isolated heart preparation

Experimental solutions were introduced to the isolated heart preparations via a multi-reservoir perfusion system. The perfusion bath (Fig. 4) was made of plexiglas throughout except for a glass cover at the face which facilitated lateral observation of the preparation through a binocular microscope. The aortal clamp (transducer clamp) was constructed of plexiglas and was attached to the force transducer with a stiff 'S-shaped' nichrome wire sheathed in polyethylene. An atrial clamp was attached to the left side of the bath and a length of 27 gauge stainless steel tubing (perfusion tube) was positioned between its jaws, protruding about 1mm past the face. The other end of the tubing extended through the left wall of the chamber to accomodate PE 20 polyethylene tubing (Clay-Adams, Persippany, New Jersey). Stimulating pulses were introduced to the preparations via the perfusion tube and were referenced to a silver wire fixed to the right side of the bath. Stimulus pulses of 5-15V and 100 msec in duration were routinely used, employing a Grass S-88 stimulator and a Grass model SIU 5 stimulus isolation unit (Grass Medical Instruments, Quincey, Massachusetts).

Following isolation of the heart, the atrium was grasped with fine forceps and forced over the perfusion tube so that the tip protruded slightly into the ventricle. The

Figure 4. A composite diagram of the bath and associated apparatus.

AB*: Agar bridge
 AG*: Ag/AgCl electrode
 C*: Cam
 CL: Clamps
 FA: Fluid aspirator tube
 FT: Force transducer
 H: Heart preparation
 HP*: High input impedance probe
 I*: Indentations
 L: Focusing lens
 LC: Clamp lever catch
 LG: Fibre optics light guide
 M*: Microelectrode
 MB: Mounting block
 MD: Micrometer drive
 MH*: Microelectrode holder

PS: Position stage
 R: Reference electrode assembly
 RB: Rubber band
 RE*: Reference electrode
 S: Spring-loaded rack
 SI: Superfusion inlet
 SL: Superfusion line
 SP: Stainless steel perfusion tube
 SR: Stimulating electrode (reference)
 ST*: Superfusion line (toxic compounds)
 T: Collimator tube
 V: 4-way valve
 VA: Viewing angle
 W: Nichrome wire

Inset: Detail of the fixed clamp used for ventricular perfusion. The clamp of the superfusion bath (not shown) had a fixed lower jaw, no indentations (I) and no cam (C). Although shown in this composite drawing, the components marked with an asterisk were unique to the superfusion bath. Scale bars = 1cm.

jaws of the clamp were closed over the atrium and a small portion of the ventricle. The distal aorta was attached to the transducer clamp, and the heart was tensioned to about 50mg using the transducer positioner (see Fig. 4).

Perfusion solutions (salines only) were introduced to the preparation via a 4-way, non-return valve (Fig. 5) (designed by Dr. S. Jacobson and Mr. C. Wilson, Carleton University). Frescon and other toxic compounds were introduced by means of a 5cc disposable syringe, bypassing the 4-way valve, using a syringe pump (model 341A, Sage Instruments, Cambridge, Massachusetts) to meter the flow.

Figure 5. A diagram of the 4-way 'flap' valve assembly.

The valve block and header were made from plexiglas. A teflon tape gasket was inserted between H and VB. Scale bar = 1cm.

An inlet on the clamp side of the bath allowed for the exchange of extracardial solutions. Superfusion solutions were introduced via a 9-way valve (identical in principle to the 4-way valve). The bath volume was 7ml, and by the use of a marker dye, it was determined that about 50ml of solution were required for complete exchange of the bath contents. Neither the perfusion nor the superfusion lines were allowed to come in contact with any suspensions or solutions other than saline or water-soluble compounds. Only the syringe method was used to introduce water-insoluble toxic compounds, thereby reducing the risk of contamination.

Flow rates could be adjusted by individual teflon needle valves at the nine superfusion reservoirs, and by adjustable tube clamps at the perfusion reservoirs. Perfusion flow rates were of the order of 0.5ml/min, while the superfusion flow rates were variable.

The bath was secured in a support base which incorporated the reference electrode manipulator and an aspirator tube which served to maintain a constant fluid level during perfusion or superfusion. The support base was attached to the translation stage which, in turn, was secured to the bottom of the Faraday cage. The preparation was illuminated from behind with a fibre optics light source (model 11-80, American Optical, Southbridge, Massachusetts). The light from the fibre guide was focused on the preparation with a double convex lens mounted in an aluminum collimator tube (see Fig. 4).

The tube was positioned with a mechanical manipulator.

b) The superfusion bath: PRM, LFS and HV strips

This bath, used to superfuse the PRM, LFS and HV strips was identical to the perfusion bath in all respects except that the perfusion tube was omitted and an extra inlet was added so as not to contaminate the main superfusion lines with toxic compounds. The fixed clamp was also slightly different from that of the perfusion bath (see Fig. 4).

The end of the PRM with the attached body wall fragment was routinely clamped in the left (fixed) clamp. The other end of the muscle was attached to the transducer clamp. The PRM was tensioned to about 150mg. The LFS, on the other hand, usually relaxed to close to zero tension, even after repeated stretching. The HV strip was tensioned to about 50mg. The interior of the HV was oriented upward allowing access to the trabeculae during microelectrode recording.

c) The open ventricle bath: open ventricle preparation

This bath consisted of a small chamber with a 45° sloped back and a viewing glass at the front face (Fig. 6). The volume of the chamber was 1ml, and 10ml of solution were required for the complete exchange of the bath contents (as determined by the use of a marker dye). Fluid entered the chamber through ports close to the bottom and was aspirated

off the top.

The open ventricle was affixed to a Sylgard mounting block with miniature tungsten staples. The block was fitted into the chamber against the sloped back, the friction of the fit having been sufficient to immobilize it.

Figure 6. A diagram of the open ventricle bath.

- B: Bath
- FA: Fluid aspirator
- HP: High input impedance probe
- I: Inlet (saline only)
- IT: Inlet (toxic compounds)
- L: Focusing lens
- M: Microelectrode
- MH: Microelectrode holder
- OV: Open ventricle preparation
- R: Reference electrode
- S: Tungsten staples
- SB: Support base
- SM: Sylgard mounting block
- T: Collimator tube

Scale bar = 1cm

4) THE SALINE SOLUTIONS

All salines were prepared in glass-distilled water and filtered through a 0.45 μ m membrane filter (Metricel GN-6, Gelman Instruments Ltd., Ann Arbor, Michigan). It was found that this filtering procedure greatly reduced contamination

of the saline solutions by airborne microorganisms. The salines were buffered with HEPES which was adjusted to pH 7.6 with either 10M NaOH or 10M KOH, depending on whether Na-free or K-free saline was desired. The addition of 10ml/l of 0.5M HEPES (stock concentration) was equivalent to adding either 2.35mM Na or 2.72mM K. The increased concentration of these ions was not corrected for except in calculations pertaining to the ionic basis of the resting potential in heart muscle cells. However, when Na-free saline was prepared, the usual complement of KCl was omitted (see Table 2).

A) Normal saline

The composition of normal *Lymnaea* saline (see Table 2) was based on flame photometric analysis of snail haemolymph. The haemolymph of 8 snails was pooled, diluted by a factor of 100 with de-ionized, double-distilled water and analyzed for Na, K, Ca and Mg concentrations with a Perkin-Elmer model 703 atomic absorption spectrophotometer (Perkin-Elmer Co., Norwalk, Connecticut). The results of this analysis are presented in Table 1.

Table 1. The ionic composition of *Lymnaea* haemolymph.

<u>Ion</u>	<u>Concentration (mM) $\pm$ S.D.*</u>
Sodium	51.10 $\pm$ 0.03
Potassium	1.57 $\pm$ 0.04
Calcium	3.47 $\pm$ 0.03
Magnesium	2.03 $\pm$ 0.03

*6 determinations of haemolymph pooled from 8 snails

The osmolarity of *Lymnaea* haemolymph was found to be 122.8 ± 1.2 (S.D.) milliosmoles (mOsm), based on an average of 6 determinations with the pooled haemolymph from 8 snails on an Osmette A osmometer (Precision Systems, Inc., Sudbury, Massachusetts). The osmolarity of normal *Lymnaea* saline was found to be 123 ± 1.2 mOsm (based on 10 determinations).

B) Substituted saline solutions

Substituted saline solutions, wherein one or more ion species of normal saline was partially or wholly replaced with a different species, were routinely used. The composition of these salines is presented in Table 2. Their ionic strengths were kept identical by manipulating the concentrations of NaCl and/or CaCl_2 .

a) Polyvalent ion substitutions

The heavy metals nickel, cobalt and manganese, and the rare earth lanthanum were used to block Ca entry into muscle cells. To achieve this, it was found that the optimum concentration ratio of the heavy metals to Ca was about 1:2.5. However, when the Ca concentration was maintained at the normal level of 3.5mM, the effect of the required 8-9mM heavy metal concentration was found to be poorly reversible as evidenced by the slow and incomplete recovery of muscle function upon return to normal saline. Good reversibility was achieved with

a heavy metal concentration of 2.5mM. Given the optimum heavy metal to Ca ratio, this necessitated the reduction of the Ca concentration from 3.5mM to 1mM. In the case of La, optimal efficacy and reversibility was found when the concentration of La was 1mM and the concentration of Ca was 2.5mM.

Other polyvalent ion-substituted salines included Ca-free saline containing 3.5mM Ni or 1mM La, and high K (30mM)/Ca-free saline containing 1mM La. The composition of these saline solutions is presented in Table 2.

Table 2. The composition of the saline solutions.

	NaCl	Normal	0.5Ca/10mM	0.5Ca/10mM ONa	10mM/2.5Ca	2.5mM/10Ca	2.5mM/10Ca	2.5Ca/10Ca	2.5mM/10Ca	10mM/10Ca	10mM/2.5Ca	10mM/30K/10Ca	30K	30K/10Ca	10Ca	0.5Ca	10Ca	2.5Ca	ONa	5Ca ²⁺
NaCl	50	39.5	0	50	50	50	50	50	52.3	49	22.3	21.6	26.9	55.3	54.5	52.8	51.5	0	5Cl 490	
KCl	1.6	1.6	0 ³	1.6	1.6	1.6	1.6	1.6	1.6	1.6	30	30	30	1.6	1.6	1.6	1.6	0 ³	1.6G	
CaCl ₂	3.5	0.5	0.5	2.5	1	0	1	1	0	2.5	0	3.5	0	0	0.5	1	2.5	3.5	3.5A	
MgCl ₂	2	10	10	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2A	
HEPES-Na ⁺	5	5	-	5	5	5	5	5	5	5	5	5	5	5	5	5	5	-	5	
HEPES-K ⁺	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	
NaSO ₄	-	-	-	1	2.5	3.5	-	-	-	-	-	-	-	-	-	-	-	-	-	
CoCl ₂	-	-	-	-	-	-	2.5	-	-	-	-	-	-	-	-	-	-	-	-	
MnCl ₂	-	-	-	-	-	-	-	2.5	-	-	-	-	-	-	-	-	-	-	-	
LaCl ₃	-	-	-	-	-	-	-	-	1	1	1	-	-	-	-	-	-	-	-	
BDAC ⁴	-	-	39.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	50	-	

Notes:

1. Concentrations given in mM
2. [Na] in 5mM HEPES = 2.35mM (e.g. total [Na] in normal saline = 52.35mM)
pH of HEPES-Na adjusted to 7.6 with 10M NaOH
3. [K] in 5mM HEPES = 2.72mM; pH of HEPES-K adjusted to 7.6 with 10M KOH
4. BDAC = Bis(2-hydroxyethyl) dimethyl ammonium chloride
5. [K] in ONa saline = 2.72mM
6. Cl = chloride salt, G = glutamate salt, Ac = acetate salt

b) *Other substituted saline solutions*

Other substituted salines were prepared by varying the proportions of the ion species of normal saline (Table 2). This group consisted of low Ca/high Mg salines (0.5mM Ca/10mM Mg and 0.5mM Ca/10mM Mg/0mM Na), Ca-free saline, Na-free saline, low Ca salines (0.5mM, 1mM or 2.5mM Ca), high K salines (30mM K and 30mM K/0mM Ca) and low Cl saline (5mM Cl).

c) *Variable [K] salines with [K] X [Cl] = constant*

In the experiments designed to investigate the ionic basis of the resting potential in *Lymnaea* heart muscle cells, salines with 1mM to 40mM K were prepared such that the product of [K] X [Cl] was constant at 60mM². Table 3a lists the composition of the stock solutions used to prepare these salines while Table 3b lists the volumes of the stock solutions required for each desired K concentration. The values in Table 3b were calculated using the following set of equations:

$$1) \text{ Volume of stock C: } \frac{V_C}{V_D} \times 50 = K_D \therefore V_C = \frac{V_D \times K_D}{50}$$

Where V_D = desired total volume
 V_C = volume of stock C
 K_D = desired [K]
 [K] in solution C = 50mM

2) Volume of stock A: since $[K] \times [Cl] = 60\text{mM}^2$, $[Cl] = \frac{60\text{mM}^2}{[K]}$

where $[Cl]$ in solution A = 63.5mM

$$\text{and } [Cl] = \frac{V_A}{V_d} \times 63.5 = \frac{60\text{mM}^2}{[K]}$$

$$\text{therefore: } V_A = \frac{60V_D}{63.5 \times K_D}$$

where V_A = volume of stock A

3) Volume of stock B: $V_B = V_A - (V_A + V_C)$

where V_B = volume of stock B

Table 3. The composition of the constant $[K] \times [Cl]$ product salines.

a) Composition of the stock solutions.

Salt (mM) ¹	Solution		
	A	B	C
NaCl	42.5	0	0
Na-glu	0	42.5	0
K-glu	0	0	50.0
CaCl ₂	0.5	0	0
CaAc ₂	0	0.5	0.5
MgCl ₂	10.0	0	0
MgAc ₂	0	10.0	10.0
HEPES-Na	5.0	5.0	5.0
[K]	0	0	50.0
[Cl]	63.5	0	0

b) Stock volumes for $[K] \times [Cl] = 60\text{mM}^2$ (final volume = 250ml).

[K] (mM)	ml		
	V _A	V _B	V _C
1	236.2	8.8	5
2	118.1	121.9	10
4	39.1	170.9	20
6	39.4	180.6	30
8	29.5	180.5	40
10	23.6	176.4	50
15	15.7	159.3	75
20	11.8	138.2	100
25	9.4	115.6	125
30	7.9	92.1	150
40	5.9	44.1	200

¹ glu = glutamate

Ac = acetate

Note elevated Mg and reduced Ca concentrations

V_A = volume of solution A

V_B = volume of solution B

V_C = volume of solution C

The constant $[K] \times [Cl]$ product salines contained elevated Mg and reduced Ca concentrations. The reason for this is discussed below in section 6-A.

d) *Helix* saline solution

Helix saline was prepared following the composition described by Kerkut & Gardner (1967), except that HEPES was substituted for Tris buffer (Table 4). The HEPES buffer was adjusted to pH 7.6 with 10M NaOH.

Table 4. The composition of *Helix* saline.

<u>Salt</u>	<u>Concentration (mM)</u>
NaCl	80
KCl	4
CaCl ₂	7
MgCl ₂	5
HEPES-Na	5

e) *Terminology*

To facilitate discussion, the various saline solutions will be abbreviated to a format wherein a number immediately preceeds the substituted ion, the number defining the concentration in mM of that ion. For example, Ca-free saline will be described as '0Ca' saline, Na-free saline as '0Na' saline, etc. Where more than one ion have been substituted, the ions and their concentrations (using the above format) will be

separated by a slash (/), e.g. the saline containing 1mM La, 30mM K and 0mM Ca will be described as '1.0La/30K/0Ca' saline, etc.

Many of the experimental agents were introduced to the preparations in substituted salines. Again, for the sake of expediency, such solutions or mixtures will be described by the format 'name of agent/type of saline'. For example, Frescon in saline containing 2.5mM Ni and 1mM Ca will be described as 'Frescon/2.5Ni/1.0Ca saline, etc.

5) THE EXPERIMENTAL COMPOUNDS

The description of the experimental compounds used in these experiments is presented in Table 5.

A) *Frescon*

Frescon (N-(triphenylmethyl) morpholine) was provided by Dr. C. Boyce of Shell Research Ltd. (Sittingbourne, England). As this compound is very hydrophobic (solubility in water = 0.02 ppm; Shell, 1974), it was dissolved in a dimethyl sulphoxide (DMSO)-based detergent formulation (blank FX 2574, Shell Chemicals Co.) to yield a stock concentration of 10^{-2} g/ml. Appropriate volumes of the stock solution were added to yield nominal 10^{-5} g/ml to 10^{-6} g/ml suspensions of fine particles approximately 1 μ m in length (Brezden & Gardner, 1980b).

COMPOUND	IUPAC NAME	M.W. (SOLVENT)	STRUCTURAL FORMULA
Proziquestel	(2-cyclohexylcarbonyl)- 12,16,7118-hexahydro- 2H-pyrazino[2,1-a]- isquinolin-4-one	312.42 (Ethanol)	
RAI 12.330A	N-(cis-2-phenylcyclopentyl)azacyclodecan-2-imine hydrochloride	376.99 (Saline)	
Sodium Pentobarbital	5-ethyl-5(1-methylbutyl)- 2,4,6(1H,3H,5H) pyrimidinetrione monosodium salt	248.26 (Saline)	

Table 5. The experimental compounds.

COMPOUND	IUPAC NAME	M.W. (SOLVENT)	STRUCTURAL FORMULA
A23187	-----	523 (Ethanol)	
Caffeine	3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione	194.91 (Saline)	
D-600	5-[(3,4-dimethoxyphenyl)ethyl]methylamino-2-methoxy-2-(trimethoxyphenyl)valeronitrile hydrochloride	521.10 (H ₂ O)	
Frescon	N-(triphenylmethyl)morpholine	329.42 (FX2574)	

B) Tritiated Frescon

Tritiated Frescon was prepared from unlabelled stock by catalytic exchange with ^3H gas (process NET-262, New England Nuclear, Boston, Massachusetts) and was dissolved in DMSO. Since the purity of the labelled Frescon was not guaranteed (i.e. hydrolysis may have occurred during the labelling process), the labelled product was purified on an alumina column following the procedure of Beynon & Wright (1975) as modified by Brezden & Gardner (1980b). The DMSO was evaporated from a 10ml aliquot of the labelled product. The resultant precipitate was dissolved in 20ml of cyclohexane (BDH Chemicals) and poured into a column containing 2g of alumina (type H basic, BDH Chemicals). The water content of the alumina had been previously adjusted to 2% v/w (cf. Beynon & Wright, 1975). The eluate was collected and mixed with 8ml of diisopropyl ether. This solution was poured back into the column, the final eluate collected and the solvent evaporated. The precipitate was re-dissolved in DMSO, and this final product was assayed by means of thin layer chromatography.

Four pre-coated glass thin layer chromatography plates (alumina G, 20 X 20cm, Analtech, Inc.) were impregnated by allowing a .5% w/v solution of paraffin in xylene to run to the top (cf. Boyce & Milborrow, 1965). The plates were dried in an oven at 60°C for 4 h and spotted with 4 μ l of a 10^{-2} g/ml solution of >99% Frescon in DMSO (2 spots per plate) and 4 μ l of the presumed ^3H -Frescon solution (2 spots per

plate). The plates were developed in a chromatography tank using cyclohexane as the mobile phase. When the solvent front came within 4cm of the top, the plates were air-dried and sprayed with a 5% w/v solution of trichloroacetic acid in acetone. The plates were heated with a blow-drier revealing intensely yellow spots of the triphenylmethyl carbonium ion derived from the triphenylmethyl moiety of Frescon. It was found that the >99% pure Frescon and the presumed ^3H -Frescon had identical R_f values (Table 6). There was no streaking, and only one spot per sample was detected.

Table 6. The R_f values of >99% pure and presumed ^3H -Frescon

Compound	$R_f \pm \text{S.D. (n=8)}$
>99% pure Frescon	0.015 ± 0.008
^3H -Frescon	0.015 ± 0.008

Tritiated Frescon was used to examine the effects of D-600 and Ni on the binding (or uptake) of this molluscicide by *Lymnaea* HV's, which were exposed to ^3H -Frescon in normal saline or in the presence of $4 \times 10^{-4}\text{M}$ D-600 or 2.5mM Ni. Hearts were pre-treated by perfusion and superfusion for 10 min with either 2.5Ni/1.0Ca saline or D-600, followed by a 10 min perfusion with 10^{-5}g/ml ^3H -Frescon/2.5Ni/1.0Ca or ^3H -Frescon/D-600, respectively, while maintaining superfusion with Frescon-free salines. At the end of 10 min the hearts were perfused and superfused with normal saline for 20 min.

The controls consisted of treating the HV preparations as above but with ^3H -Frescon in normal saline. Perfusion rates were kept constant at about 0.5ml/min. The HV's were continuously superfused so as to prevent Frescon from coming in contact with the outside of the ventricle. The Frescon particles, introduced at the atrial end of the heart, were made visible by the diffracted light and could be seen leaving the aorta in a steady stream which was directed away from the ventricle by the superfusate and was aspirated from the bath opposite the point of entry.

Following the above treatments the atrium and aorta of the HV were discarded, the ventricle was weighed and dissolved in 1ml of NCS tissue solubilizer (Amersham Corp., Arlington Heights, Illinois) in scintillation counting vials containing 0.2ml of distilled water. When the tissue had completely dissolved, 10ml of scintillation fluid (4g PPO and 0.2g POPOP per litre of toluene) were added and the samples counted on a Beckman LS 7500 liquid scintillation counter (Beckman Instruments Co., Montreal, Quebec).

C) *The divalent cation ionophore A23187*

A23187 was obtained from Calbiochem-Behring Corp. (La Jolla, California). A 2×10^{-3} g/ml stock solution was prepared by dissolving 2mg of the ionophore in 1ml of absolute ethanol. The ionophore formed a fine particulate suspension upon addition to saline. Nominal concentrations of 10^{-5} g/ml to

2×10^{-5} g/ml were used.

D) Caffeine

The methylxanthine caffeine (Sigma Chemicals Co.) was dissolved directly in saline to a concentration of 2×10^{-2} M.

E) D-600

The calcium entry blocker D-600 was obtained from Drs. Oberdorf and Sharma (Knoll AG, Ludwigshafen, W. Germany). A 4×10^{-2} M stock solution was prepared in de-ionized, distilled water. Final concentrations in saline ranged from 10^{-4} M to 5×10^{-4} M.

F) RMI 12330A

The adenylyl cyclase inhibitor RMI 12330A was obtained from Dr. W. Albrecht of Merrel Dow Pharmaceuticals (Cincinnati, Ohio). This compound was dissolved directly in saline to a concentration of 10^{-3} M.

G) Sodium pentobarbital

Sodium pentobarbital ("Somnotol", M.T.C. Pharmaceuticals, Hamilton, Ontario) was added to saline to a final concentration of 5×10^{-3} M.

H) Praziquantel

The anthelmintic praziquantel (PQ) was obtained from Dr. P. Andrews (Bayer AG, W. Germany). A stock solution of $3.2 \times 10^{-5} \text{ M}$ was made by dissolving 100mg of PQ in 10ml of absolute ethanol. A concentration of $1.6 \times 10^{-6} \text{ M}$, made by dissolving 1ml of stock solution in 19ml saline, was routinely used.

6) THE EXPERIMENTAL REGIMES

A) *The effect of varying $[K]_e$ on the membrane potential*

In these experiments, the response of the transmembrane potential difference (membrane potential, V_m) in individual *Lymnaea* ventricle muscle cells to changes in the extracellular K concentration ($[K]_e$) was measured, using the open ventricle preparation (section 2-B-b). The $[K]_e$ was varied in a random sequence. After each new $[K]_e$ the preparation was returned to 1mM K saline (baseline V_m). The experiment was considered successful only if a complete recovery of the baseline V_m was achieved after each change in $[K]_e$. However, in early experiments elevated K concentrations resulted in a contraction of the heart muscle cells. As a result, it was not possible to maintain successful microelectrode insertion. Various mechanical means (e.g. 'floating' microelectrodes, more rigid preparation mounting, etc.) were not successful in eliminating this problem,

probably because of the extreme fragility of this tissue. Therefore, the [Mg] was elevated to 10mM and the [Ca] was lowered to 0.5mM to inhibit contraction. Following this procedure, successful microelectrode impalement could be maintained through many solution changes.

A further criterion for an acceptable experiment was that the resting potential in 1mM K_e must have been more negative than or equal to -50mV. Cells with more positive resting potentials were judged to be damaged.

B) The perfusion and superfusion experiments

The preparations and their mounting procedures have been described in sections 2-C and 3-C. After mounting the preparations in their respective baths, they were allowed to stabilize after tensioning. The experiments were not started until after at least 5 min of stable muscle tension was recorded. Preparations which did not exhibit stable tensions at rest were discarded.

C) The concentration of calcium in 'Ca-free' saline

The calcium content of the distilled water used to make up the salines in these experiments was compared to the calcium content of de-ionized, double-distilled water (provided by Dr. S. Jacobson, Carleton University). The determinations were carried out on a Perkin Elmer 703 atomic absorption

spectrophotometer (Perkin Elmer Ltd., Montreal, Quebec). In addition, the Ca content of the 'Ca-free' saline was determined (Table 7).

Table 7. The calcium content of distilled water and 'Ca-free' saline.

	[Ca] (M)*
Distilled H_2O (dH_2O)	ND
De-ionized, double-distilled H_2O (ddH_2O)	ND
Ca-free saline (in dH_2O)	8×10^{-6}
Ca-free saline (in ddH_2O)	8×10^{-6}

*ND = not detectable; limit of detection = $10^{-8} M$

D) Precautions against contamination

Considerable precautions were taken to avoid contamination. Apart from segregating the toxic and non-toxic fluid lines, after each experiment, all of the components which came in contact with a toxic compound were cleaned by ultrasonication in a warm 5% solution of Extran 300 detergent (BDH Chemical Co.) in distilled water. Perfusion or superfusion tubing which had been contaminated was discarded.

RESULTS

The majority of the data presented are based on measurements of muscle tension. Changes in tension in response to a test substance are defined in terms of a deviation from resting tension. For the heart ventricles (HV's) and the foot strips of *Lymnaea* (LFS's) this deviation is plotted in mg tension/mg tissue (wet weight), and for the penis retractor muscles (PRM's) in g tension/mg tissue (wet weight). Note that the PRM's are capable of generating much greater tension per unit weight than either the HV's or LFS's.

Strictly speaking, tension should be defined by units of force, not mass. However, mass units were preferred as they provide a more intuitive feel for the degree of force generated. In fact, a 'tension' of '1mg' represents a force of 9.8×10^{-6} Newtons, and '1g' represents a force of 9.8×10^{-3} Newtons.

All of the figures, unless otherwise noted, represent the results of a single experiment: 'n' designates the number of repetitions of that particular experimental protocol.

Throughout the discussion, whenever a value is followed by ' $\pm m$ ', m is always the standard deviation of the given value.

1) CONTROL EXPERIMENTS

Several of the experimental compounds, being water-insoluble, were dissolved in ethanol (praziquantel, A23187), DMSO (^3H -Frescon) or blank FX 2574 emulsifier (unlabelled Frescon). In control experiments, a 1% solution of either DMSO or blank FX 2574 had no effect on the resting tension of either *Helix* or *Lymnaea* musculature (Fig. 7). A 10^{-5} g/ml suspension of Frescon, however, contained only 0.1% of these emulsifiers. Only 5% ethanol caused a slight, transient contraction in the *Lymnaea* PRM. This amount of ethanol was present in the praziquantel/saline solutions.

Figure 7. The effects of blank FX 2574, ethanol and DMSO on the musculature of *Helix aspersa* and *Lymnaea stagnalis*.

n=4 for each group. Ethanol and DMSO controls for *Lymnaea* only.
Note change in time scale.

2) THE EFFECT OF FRESCON ON *Lymnaea* MUSCULATURE

A) *The heart ventricle*

The perfusion of the isolated *Lymnaea* heart with a 10^{-5} g/ml suspension of Frescon induced a spontaneous ventricular contraction within about 30 sec of exposure which, after having reached a maximum tension, declined to a level well above resting tension (the 'residual' contracture). This residual contracture was not reversed by perfusion with normal saline (Fig. 8). The mean maximum tension reached with perfusion of the heart ventricle (HV) with Frescon was 171 ± 65

Figure 8. Frescon-induced contracture of the *Lymnaea* heart ventricle.

A small transient contraction often appeared when the Frescon was flushed from the heart by perfusion with normal saline (arrow). Note change in time scale. $n=20$

mg/mg tissue and the mean magnitude of the residual contracture was 46 ± 20 mg/mg tissue ($n=20$).

In contrast to the ventricle, the atrium did not appear to be affected by Frescon. This could be observed when the isolated heart was immersed in a 10^{-5} g/ml suspension of this molluscicide in a small petri dish. Eventually, the Frescon diffused into the heart causing the ventricle to contract whereas the atrium often continued to beat in a normal fashion.

Extracardial application of Frescon was ineffective in producing contracture in the HV. When the atrial and aortal openings were blocked by the bath preparation clamps, superfusion with a 10^{-5} g/ml suspension of Frescon for up to 1 h had no effect on the resting tension.

B) The penis retractor muscle

The PRM also responded to Frescon with a strong contracture within about 30 sec of exposure. Unlike the ventricular response, the PRM contracture was maintained at a high level. Prolonged superfusion with normal saline did not reverse it (Fig. 9).

The response of the PRM to Frescon appeared to be dose-dependent. A 10^{-5} g/ml suspension induced a more powerful contracture than did 10^{-6} g/ml. The mean maximum tension for exposure to 10^{-5} g/ml Frescon was 1774 ± 621 mg/mg tissue ($n=22$), and for 10^{-6} g/ml it was 909 ± 365 mg/mg tissue ($n=8$). The magnitude of the residual contraction for 10^{-5} g/ml Frescon was

Figure 9: Frescon-induced contracture of the penis retractor muscle of *Lymnaea*.

● n=22, ▲ n=8; Note change in time scale

1528 $\pm$ 643 mg/mg tissue, and for 10⁻⁶g/ml Frescon, it was 847 $\pm$ 342 mg/mg tissue. The difference between these values is significant at the $p < 0.001$ level (Student's t statistic). In addition to the difference in generated tension, the latency of response of the PRM to 10⁻⁶g/ml Frescon was about twice as long as that for 10⁻⁵g/ml (about 1 min vs. about 30 sec, respectively).

c) The foot strip.

The *Lymnaea* foot strip (LFS) responded to Frescon with a relatively slowly developed, sustained contracture, with a response latency of 2-4 min (Fig. 10). The mean maximum tension developed was 102 ± 82 mg/mg tissue ($n=10$), and was also irreversible with normal saline. The response of this tissue to Frescon was generally more variable than in either of the previous examples.

Figure 10. Frescon-induced contracture of the foot strip of *Lymnaea*

n=10

3) THE EFFECT OF FRESCON ON *Helix* MUSCULATURE

A) *The heart ventricle*

Perfusion or superfusion of the *Helix* heart with up to a 10^{-4} g/ml suspension of Frescon had no effect on either the resting tension or on the spontaneous contractions of the ventricle.

B) *The penis retractor muscle*

The *Helix* PRM was affected by Frescon, although the time course and the profile of the response were considerably different from those in *Lymnaea*. Spontaneous contractions in response to a 10^{-5} g/ml or 2×10^{-5} g/ml suspensions occurred in less than half of the preparations, and the contractions were transient, lasting about 24 min (Fig. 11).

Those preparations which did not respond to Frescon with spontaneous contractions were, nevertheless, affected as evidenced by the large increase in magnitude and duration of stimulus-evoked contractions. Repeated, transient contractions could be evoked with electrical stimuli over a period of more than one hour following Frescon treatment. Subsequent washing with normal saline for up to one hour did not restore normal activity. Eventually, a single stimulus induced rhythmic, oscillatory contractions with a progressively decreasing relaxation component, culminating in a sustained and irreversible contracture (Fig. 12).

Figure 11. Spontaneous, transient contraction induced by Frescon in the *Helix* penis retractor muscle.

n=3

Figure 12. Frescon-induced prolongation of stimulus-evoked contractions in the *Helix* penis retractor muscle.

n=4. Note changes in time scale. Arrows denote points of electrical stimulations (10V, 100msec).

4) THE IONIC BASIS OF THE RESTING POTENTIAL IN *Lymnaea* HEART VENTRICLE MUSCLE CELLS

A) The potassium and chloride dependence of the resting potential

According to Boyle & Conway (1941) and Hodgkin & Horowicz (1959), the membrane of the muscle cells of the frog *Rana temporaria* is permeable to both potassium and chloride, the concentration gradients of these two ions being associated with the membrane potential. However, equivalent information for molluscan muscles is, for the most part, unavailable. The resting potential (V_r) of those molluscan muscles studied appears to be more or less dependent on $[K]_e$ (see Introduction, section 5-A) although the extent of the contribution by Cl is not known. However, Hagiwara et al. (1964) have shown that the barnacle muscle fibres are permeable to Cl, suggesting that this ion, as well as K, may contribute to the V_r of some molluscan muscles.

Lymnaea heart ventricle muscle cells also appear to be permeable to Cl. A reduction of $[Cl]_e$ from 62.6mM to 5mM resulted in a transient depolarization of the membrane potential (V_m) (Fig. 13). This depolarization was of the order of 6mV in magnitude and V_m reverted to a level which was 2mV less negative than the original V_r after about 3 min in the reduced Cl saline. Restoration of the original $[Cl]_e$ induced a hyperpolarization of about 7mV, followed by a repolarization of the V_m to a stable level about 1mV more negative than the original

V_r . These results are consistent with those of Hodgkin & Horowicz (1959) insofar as changes in $[Cl]_e$ produced a transient change in the V_m but the magnitude of the change in V_m was lower than expected, and the original V_r was not restored following the changes in $[Cl]_e$.

If the muscle cell membrane is freely permeable to both K and Cl, the Donnan equilibrium theory holds that there should be no movement of KCl across the membrane during variations in $[K]_e$ or $[Cl]_e$ if their product is kept constant (Boyle & Conway, 1941). If such is the case, and if the mem-

Figure 13. The effect of reduced chloride concentration on the membrane potential of *Lymnaea* ventricle muscle cells.

Note change in time scale. $n=8$

brane is impermeable to other ion species, the membrane potential should be a function of the equilibrium potentials of K and Cl, i.e.,

$$E_K = \frac{RT}{F} \ln \frac{[K]_e}{[K]_i} \quad \text{and} \quad E_{Cl} = \frac{RT}{F} \ln \frac{[Cl]_i}{[Cl]_e}$$

where E_K and E_{Cl} are the potassium and chloride equilibrium potentials, R is the gas constant, T is absolute temperature, F is Faraday's constant and $[]_i$ and $[]_e$ denote intracellular and extracellular concentrations, respectively. If K and Cl are passively distributed then $V_m = E_K = E_{Cl}$. Thus, under conditions of a constant $[K]_e \times [Cl]_e$ product, V_m should conform to the relation

$$V_m \text{ (mV)} = 58 \log \frac{[K]_e}{[K]_i} \quad (1)$$

where a decade change in $[K]_e$ should be associated with a 58mV change in V_m .

In *Lymnaea* HV muscle cells, a straight line relationship between V_m and $[K]_e$ was observed only at K concentrations above 6-10mM, where a decade change in $[K]_e$ was associated with a 50.4mV change in V_m . In 20 successful experiments, the slope of the straight line portion of $[K]_e$ vs. V_m varied from 44mV to 59mV (mean = 50.4 ± 3.8 mV). Figure 14 shows the results of an experiment where a 54mV slope was obtained between 6mM and 30mM $[K]_e$.

In an attempt to account for the deviation observed

from the predicted behaviour of V_m at low $[K]_e$, the equation of Hodgkin & Katz (1949) as applied by Moreton (1968) and Sattelle (1974) was used. The Hodgkin-Katz equation is given by

$$V_m = \frac{RT}{F} \ln \left(\frac{P_K [K]_e + P_{Na} [Na]_e + P_{Cl} [Cl]_i}{P_K [K]_i + P_{Na} [Na]_i + P_{Cl} [Cl]_e} \right) \quad (2)$$

where P_x denotes the membrane permeability to the ion species x (the other terms have been previously defined). This equation

Figure 14. The relation between the membrane potential and the extracellular potassium concentration in *Lymnaea* heart ventricle muscle cells.

Data are taken from a single experiment. $n=20$

The solid line denotes the expected results using the data from Fig. 15 and eqn. (1). The dashed line denotes the expected results using the data from Fig. 15 and eqn. (5). See text for further explanation.

can be rearranged to remove the logarithm, giving

$$e^{VF/RT} = \frac{P_K[K]_e + P_{Na}[Na]_e + P_{Cl}[Cl]_i}{P_K[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_e} \quad (3)$$

Assuming that the permeability of the membrane to K is much greater than to Na, and that the $[Na]_i$ is small, the value of the term $P_{Na}[Na]_i$ may be neglected since it would be much smaller than the value of the term $P_K[K]_i$. Also, the chloride terms may be neglected since there should be no movement of Cl across the cell membrane if a constant $[K]_e \times [Cl]_e$ product is employed (Boyle & Conway, 1941). Thus, the Cl ions should always be at the same equilibrium potential as the K ions. Consequently, only the potassium terms need be considered, and equation (3) can be reduced to

$$e^{VF/RT} = \frac{[K]_e}{[K]_i} + \frac{P_{Na}[Na]_e}{P_K[K]_i} \quad (4)$$

This equation predicts a linear relationship between $e^{VF/RT}$ and $[K]_e$ if $[K]_i$ and the ratio P_{Na}/P_K remain unchanged (cf. Moreton, 1968). The slope of the line gives an estimate of $[K]_i$, and the value of the term P_{Na}/P_K is given by the Y-intercept.

The data from Fig. 14 were used to plot $e^{VF/RT}$ against $[K]_e$, giving a value for $[K]_i$ of 41.4mM, and a P_{Na}/P_K ratio of 0.04 (Fig. 15). Substituting these values in equation (4) and rearranging gives

$$V \text{ (mV)} = 58 \log \left(\frac{[K]_e}{41.4} + 0.04 \frac{[Na]_e}{41.4} \right) \quad (5)$$

Figure 15. The relationship between $e^{VF/RT}$ and the extracellular potassium concentration in *Lymnaea* ventricle muscle cells.

The data for this figure were taken from the data for Figure 14. See text for explanation.

where $[Na]_e$ was known for each value of $[K]_e$. The curve predicted by this equation is represented by the dashed line in Fig. 14, and very closely approximated the curve of the experimentally determined points.

The above calculations were performed for each of the 20 successful experiments. The estimated $[K]_i$ ranged from 27.8mM to 77.3mM (mean = 51.5 ± 14.6 mM) and the ratio of P_{Na}/P_K ranged from 0.01 to 0.14 (mean = 0.07 ± 0.03). In most cases, the experimental points were very close to those pre-

dicted by equation (4). The differences between the observed and predicted values for V_m at various $[K]_e$ are presented in Table 8.

Table 8. The deviation between the predicted and observed values for the membrane potential of *Lymnaea* heart ventricle muscle cells at various extracellular potassium concentrations.

$[K]$ (mM)	Observed V_m ($\pm$ S.D.)	Expected V_m ($\pm$ S.D.)
1	-62.2 (5.2)	-63.0 (9.8)
2	-58.7 (5.4)	-55.3 (14.0)
4	-52.5 (5.4)	-51.1 (7.9)
6	-45.1 (5.5)	-44.4 (7.0)
8	-38.4 (5.8)	-40.3 (6.7)
10	-31.6 (6.0)	-34.2 (6.4)
15	-24.8 (6.6)	-27.0 (5.9)
20	-19.5 (6.3)	-22.7 (5.6)
25	-15.5 (6.3)	-17.5 (6.2)
30	-11.0 (6.3)	-12.9 (6.0)
35	-7.8 (5.3)	-8.6 (5.5)
40	-5.5 (5.9)	-5.3 (6.3)

The predicted values are from equation (4) (see text). $n=20$

The mean resting potential in normal saline (1.6mM K) was found to be -61.2 ± 3.5 mV ($n=30$). The mean estimated $[K]_i$ of 51.5mM suggests that the V_r of *Lymnaea* HV cells should be about -87mV in normal saline if the membrane behaves as an ideal potassium electrode. This is about 25mV more negative than the observed V_r . However, using equation (4), the mean

$[K]_i$ of 51.5mM and the mean value of 0.07 for the ratio P_{Na}/P_K , the predicted resting potential in normal saline would be -57.4mV, about 4mV less negative than the observed V_r . Therefore, it appears that V_m can be reasonably well predicted by introducing a term for sodium permeability.

B) The effect of Na-free saline on the membrane potential

If the resting membrane of *Lymnaea* HV cells is permeable to Na, a hyperpolarization of V_m would be expected upon removal of the extracellular Na (cf. Sattelle, 1973), since the Na concentration gradient would be reversed, orientating the Na current outward. However, such a hyperpolarization was not observed when BDAC was substituted for Na in normal saline. In fact, the exposure of the HV muscle cells to 0Na saline resulted in a gradual depolarization of V_m and an eventual contraction of the cells. This depolarization could be reversed and the contraction prevented if the cells were exposed to normal saline shortly after the onset of depolarization. If, however, the cells were pre-treated with 0.5Ca/10Mg saline, the subsequent exposure to 0Na/0.5Ca/10Mg saline resulted in a transient hyperpolarization of V_m (Fig. 16). Thus, it would appear that the removal of Na_e results in the expected hyperpolarization only if the influx of Ca_e is prevented, although even 0.5Ca/10Mg saline did not prevent the contraction induced by the removal of Na_e .

Figure 16. The effect of Na-free saline on the membrane potential of *Lymnaea* heart ventricle muscle cells under normal conditions and in the presence of low Ca-high Mg saline.

Data are from a single cell. Intracellular recording could not be maintained following the exposure to Na-free saline even after pre-treatment with low Ca/high Mg due to an eventual contraction which followed the hyperpolarization. Note changes in time scale. $n=8$

C) The effect of Frescon on the resting potential.

The principle objective of the above experiments was to determine if Frescon has any effect on the ionic mechanisms underlying the membrane potential in muscle cells. It was plausible to suspect that Frescon contracture could result from a depolarization of the cell membrane with a consequent contraction of the muscle cells, possibly by affecting one or more of

the ionic mechanisms which regulate the membrane's electrical properties. However, it was found that Frescon had no effect on the membrane potential in the presence of 0.5Ca/10Mg saline (Fig. 17).

Figure 17. The effect of Frescon on the membrane potential of *Lymnaea* heart ventricle muscle cells in the presence of low Ca-high Mg.

Data are from a single cell. Note change in time scale. $n=6$

However, when Frescon was introduced in normal saline, a small (ca. 2mV) depolarization was often observed (Fig. 18). The significance of this event is not clear, as the Frescon-induced contracture made it very difficult to maintain microelectrode impalement. Consequently, the observed fluctuations in V_m following Frescon treatment may have been due to changes in the physical state of the recording parameters alone, e.g. when the muscle contracted, the distortion of the microelectrode tip may have caused a change in the tip potential, although

the consistency of the hyperpolarizing response may reflect a genuine physiological phenomenon (see Discussion).

Figure 18. The effect of Frescon on the membrane potential of *Lymnaea* heart ventricle muscle cells in normal saline.

Data are from a single cell. $n=6$

During these experiments, it was observed that Frescon-induced contractures were prevented by 0.5Ca/10Mg saline. Since these conditions inhibit the influx of Ca into the muscle cells and/or interfere with Ca at the level of the contractile apparatus (see Discussion), it was plausible to propose that Frescon may act by somehow altering membrane Ca permeability and/or Ca regulation in muscle cells. The following experiments were designed to investigate these possibilities.

5) THE EFFECT OF CALCIUM CHANNEL BLOCKERS ON FRESCON-INDUCED CONTRACTURES IN *Lymnaea* MUSCULATURE

A) Low calcium-high magnesium saline

Perfusion of the *Lymnaea* HV with 0.5Ca/10Mg saline induced a slight relaxation of muscle tension. After 10 min of exposure to this saline, the HV responded to Frescon/0.5Ca/10Mg saline with only a very slight increase in tension which did not exceed the resting level. Subsequent introduction of normal saline brought about a Frescon contracture (Fig. 19), suggesting that this molluscicide had previously bound to its

Figure 19. Inhibition of Frescon contracture in the *Lymnaea* heart ventricle by low Ca-high Mg saline.

n=8

'target', but was prevented from exerting its effect by the reduced Ca and elevated Mg concentrations. The reduction of Ca alone to 0.5mM did not prevent Frescon-induced contracture, although the response was considerably reduced compared with the response in normal saline (Fig. 20); suggesting that the elevated Mg concentration also contributed to the previously observed, almost total inhibition of Frescon contracture.

Figure 20. Partial inhibition of Frescon contracture in the *Lymnaea* heart ventricle by 0.5mM calcium saline.

Note changes in time scale. [Ca] in normal saline = 3.5mM
n=4

B) The heavy metals nickel, cobalt and manganese, and the rare earth lanthanum

The heavy metals are believed to act by inhibiting the influx of Ca into cells. If Frescon acts by somehow promoting this influx, e.g. by increasing the cell membrane permeability to Ca through interference with existing Ca channels, then the heavy metals should reduce or prevent Frescon contracture. Pre-treatment of the HV with 2.5Ni/1.0Ca saline generated results which were virtually identical to those obtained with 0.5Ca/10Mg saline. Nickel almost entirely prevented Frescon contracture, although the contracture was expressed upon restoration of normal saline (Fig. 21). Again it would appear

Figure 21. Inhibition of Frescon contracture by nickel in the *Lymnaea* heart ventricle.

n=5

that Frescon had bound to its site of action but was prevented from exerting its effect by the presence of Ni in the extracellular phase.

If the heavy metals act by interfering with a Frescon-induced increase in sarcolemmal Ca permeability, then they should also be able to reverse Frescon contracture. Figure 22 shows typical results of experiments designed to investigate the effects of Ni, Co and Mn on Frescon contracture in the PRM. All three of these ions were found to almost completely and reversibly relax this contracture, although relaxation to resting levels was rarely observed. Note that 1mM Ca alone had

Figure 22. Relaxation of Frescon contracture in the *Lymnaea* penis retractor muscle by nickel, cobalt and manganese.

Note change in time scale. Ni:n=4, Mn:n=4, Co:n=5, 1mM Ca:n=4

a far weaker relaxing effect on Frescon contracture than the 1mM Ca/heavy metal combinations. The above treatments were readily reversible as evidenced by the recovery of Frescon contracture upon the restoration of normal saline.

Lanthanum also antagonized Frescon-induced contracture. The exposure of Frescon-treated PRM's to 1.0La/2.5Ca saline reduced muscle tension to close to resting levels. The reduction in Ca alone to 2.5mM had only a slight relaxing effect. Lanthanum-induced relaxation was also reversible upon restoration of normal saline (Fig. 23).

Figure 23. Relaxation of Frescon contracture in the *Lymnaea* penis retractor muscle by lanthanum.

C) D-600

Pre-treatment of *Lymnaea* HV's with the Ca entry blocker D-600 prevented Frescon contractures (Fig. 24), but only at the relatively high concentration of 4×10^{-4} M, and only if the concentration of Frescon was reduced to 5×10^{-6} g/ml. If the concentration of the molluscicide was raised or the concentration of D-600 lowered, Frescon contracture was not prevented although it was inhibited to various degrees depending on the D-600:Frescon ratio. For example, if the Frescon concentration was raised to 10^{-5} g/ml while maintaining the concen-

Figure 24. Prevention of Frescon contracture in the *Lymnaea* heart ventricle by D-600.

Control = 1% v/v blank EX 2574. D-600:n=10, Control:n=5

tration of D-600 at 4×10^{-4} g/ml, the mean tension generated by the HV was 41.8 ± 8 mg/mg tissue, which is significantly lower than the tension generated in the absence of D-600 ($p < 0.001$, Student's *t* statistic). The mole to mole ratio of D-600 to Frescon for complete inhibition was about 50.

Inhibition of Frescon contracture by the heavy metals or La could be removed simply by restoring normal saline to the preparations. However, this was not the case with D-600. After the HV had been exposed to D-600 and Frescon, perfusion with normal saline increased the muscle tension to only slightly above resting levels, although if a 5×10^{-6} g/ml suspension of Frescon was re-introduced, the HV contracted in a typical manner. Once Frescon contracture had set in, D-600 was ineffective in relaxing the muscle, again in contrast to Co, Mn, Ni or La.

These results suggest that D-600 may have prevented Frescon contracture by competing for Frescon binding sites. If this was the case, perfusion with normal saline may have simply washed away the Frescon as well as the D-600. In order to test this possibility, ^3H -Frescon was used for binding studies (see Methods, section 5-B). Treatment of *Lymnaea* HV's with a 10^{-5} g/ml suspension of Frescon alone resulted in a count of 639 ± 59 DPM/mg tissue (DPM = disintegrations per minute). Pre-treatment with D-600 followed by perfusion with ^3H -Frescon/ 4×10^{-4} M D-600 reduced the count to 94 ± 43 DPM/mg tissue ($p < 0.001$, Student's *t* statistic). However, pre-treatment with

2.5Ni/1.0Ca saline prior to exposure to ^3H -Frescon/2.5Ni/1.0Ca resulted in a count of 537 ± 180 DPM/mg tissue, which was not significantly different from treatment with ^3H -Frescon alone ($p > 0.80$, Student's t statistic). Thus, D-600 significantly reduced labelling of the HV by ^3H -Frescon whereas 2.5Ni/1.0Ca saline did not (Table 9).

Table 9. The effects of D-600 and nickel on ^3H -Frescon labelling of *Lymnaea* heart ventricles

10	Perfusion time (min)		20	Count DPM/mg $\pm$ S.D.	n
	20	10			
Normal Saline		$10^{-5}\text{g/ml } ^3\text{H-Frescon}$	Normal Saline	639 ± 59	8
Normal Saline	2.5mM Ni 1.0mM Ca	2.5mM Ni 1.0mM Ca $10^{-5}\text{g/ml } ^3\text{H-Frescon}$	Normal Saline	537 ± 180	4
Normal Saline	$4 \times 10^{-4}\text{M D-600}$	$10^{-5}\text{g/ml } ^3\text{H-Frescon}$ $4 \times 10^{-4}\text{M D-600}$	Normal Saline	94 ± 43	10

DPM = disintegrations per minute. The results are the average of 'n' experiments

6) THE EFFECT OF CALCIUM-FREE SALINE ON FRESCON CONTRACTURES IN *Lymnaea* MUSCULATURE: A COMPARISON WITH THE ACTION OF CAFFEINE

Since Ca channel blockers interfere with Frescon-induced contracture in *Lymnaea* musculature, it is possible that this molluscicide promotes an increase in free sarcoplasmic Ca

either by increasing the permeability of the sarcolemma to this ion, or by releasing intracellular Ca stores. The latter possibility must be considered since it has not been unequivocally established that the Ca blocking action of the heavy metals or La is restricted to the cell membrane. Therefore, these agents may interfere with Frescon action at intracellular sites, although the antagonistic properties of D-600 would appear to favour a peripheral site of action for this molluscicide.

If the action of Frescon can be attributed to an increased influx of extracellular Ca, then the removal of this ion from the extracellular phase should inhibit Frescon-induced contracture. This was found to be the case. The exposure of *Lymnaea* HV's to 0Ca saline was followed by perfusion with 10^{-5} g/ml Frescon/0Ca. Frescon contracture was prevented under these conditions. The subsequent restoration of extracellular Ca, however, reversed the inhibitory action of 0Ca saline, promoting contracture of the HV (Fig. 25). Qualitatively identical results were obtained with the PRM (Fig. 26).

It may still be argued that the site of action for Frescon could be intracellular, but that internal Ca stores alone are insufficient to produce a contraction. In order to investigate this possibility, the effects of the methylxanthine, caffeine, on *Lymnaea* muscles were investigated. Weber (1968) and Weber & Herz (1968) have shown that caffeine releases Ca from the sarcoplasmic reticulum, but not from mito-

Figure 25. The effect of Ca-free saline on the actions of Frescon and caffeine in the *Lymnaea* heart ventricle.

Frescon:n=6, Caffeine:n=5, Control (1% v/v blank FX 2574):n=4

chondria. Bianchi (1962) provided evidence that caffeine readily penetrates into the cytoplasm. Thus, if internal Ca stores are sufficient for muscular contraction, caffeine should be able to initiate a contraction in the absence of extracellular Ca. Figures 25 and 26 present the results of experiments showing that 2×10^{-2} M caffeine was, in fact, able to induce contraction in 0Ca saline in the PRM and HV. The mean maximum tension induced by caffeine in the HV was 114 ± 35 mg/mg tis-

Figure 26. The effect of Ca-free saline on the actions of Frescon and caffeine in the *Lymnaea* penis retractor muscle.

Frescon:n=5, Caffeine:n=4, Control (1% v/v blank FX 2574):n=4

sue (n=10), and in the PRM it was 1537 ± 225 mg/mg tissue (n=18). This was in contrast to the effect of 0Ca saline on Frescon action, which was to completely inhibit contracture, suggesting that Frescon does not act by releasing sarcoplasmic Ca stores.

This was investigated further by examining the effect of repeated applications of caffeine on Frescon action. Caffeine-induced contraction was progressively reduced upon successive treatments of the PRM with 2×10^{-2} M caffeine/0Ca (each treatment was followed by a wash with 0Ca saline). Eventually, the

PRM no longer responded to caffeine, suggesting that the sarcoplasmic Ca stores were depleted or at least reduced to a level whereby further release was of insufficient quantity to affect the contractile apparatus. A muscle thus treated will henceforth be referred to as 'caffeine- Ca_s -depleted' (Ca_s = SR calcium). Following repeated caffeine application, the caffeine- Ca_s -depleted muscle was exposed to 10^{-5} g/ml Frescon/0Ca. As expected, the muscle failed to respond to this treatment. However, very shortly after exposure to normal saline, the muscle generated a typical Frescon contracture (Fig. 27). These experiments strongly implicate the extracellular

Figure 27. The effect of depletion of sarcoplasmic reticular calcium stores with repeated caffeine applications on the action of Frescon in the *Lymanaea* penis retractor muscle.

The first number in the caffeine 'frame' represents the exposure number, while the second gives the duration (in min) of a 0Ca saline wash. Each caffeine exposure was 10 min long. Note changes in time scale. n=6

compartment as the Ca source during Frescon action.

Following the exposure of the PRM to normal saline, the application of caffeine potentiated the Frescon contraction, suggesting that the internal Ca stores were replenished during the exposure to Frescon in normal saline. In five out of six experiments of this nature, a substantial relaxation of tension was observed following the post-Frescon caffeine treatment. A possible explanation for this relaxation will be considered in the following section.

The controls for the above series of experiments showed that the introduction of normal saline alone to a caffeine- Ca_s -depleted muscle had no effect on the resting tension (Fig. 28). Therefore, it seems likely that the contraction observed upon

Figure 28. The effect of normal saline on the *Lymnaea* penis retractor muscle following the depletion of sarcoplasmic reticular calcium with caffeine.

The first number in the caffeine 'frame' represents the exposure number, while the second gives the duration (in min) of a 0Ca saline wash. Note changes in time scale. n=4

exposure of the PRM to normal saline following caffeine/Frescon/0Ca treatment (Fig. 27) was due to the action of Frescon alone. After caffeine- Ca_s -depletion, the exposure of the PRM to normal saline for a short time (2 min) partly restored the ability of the muscle to respond to a further caffeine/0Ca exposure (Fig. 28), underlying the reversible nature of Ca_s -depletion.

The assumption that caffeine reduced or depleted sarcoplasmic reticular stores in the muscles examined here was not substantiated experimentally. It may have been possible that enough Ca was present in the '0Ca' saline to initiate muscle contraction (see section 9), and that caffeine was more efficacious than Frescon in mobilizing this Ca. If such is the case, then it might be argued that the addition of a Ca channel blocker to 0Ca saline would reduce or prevent caffeine contraction (if caffeine acts via Ca channels in the sarcolemma). The progressive diminution of the muscle's response upon successive caffeine/0Ca treatments tends to argue against such a possibility (see Discussion) but, nevertheless, the PRM was exposed to Frescon/3.5Ni/0Ca and to caffeine/3.5Ni/0Ca after pre-treatment with 3.5Ni/0Ca saline. These conditions completely inhibited the action of Frescon, but the caffeine-induced contraction was not affected (Fig. 29). Again, the subsequent introduction of normal saline brought about a contracture in the PRM, most likely resulting from the previous treatment with Frescon/3.5Ni/0Ca. Therefore, unless caffeine acts via a

Figure 29. The inhibition of Frescon contracture, but not caffeine contraction by 3.5mM Nickel/Ca-free saline in the *Lymnaea* penis retractor muscle.

n=4

nickel-insensitive Ca permeability increase at the level of the cell membrane, it does not seem likely that the very low extracellular Ca concentration (in '0Ca' saline) was sufficient to account for the action of this agent.

Considering the data presented thus far, the most likely working hypothesis would be that Frescon initiates and maintains increased intracellular Ca concentrations by promoting an increase in Ca permeability at the level of the muscle cell membrane, causing a sustained contracture.

7) A COMPARISON OF THE ACTION OF FRESCON TO THE ADENYL
CYCLASE INHIBITOR RMI 12330A

In the previous section, it was shown that the treatment of a Frescon-contracted PRM with caffeine resulted in a further increase in muscle tension which was followed by a substantial, but transient, relaxation. In addition to its action on the sarcoplasmic reticulum, caffeine also inhibits phosphodiesterase (Robison et al., 1968). Such an inhibition would be expected to result in an increased level of cAMP which may, in turn, stimulate Ca pumps, thereby reducing the Ca concentration at the level of the contractile apparatus (see Introduction, section 4-B-b), causing relaxation of the muscle. Alternatively, it may be postulated that reduced levels of cAMP may potentiate, or even initiate muscle contraction by inhibiting the removal of free Ca from the vicinity of the contractile apparatus via a reduction in Ca pump activity. Following this argument, it is possible that Frescon action may be attributed to the inhibition of adenyl cyclase. In order to investigate this possibility, the action of Frescon was compared to the action of a known adenyl cyclase inhibitor, RMI 12330A (cf. Grupp et al., 1980).

The exposure of PRM's to 10^{-3} M RMI 12330A initiated a contracture which reverted to resting levels after about 24 min (Fig. 30). Following this exposure, the muscles became insensitive to electrical stimulation, caffeine treatment or Frescon, i.e., RMI 12330A appeared to have caused irreversible

Figure 30. Contracture induced by the adenyl cyclase inhibitor RMI 12330A in the *Lymnaea* penis retractor muscle in normal and Ca-free saline.

Note change in time scale. $n=8$ for each group.

cell damage at the concentration used. Besides the transient nature of the response of the PRM to RMI 12330A treatment, an additional difference from that of Frescon action was that the contracture induced by the adenyl cyclase inhibitor was neither prevented nor reduced in 0Ca saline (Fig. 30). Therefore, it would appear that the actions of Frescon and of RMI 12330A are expressed via different mechanisms. It should be noted that RMI 12330A was ineffective at concentrations of, or lower than, 10^{-4} M. The concentration used in the present experiments was chosen to initiate a contracture of a magnitude comparable to

that induced by a 10^{-5} g/ml suspension of Frescon.

The inactive analog of RMI 12330A, RMI 12119A, was unfortunately no longer available, precluding a more meticulous investigation of this adenylyl cyclase inhibitor.

8) A COMPARISON OF THE ACTION OF FRESCON TO THE DIVALENT CATION IONOPHORE A23187

A) Contractures induced in *Lymnaea* musculature by A23187

As a corollary to the hypothesis that Frescon induces an increase in sarcolemmal Ca permeability, a Frescon-like contracture should be mimicked by known Ca ionophores. The action of one such ionophore, A23187, on *Lymnaea* HV's and PRM's was examined under various extracellular ionic conditions.

The perfusion of the HV with a 10^{-5} g/ml suspension of A23187 in normal saline induced a contracture which was very similar in time course, magnitude and profile to that induced by 10^{-5} g/ml Frescon (Fig. 31). The maximum tension developed by the HV in response to A23187 treatment was 164 ± 63 mg/mg tissue and the residual tension was 40 ± 23 mg/mg tissue (n=6).

The PRM also responded to A23187 treatment with a contracture (Fig. 32). The ionophore concentration was increased to 2×10^{-5} g/ml in order to more closely approximate the magnitude of the contracture achieved with 10^{-5} g/ml Frescon in this

Figure 31. The effect of the calcium ionophore A23187 on the *Lymnaea* heart ventricle.

n=6

muscle. The mean maximum tension generated by the ionophore was 1017 ± 334 mg/mg tissue (n=6).

The A23187-induced contracture was completely inhibited by the subsequent exposure to 2.5Ni/1.0Ca saline, but not by normal saline or 1.0Ca saline alone. However, after Ni treatment, the introduction of normal saline did not restore the ionophore-induced contracture, in contrast to the restoration by normal saline of the Ni-inhibited Frescon-induced contracture. After Ni treatment of the A23187-contracted PRM, subsequent contracture could be restored only by an additional exposure to a fresh suspension of this ionophore (Fig. 32).

Figure 32. The effect of the calcium ionophore A23187 on the *Lymnaea* penis retractor muscle in normal saline and in the presence of nickel ions.

Unlike Frescon, A23187 induced a substantial contraction in 0Ca saline (mean maximum tension = 367 ± 66 mg/mg tissue, $n=5$). Pre-treatment of the PRM with 0Ca saline for 40 min was followed by exposure to 2×10^{-5} g/ml A23187/0Ca. The latter treatment was associated with a strong contracture which was reduced by about one-third by exposure to 0Ca saline alone. The subsequent introduction of normal saline resulted in approximately a two-fold increase in muscle tension, which was again reduced by the removal of extracellular Ca, but only 2.5Ni/1.0Ca saline was able to completely restore the muscle tension to the resting level (Fig. 33).

Since A23187-induced contracture was not prevented but

Figure 33. The effects of Ca-free saline and nickel ions on the A23187-induced contracture in the *Lymnaea* penis retractor muscle.

Note changes in time scale. n=6

was reduced by ^{Na}0Ca saline, it appears that this ionophore acts at intracellular sites as well as at the level of the sarcolemma. However, since the inhibition of A23187 action by Ni was complete, it seems that this heavy metal ion can penetrate into the interior of the muscle cell. In order to investigate this possibility, the action of La was compared to the action of Ni on A23187-induced contracture.

There is some evidence that La does not penetrate beyond the level of the cell membrane (Laszlo et al., 1952). If La can inhibit A23187-induced contracture, and if it does not penetrate to the interior of the cell, then La inhibition should be similar in magnitude to that of 0Ca saline alone.

If the ionophore has an intracellular as well as a peripheral site of action, and if Ni, but not La, can penetrate the sarcolemma, then Ni alone should reduce the A23187 contracture to the resting level, whereas La, by virtue of its impermeability, would inhibit only the sarcolemmal component of the ionophore-induced increase in Ca permeability. The following experiment was designed to test this supposition.

Lymnaea PRM's were treated with a 2×10^{-5} g/ml suspension of A23187 in normal saline. After A23187 treatment, a wash with normal saline resulted in a transient reduction in tension, suggesting that some of the ionophore had been washed out. However, the subsequent increase in tension indicated that enough ionophore remained in the cell membrane to maintain a progressive influx of Ca into the muscle cells (Fig. 34a). The exposure of the ionophore-treated PRM to either 1.0La/2.5Ca (Fig. 34a) or to 0Ca saline (Fig. 34b) resulted in a reduction of muscle tension. The subsequent exposure of a La-treated PRM to 0Ca saline (Fig. 34a) or a 0Ca-treated PRM to 1.0La/2.5Ca saline (Fig. 34b) had no further effect on muscle tension, i.e., the magnitude of relaxation of an ionophore-treated muscle was the same in both 1.0La/2.5Ca and 0Ca salines. The slight transient increase in tension which was associated with the introduction of 1.0La/2.5Ca saline following 0Ca-induced A23187 inhibition (Fig. 34b) may have been due to the translocation of Ca ions across the cell membrane before the inhibitory action of La was expressed. In contrast

to the action of La, 1.0Ni/2.5Ca saline always reduced the A23187-induced contracture to the resting level (Fig. 34).

Figure 34. A comparison of the effects of lanthanum, Ca-free saline and nickel on A23187-induced contracture in the *Lymnaea* penis retractor muscle.

Figures a and b represent different experiments. Note changes in time scale. $n=4$ for each group

If A23187 does penetrate into the muscle cell, the depletion of intracellular Ca stores should reduce or prevent its action. To test this hypothesis, caffeine- Ca_s -depleted PRM's were exposed to A23187/0Ca. The A23187 contracture was considerably reduced (but not entirely prevented) (Fig. 35). The mean maximum tension generated by the ionophore in a caffeine- Ca_s -depleted PRM was 103 ± 34 mg/mg tissue ($n=4$), which

was significantly lower than that in 0Ca saline alone (cf. Figs. 26 & 35) ($p < 0.01$, Student's t statistic). An interesting result of these experiments was that after exposure of the caffeine- Ca_s -depleted PRM to the ionophore, the previously abolished response of the muscle to caffeine/0Ca was partially restored, suggesting that A23187 did not prevent accumulation of Ca by the caffeine-sensitive compartment. Following the above treatments, the exposure of the PRM to normal saline resulted in a contracture, probably due to the restoration of the sarcolemmal component of A23187 action (Fig. 35).

Figure 35. Reduction of A23187-induced contracture in the *Lymnaea* penis retractor muscle by successive exposures to caffeine.

The first number in the caffeine 'frame' represents the exposure number, while the second gives the duration (in min) of a 0Ca saline wash. Note changes in time scale. $n=4$

B) The action of A23187 on the heart ventricle of *Lymnaea* in the presence of D-600

It has been shown that D-600 can prevent or reduce Frescon contractures, the extent of inhibition being determined by the D-600:Frescon ratio. However, D-600 neither prevented nor reduced A23187-induced contracture in the *Lymnaea* HV (Fig. 36).

Lymnaea HV's were pre-treated with 4×10^{-4} M D-600 prior to exposure to 10^{-5} g/ml A23187 (also in D-600). The mean maximum tension generated under these conditions was 157 ± 23 mg/mg tissue, which was not significantly different from the mean maximum tension generated by the ionophore in the absence of D-600 ($p > 0.85$, Student's *t* statistic).

Figure 36. The lack of an inhibitory action by D-600 on A23187 in the *Lymnaea* heart ventricle.

In summary, the following differences were observed between the actions of A23187 and Frescon on *Lymnaea* musculature:

- 1) A23187-induced contracture was irreversibly abolished by 2.5Ni/0Ca saline, whereas Frescon contracture was reversibly inhibited by this heavy metal.
- 2) Frescon contracture was prevented with 0Ca saline, whereas A23187 contracture was only reduced in magnitude.
- 3) Lanthanum was much more effective in reducing Frescon-induced than A23187-induced contractures in the PRM (compare Figures 23 and 34).
- 4) D-600 blocked or reduced Frescon contractures but did not have any effect on A23187 contractures.

9) AN EXAMINATION OF THE CONSEQUENCES OF THE PRESENCE OF RESIDUAL CALCIUM IN 'CALCIUM-FREE' SALINE

It was found that the concentration of residual calcium in the '0Ca' saline used throughout these experiments was about 8×10^{-6} M. Since A23187 and caffeine were both shown to induce contraction in 0Ca saline, there is a possibility that the concentration of Ca in this saline may have been high enough to account for the observed effects. This possibility is especially *à propos* for the A23187-induced contracture in the PRM even after caffeine- Ca_s -depletion.

In order to investigate the degree of effect the residual Ca levels in 0Ca saline may have had on muscle contraction, the action of 30K/0Ca saline on PRM contracture was

examined. The exposure of the PRM to 30K saline (3.5mM Ca) resulted in a relatively prolonged contracture (K-contracture) which averaged 1200 ± 165 mg/mg tissue (Fig. 37 and 46). If the muscle was pre-treated with 0Ca saline for 30 min and then exposed to 30K/0Ca saline, the magnitude of the K-contracture was reduced by about 95% to 57 ± 41 mg/mg tissue, and its duration was considerably reduced (Fig. 37). In two out of eight preparations, this contraction was entirely prevented. Therefore, unless caffeine or A23187 increase the sarcolemmal permeability to Ca by much more than 30K saline, it would appear that the Ca concentration in 0Ca saline was sufficient to

Figure 37. The reduction of potassium-induced contracture by Ca-free saline in the *Lymanaea* penis retractor muscle.

In this experiment, the introduction of 0Ca saline alone induced a transient contraction of the PRM. Note changes in time scale. n=8

account for only about 4% of the total contractile response observed during caffeine/0Ca treatment of the PRM, and for only about 16% of the total response of the PRM to A23187/0Ca.

It was also found that caffeine- Ca_s -depletion of the PRM invariably prevented any contraction in 30K/0Ca saline (Fig. 38). As this was in contrast to the results obtained with A23187 under identical conditions (see Fig. 35), the ionophore induced contracture could not be attributed to the presence of extracellular Ca alone (assuming that the increase in Ca permeability caused by A23187 was no greater than that for (30K saline). However, both 30K/0Ca and A23187/0Ca treatment of

Figure 38. The prevention of potassium-induced contracture by successive exposures of the *Lymnaea* penis retractor muscle to caffeine.

The first number in the caffeine 'frame' represents the exposure number, while the second gives the duration (in min) of a 0Ca saline wash. Note changes in time scale. $n=5$

a caffeine- Ca_s -depleted PRM partially restored the previously abolished caffeine/0Ca-induced contractile response (Figs. 35 & 38). Therefore, an extracellular source of Ca may have been at least partly responsible for replenishing caffeine-depleted stores during the action of A23187/0Ca (see Discussion, p. 175).

In the experiment represented by Figure 37, the exposure of the PRM to 0Ca alone caused a transient contraction. This was not an isolated event. The exposure of either the HV or the PRM to 0Ca saline sometimes induced a contraction, although the majority of the preparations were unaffected by this treatment. It was observed that 0Ca-induced contractions were grouped over a period of time, i.e., during some times most of the preparations responded to 0Ca saline with a contraction, whereas most of the time this contraction was not evident.

10) A COMPARISON OF THE ACTION OF THE ANTHELMINTIC PRAZIQUANTEL AND FRESCON ON THE *Lymnaea* PENIS RETRACTOR MUSCLE

Praziquantel (PQ) has been reported to increase the permeability of the sarcolemma of schistosome musculature to Ca (Fetterer et al., 1980). As this is the proposed mode of action for Frescon, it was of interest to compare the effects of these two agents on *Lymnaea* musculature.

The exposure of the PRM to 1.6×10^{-6} M PQ induced a

contracture which was very similar in time course and magnitude to that induced by 10^{-5} g/ml Frescon. However, in contrast to Frescon contracture, the action of PQ was completely reversed by washing the preparation with normal saline. Moreover, the PQ-induced contracture was not prevented by 0Ca saline although the contracture was considerably reduced in magnitude and duration. The re-introduction of PQ/normal saline restored the original contracture (Fig. 39). The reduction of the magnitude of PQ contracture in 0Ca saline was observed only when the PRM was previously treated with PQ. When the muscle was exposed directly to PQ/0Ca, i.e., without a prior PQ treatment, the magnitude of the contracture was not reduced, although the duration was shortened (e.g. see Fig. 41). The mean maximum tension developed in the penis retractor muscle by 1.6×10^{-6} M PQ in normal saline was $1727 \pm$

Figure 39. The effects of normal and Ca-free saline on the praziquantel-induced contracture in the *Lymnaea* penis retractor muscle.

247 mg/mg tissue (n=9), whereas the mean maximum tension for the same concentration of PQ in 0Ca saline (without previous exposure to PQ) was 1754 ± 236 mg/mg tissue (n=5). The difference between these two values is not statistically significant ($p > 0.85$, Student's t statistic). Pre-treatment of the PRM with PQ/normal saline, however, reduced the mean maximum response of the PRM in PQ/0Ca to 624 ± 49 mg/mg tissue (n=5), which is significantly lower than that in normal saline ($p < 0.001$, Student's t statistic).

These results suggest an intracellular, as well as a sarcolemmal site for PQ action, and it would appear that the PQ-sensitive intracellular Ca stores can be partially depleted by successive treatments with PQ. As this latter action is very similar to that of caffeine, it was thought that perhaps both caffeine and PQ affect the same Ca stores. In support of this possibility, it was found that PQ/0Ca contractures can be completely eliminated by caffeine- Ca_s -depletion. Following successive treatments of the PRM with caffeine/0Ca, the exposure to 1.6×10^{-6} M PQ/0Ca had no effect on resting tension (Fig. 40).

In order to further examine a possible intracellular site for PQ action, and to assess the consequences of a possible involvement of the residual Ca in 0Ca saline during the action of PQ/0Ca, La was used to block the influx of extracellular Ca. Pre-treatment of the PRM with 1.0La/0Ca saline prevented contraction in 30K/1.0La/0Ca saline, but did not prevent

Figure 40. Prevention of praziquantel-induced contracture in Ca-free saline by successive treatments of the *Lymanaea* penis retractor muscle with caffeine.

The first number in the caffeine 'frame' represents the exposure number, while the second gives the duration (in min) of a 0Ca saline wash. Note change in time scale. $n=4$

PQ-induced contracture (Fig. 41). Although the maximum tension by the PRM in response to PQ/1.0La/0Ca was not reduced when compared to the response to PQ/normal saline, 1.0La/0Ca did reduce the duration of the response and increased the rate of decay of muscle tension.

It may be argued that PQ induces a La-insensitive increase in membrane Ca permeability, and that the residual levels of Ca in 0Ca saline might still have been sufficient to account for the PRM contracture following PQ/1.0La/0Ca treat-

Figure 41. The effects of lanthanum on praziquantel- and potassium-induced contracture in the *Lymnaea* penis retractor muscle.

In this experiment, the exposure of the PRM to 1.0La/0Ca saline resulted in a small, transient contraction. Note changes in time scale. n=4

ment. This, however, appears not to be the case. A PRM was treated with PQ/normal saline (initiating a sustained contracture) and subsequently exposed to PQ/1.0La/2.5Ca saline. Within several minutes, the muscle relaxed to slightly above control levels, even though PQ and Ca were still present in the extracellular phase (Fig. 42). Therefore, if La almost completely reversed PQ-induced contracture in the presence of 2.5mM Ca, it would be expected that it should also have inhibited contracture in 0Ca saline (if only extracellular Ca was involved), especially after pre-treatment of the PRM with 1.0La/0Ca saline (cf. Fig. 41).

Figure 42. Inhibition of praziquantel-induced contracture by lanthanum in the *Lymanaea* penis retractor muscle.

Note change in time scale. n=4

In summary, the major differences between PQ and Frescon actions were found to be the following:

- 1) Unlike Frescon-induced contracture, PQ-induced contracture was not prevented by 0Ca saline, and the action of PQ could not be attributed to the presence of residual Ca in the extracellular medium.
- 2) PQ contracture was reversible if washed away with normal saline, whereas Frescon contracture was not.
- 3) PQ appears to affect the caffeine-sensitive Ca stores (as the depletion of these stores prevents PQ-induced contracture in 0Ca saline), whereas Frescon does not (cf. Figs. 27 & 40).

11) THE RELATION BETWEEN THE ACTIONS OF FRESCON, SODIUM
PENTOBARBITAL AND ELEVATED POTASSIUM CONCENTRATION
ON *Lymnaea* MUSCULATURE

The experimental evidence presented thus far is consistent with the hypothesis that Frescon increases the permeability of the snail muscle cell membrane to Ca. Several mechanisms may be postulated to account for such an action. Frescon may act by

- 1) blocking open existing Ca channels (i.e., either PDC or ROC channels, or both),
- 2) creating new channels by acting as an ionophore, or
- 3) inhibiting membrane-bound mechanisms which are instrumental in maintaining low intracellular Ca concentrations, e.g. Ca-ATPase or the Na-Ca exchange process. Such an inhibition could lead to elevated intracellular Ca levels and subsequent contracture.

The first possibility is considered in this section. The second requires further investigation, although the differences between A23187 and Frescon action tend to argue against such a role for this molluscicide (however, see Discussion, section 4). The third possibility has been examined only in a partial and preliminary fashion (see following section).

The anaesthetic sodium pentobarbital (NAPB) reportedly inhibits potential-dependent Ca channels (Blaustein & Ector, 1975). If Frescon acts to block open these channels, then it might be possible for NAPB to either prevent or partially inhibit the action of this molluscicide.

Lymnaea PRM's were exposed to 5×10^{-3} M NAPB for 30 min. This treatment greatly inhibited the contracture in 30K saline, but did not prevent contracture induced by 10^{-5} g/ml Frescon (Fig. 43). However, the rate of muscle tension development was considerably reduced. In the presence of NAPB, half-maximal tension following Frescon exposure was reached in just over 4 min, whereas in the absence of NAPB, half-maximal tension was reached in less than 1 min. Moreover, the introduction of normal saline to the NAPB/Frescon-treated PRM resulted in a further increase in muscle tension, suggesting that NAPB did have some inhibitory effect on Frescon.

It would be expected that if Frescon acts to block open

Figure 43. The effect of sodium pentobarbital on Frescon- and potassium-induced contracture in the *Lymnaea* penis retractor muscle.

Note changes in time scale. n=5

PDC channels, then the exposure of a Frescon-treated PRM to 30K saline should result in little, if any, further increase in tension. However, exposure of Frescon-contracted PRM's to 30K saline resulted in a considerable potentiation of contracture, although this potentiation was transient (Fig. 44). Similar results were obtained for the *Lymnaea* HV, but the post-Frescon exposure of this muscle to 30K saline resulted in a much more pronounced potentiation of contracture (relative to the residual tension) (Fig. 45). The treatment of a Frescon-treated PRM with 30K saline resulted in an initial relaxation of ten-

Figure 44. Potentiation of Frescon-induced contracture in the *Lymnaea* penis retractor muscle by high potassium saline.

Note change in the time scale. n=5

Figure 45. Potentiation of Frescon-induced contracture in the *Lymnaea* heart ventricle by high potassium saline.

Note change in time scale, n=4

sion which was followed by a transient potentiation of contracture. The final residual tension in 30K saline was always about 15% less than the original residual tension in Frescon alone. The relaxation in tension following 30K treatment was not evident in Frescon-treated HV's.

In a variation of the above experiments, PRM's were first exposed to 30K saline and subsequently to Frescon. The exposure to 30K saline alone resulted in a relatively prolonged contracture which approached resting levels after about 16 min. The addition of Frescon/30K at this point induced a slowly-

Figure 46. Frescon-induced contracture of a potassium-contracted *Lymnaea* penis retractor muscle.

Note change in time scale. n=4

developed, sustained contracture (Fig. 46).

If Frescon acts on PDC channels, it might be expected that a measurable depolarization of V_m would result from the Frescon-induced influx of Ca. That such a depolarization does not occur has already been shown (see Fig. 18). However, the earlier experiment did not include the simultaneous measurement of muscle tension.

Lymnaea HV strips were exposed to 10^{-5} g/ml Frescon while measuring the tension and V_m simultaneously. The results show that V_m did not change during contracture of the muscle (Fig. 47). It is difficult to interpret these results since the amount of Ca influx required to promote a measurable change in

Figure 47. The simultaneous measurement of tension and membrane potential in the *Lymnaea* heart ventricle strip preparation.

n=5

V_m is not known (see Discussion, section 5-a). However, the results as a whole suggest that although Frescon may have some effect on PDC channels, its action cannot be wholly attributed to such an action. Certainly, in the PRM at least, 30K-induced and Frescon-induced contractures were considerably different with respect to their contractile profiles, and the actions of these two agents were not mutually exclusive. Therefore, if Frescon does act on Ca channels, the ROC channels seem to be better candidates for the action of this molluscicide.

12) A COMPARISON OF THE ACTIONS OF FRESCON AND SODIUM-FREE SALINE ON *Lymnaea* MUSCULATURE

The exposure of the PRM to 0Na saline resulted in a contracture which was very similar to that induced by Frescon. Presumably, this contracture is the result of an inhibition of the Na-Ca exchange mechanism (Baker, 1972). If Frescon acts in the same manner, then the exposure of a Frescon-treated muscle to 0Na saline should not potentiate the contracture (but only if Frescon inhibits the Na-Ca exchange as completely as 0Na saline).

The exposure of a Frescon-treated PRM to 0Na saline resulted in a potentiation of the contracture (Fig. 48). The

Figure 48. Potentiation of Frescon-induced contracture by sodium-free saline in the *Lymnaea* penis retractor muscle.

n=4

sustained portion of the Frescon-induced contracture was greater in magnitude than that in ONa saline alone, suggesting that Frescon is the more potent of the two. The sustained magnitude of the Frescon/ONa-induced contracture was greater than that induced by either Frescon or ONa saline alone. These results suggest either that Frescon does not act via an inhibition of the Na-Ca exchange, or if Frescon does act via this mechanism, it does not inhibit it as effectively as ONa saline. However, in view of the above results, the latter possibility does not seem as likely as the former.

DISCUSSION

1) THE EFFECT OF FRESCON ON SNAIL MUSCULATURE

Frescon has been shown to cause contracture in isolated smooth (LFS and PRM) and cross-striated (HV) muscles of *Lymnaea*, and in a smooth muscle (PRM), but not the HV, of *Helix*. In the case of *Lymnaea*, the onset of this contracture was rapid and irreversible; having occurred within 20-30 sec of exposure of the muscles to a 10^{-5} g/ml suspension of Frescon. The magnitude (force per unit weight) of the Frescon-induced contracture was much greater in the PRM than in either the LFS or HV. The LFS, however, is not a discrete muscle, consisting of muscle cells diffusely embedded in a collagenous matrix, and aligned in several directions (Plesch et al., 1975). Therefore, the additive force of their simultaneous contraction would not be expected to be generated along a single vector. Consequently, the measured force during contracture of the LFS would be less than that of the PRM, a discrete muscle which contains many more muscle cells per unit volume than the LFS. The HV, on the other hand, is a cross-striated muscle and as such, contains much shorter contractile filaments than the PRM. As a result, it cannot generate as great a tension as the smooth muscle (Rüegg, 1971; Plesch, 1977).

Both the PRM and the LFS were able to maintain Frescon contractures at a much higher level, relative to maximum,

than the HV. In the latter organ, the Frescon-induced contracture dropped within a few minutes to a new stable level which was less than 50% of maximum, although it never returned to control levels. On the other hand, both the PRM and the LFS were able to maintain tensions which were 85-95% of maximum. In the case of many molluscan smooth muscles, slow relaxation has been associated with the presence of paramyosin (see Introduction, p. 11). This protein does not occur in appreciable amounts in molluscan 'striated' muscle (Rüegg, 1971). However, that paramyosin-dependent prolonged contracture ('catch') was invoked by Frescon in the PRM and LFS, but not the HV, is not likely as catch is not affected by the removal of extracellular Ca (Ca_e) (Rüegg, 1971), whereas Frescon contracture was prevented under these conditions (see Figs. 25 & 26).

It may be that the HV muscle cells are more efficient at reducing the Frescon-induced increase in intracellular Ca (Ca_i) levels (see later), and/or the heart, but not the PRM or LFS, may have a series-elastic component which relaxes following extreme contracture. Some muscles show a decrease in the phase advance of sinusoidal tension changes relative to applied sinusoidal length changes, indicating a decrease in structural viscosity (Rüegg, 1971). A decrease in this 'viscoelastic' component during muscle stretch (or generated tension as in the case of the Frescon-treated HV) may have been responsible for at least a part of the relaxation of the

Frescon contracture in the HV. Furthermore, smooth muscles in general can maintain higher tensions per unit of metabolic energy than striated muscles. The greater energy economy of smooth muscle tension has been attributed to a decreased rate of ATP splitting by Ca-activated ATPases in the contractile filaments (Rüegg, 1971). The slow relaxation of smooth muscles may be due to a relatively slow rate of Ca uptake by the sarcoplasmic reticulum (SR) (Hasselbach, 1964, 1968) and/or to a slower rate of turnover of actin-myosin linkages (Lowy & Millman, 1963). However, relaxation of the Frescon-treated HV can be attributed to a more efficient uptake of Ca by intracellular organelles only if the capacity of these stores is sufficient to maintain reduced Ca_i levels during the prolonged increase in the Frescon-induced influx of Ca_e . At present, there is insufficient information to resolve this question. Nevertheless, a combination of some or all of the factors described above may have contributed to the differences in the duration and the response profile of Frescon-induced contractures in the molluscan muscle types thus far examined.

The PRM of *Helix* responded to Frescon differently from its counterpart in *Lymnaea*. Frescon contractures in the *Helix* PRM took 2-5 min to develop and were initially transient, returning to resting tension within about 20 min. Powerful transient contractures could be evoked over a period of more than 1h by electrical stimulation before a final irreversible

and sustained contracture was generated (Fig. 12). A further difference between the two genera was that the *Helix* HV was not sensitive to either perfusion or superfusion with up to 10^{-4} g/ml Frescon, whereas the *Lymnaea* HV was sensitive to perfusion with a concentration as low as 10^{-6} g/ml (lower concentrations were not tried). However, the *Lymnaea* HV also did not respond to extracardial application of Frescon.

Molluscan hearts have an epicardial sheath covering the muscle bundles on the outside, but not on the inside where the trabeculae are directly exposed to the chamber interior (Hill & Welsh, 1966). It is very probable that this sheath acts as a diffusion barrier to extracardial Frescon in *Lymnaea*, but unless the *Helix* HV has a comparable diffusion barrier covering the individual trabeculae inside the heart chamber, its lack of response to intracardial Frescon cannot be attributed to such a barrier but rather to a basic physiological difference (e.g. different Ca channel 'receptors', see later) from *Lymnaea* cardiac tissue.

Interestingly, *Helix* is not affected either by ingesting Frescon-treated lettuce leaves (Shell, 1974) or by direct exposure to a 10^{-5} g/ml suspension of Frescon (Gardner & Moreton, unpublished observations). Since the isolated PRM of this animal is affected by this molluscicide, a likely explanation for the immunity of the whole animal is the existence of a set of diffusion barriers which prevent Frescon from reaching sensitive tissues. These barriers would, of course,

have to be absent in *Lymnaea*. However, diffusion barriers alone cannot entirely account for the lack of molluscicidal effectiveness of Frescon against *Helix*. In addition to a lack of an effect of intracardial Frescon on the HV of this animal, the isolated brain, in which all diffusion barriers have been removed, is also insensitive to Frescon (Gardner & Moreton, unpublished observations), whereas the electrophysiological activity of the isolated *Lymnaea* brain is disrupted by this molluscicide (Moreton & Gardner, 1976). Clearly, target sites for Frescon are not available in all molluscan tissue.

2) THE EFFECT OF FRESCON ON THE RESTING POTENTIAL OF *Lymnaea* HEART VENTRICLE MUSCLE CELLS

At the beginning of these investigations, it was surmised that the action of Frescon on snail musculature might be explained by an alteration of normal electrophysiological parameters. For example, if this molluscicide altered Na and/or K permeability in such a way as to induce a sustained depolarization, it would be expected that Ca would enter the cell via activated potential-dependent Ca (PDC) channels, thereby inducing contracture. However, the paucity of information on the ionic basis of the resting potential (V_r) in molluscan muscles in general, and the complete lack of it for *Lymnaea* in particular, prompted an investigation of the

electrophysiology of *Lymnaea* heart ventricle muscle cells (HVMC's).

A) *The ionic basis of the resting potential*

The mean V_r in *Lymnaea* HVMC's ($[K]_e = 1.6\text{mM}$) was found to be $-61.2 \pm 3.5\text{mV}$, which is substantially less negative than that in vertebrate cardiac muscle (-90mV to -100mV ; Weidmann, 1956). The average $[K]_i$ was estimated to be $51.5 \pm 14.6\text{mM}$ (ranging from 27.8mM to 77.3mM), which is considerably lower than that of vertebrate cardiac muscles ($>100\text{mM}$; Kunze & Russell, 1981; Walker, 1981). However, apart from the low V_r and the low $[K]_i$, the ionic basis of the resting potential in the HVMC's was found to be similar to that of vertebrate skeletal muscle in that the HVMC membranes appear to be permeable to both K and Cl, since changes in the extracellular concentration of either of these ions resulted in a change in the membrane potential (V_m) (Figs. 13 & 14). They also appear to have a resting permeability to Na (Fig. 16), which may explain their relatively low V_r (cf. Hodgkin & Horowicz, 1959).

a) *Variations of $[Cl]_e$, with constant $[K]_e$*

When $[Cl]_e$ was changed from 62.6mM to 5mM , the HVMC membrane was depolarized from -58mV to -52mV (using the results in Fig. 7). This depolarization was transient and the membrane potential returned to -56mV (2mV less negative than

the original V_r) after about 3 min. This lower V_m could not be accounted for by membrane damage as restoration of the original $[Cl]_e$ caused the HVMC membrane to hyperpolarize from -56mV to -63mV. This hyperpolarization was also transient, and the V_m returned to -59mV after about 2.5 min., 1mV more negative than the original V_r . These results are consistent with those expected if the membrane is permeable to both K and Cl. Assuming little or no membrane permeability to other ion species at equilibrium, then $V_r = E_{Cl} = E_K$ (where E_{Cl} and E_K are the Cl and K equilibrium potentials, respectively). Therefore,

$$E_K = V_r \text{ (mV)} = 58 \log ([K]_i/[K]_e) \quad (6)$$

and

$$E_{Cl} = V_r \text{ (mV)} = 58 \log ([Cl]_e/[Cl]_i) \quad (7)$$

Initially, when $[Cl]_e$ was changed from 62.5mM to 5mM (keeping $[K]_e$ constant), the E_{Cl} should have changed from -58mV to -6mV (using equation (7)), whereas E_K , by virtue of the constant $[K]_e$ (and assuming $[K]_i$ did not change immediately), should have remained unchanged (cf. Hodgkin & Horowicz, 1959). As a result, V_m would take up a new value between E_{Cl} and E_K at which K and Cl ions leave the cell at the same rate, along with water to preserve osmotic equilibrium, until $[K]_e \times [Cl]_e = [K]_i \times [Cl]_i$ (Boyle & Conway, 1941). The hyperpolarization of the membrane upon restoration of the original $[Cl]_e$ could be explained in the same way except that in this case, E_{Cl} would be initially more negative than V_m and KCl and water

would enter the cell to restore the Donnan equilibrium. However, in the present experiments, V_m never changed by the predicted amount during the reduction of $[Cl]_e$. In the above example, V_m should have changed from -58mV to -26mV, whereas only a 6mV depolarization was observed. However, the full magnitude of the expected change in V_m can only be measured when $[Cl]_e$ is changed very rapidly (Hodgkin & Horowicz, 1959). In the present experiments, about 1 min was required to completely exchange the bath contents (faster flow rates dislodged the microelectrode). Such slow changes in $[Cl]_e$ would be associated with a gradually changing E_{Cl} and E_K along with a continuous re-equilibration of K and Cl across the cell membrane. Under these conditions, V_m would always remain closer to E_K since $[K]_i \gg [Cl]_i$ (see below), and the full magnitude of the expected change would not be observed. In fact, if the rate of reduction of $[Cl]_e$ is slow enough, no detectable change in V_r should occur if $[K]_i$ is high compared to $[Cl]_i$, as each incremental change in $[Cl]_e$ would result in a rapid restoration of the Donnan equilibrium between K and Cl (where $[K]_e \times [Cl]_e = [K]_i \times [Cl]_i$). If $[Cl]_i$ is very low compared to $[K]_i$, only a very small outward movement of Cl would be required to re-establish this equilibrium. A similar movement of K, which would accompany Cl, would leave E_K , and therefore the original V_r , practically unchanged. For example, Hodgkin & Horowicz (1959) reported that the V_r in frog skeletal muscles was quickly restored following changes induced by lower-

ing or restoring $[Cl]_e$, and attributed the restoration of V_r to an insignificant change in $[K]_i$ during variations in $[Cl]_e$.

However, the hysteresis in V_m following a change in $[Cl]_e$ was observed in all of the HVMC's examined, i.e., the original V_r was not restored within the 2-3 min time frame of the experiment, and always stabilized at a level about 2mV less negative (following a reduction of $[Cl]_e$) and about 1mV more negative (following the restoration of the original $[Cl]_e$) than the control level. In view of the above, it would appear that $[K]_i$ did change in the HVMC's. For example, the lowest estimate for $[K]_i$ was 27.8mM. By the Donnan equilibrium this would necessitate a $[Cl]_i$ of 3.6mM. Thus, ionic and physiological parameters for an HVMC might be the following (note that all the parameters are within experimentally observed values).

$$V_r = -58mV$$

$$[K]_e = 1.6mM$$

$$[K]_i = 27.8mM$$

$$[Cl]_e = 62.6mM$$

$$[Cl]_i = 3.6mM$$

$$[K]_e \times [Cl]_e = [K]_i \times [Cl]_i = 100mM^2$$

$$\alpha = P_{Na}/P_K = 0.023 \text{ (see section 2-A-c, fitted to yield } V_r = -58mV \text{ using equation (8), } [Na]_e = 52.35mM \text{)}$$

According to Boyle & Conway (1941), when $[Cl]_e$ is reduced from 62.6mM to 5mM ($[K]_i \times [Cl]_i = 8.0mM^2$), KCl will move out

of the muscle fibre until $[K]_i = 24.5\text{mM}$ and $[Cl]_i = 0.33\text{mM}$ (where $[K]_e \times [Cl]_e = [K]_i \times [Cl]_i = 8\text{mM}^2$). This represents a significant change in $[K]_i$. As a result of this new equilibrium and the new $[K]_i$, the new V_m would be -54.6mV (using equation (8)), almost 4mV less negative than the original value of -58mV . On the other hand, it can be shown that if $[K]_i = 77.3\text{mM}$ (the highest estimated value for HVMC's) and $[Cl]_i = 1.3\text{mM}$ ($[K]_e \times [Cl]_e = [K]_i \times [Cl]_i = 100.16\text{mM}^2$); $\alpha = 0.115$, fitted to equation (8) to yield $V_r = -58\text{mV}$, a reduction of $[Cl]_e$ from 62.6mM to 5mM would result in a reduction of $[K]_i$ of less than 0.01mM , and the V_r at the new equilibrium would be virtually unchanged.

It was also observed that upon restoration of the original $[Cl]_e$ following its reduction, V_m stabilized at a level which was 1mV more negative than the original V_r . This may be experimentally insignificant, or it may reflect a genuine phenomenon: possibly a greater inward permeability to K than to Cl. Since the restoration of $[Cl]_e$ is initially associated with the establishment of E_{Cl} which is more negative than either V_r or E_K , K ions may flow down this new electrochemical gradient more readily than Cl ions. For example, some muscle cells, as well as the squid axon, exhibit the property of 'anomalous rectification' where inward K flux is increased under the influence of a more negative V_m , in contrast to 'normal' K channels which exhibit the opposite response (Katz, 1949; Noble, 1962; Armstrong & Binstock, 1965; Armstrong,

1975). A similar phenomenon in molluscan muscle cells might account for the hysteresis observed during the restoration of the original $[Cl]_e$, a treatment which would be expected to generate a more negative V_m which, in turn, might enhance the inward movement of K, but not the inward Cl movement. Consequently, at the new equilibrium $[K]_i$ may be greater than it was before, resulting in a more negative V_m .

b) *Variations of $[K]_e$ with a constant $[K]_e \times [Cl]_e$ product*

If the HVMC's are freely permeable to only K and Cl, and if the $[K]_e \times [Cl]_e$ product is kept constant, then V_m should conform to the relation described by equation (6). In the present experiments, V_m was found to be relatively insensitive to changes in $[K]_e$ at concentrations below 6mM. An average slope of 50.4 ± 3.8 mV per decade change in $[K]_e$ (K-slope) was obtained for values of $[K]_e$ between 6mM and 40mM. This is about 8mV lower than predicted by equation (6), suggesting that the HVMC's are permeable to some ion(s) other than K or Cl. It is unlikely that changes in $[Cl]_i$ or $[K]_i$ contributed to the reduction of the K-slope, since constant $[K]_e \times [Cl]_e$ product salines were always employed. Occasionally, K-slopes of close to 58mV were observed, although the majority were of the order of 48mV to 52mV. These low values could not be attributed to experimental error, since low K-slopes were obtained even after the techniques of recording from the HVMC's had become well-practiced.

c) Alteration of $[Na]_e$ with $[K]_e$ and $[Cl]_e$ constant

In all cases, V_r was considerably lower than that predicted by equations (6) or (7). This type of deviation could be explained if the HVMC membrane has a small resting permeability to Na. If such is the case, the removal of Na_e should result in a hyperpolarization of the cell membrane, since the Na electrochemical gradient would be oriented outward and Na should leave the cell. However, when the HVMC's were exposed to Na-free saline, the membrane depolarized and the cells contracted. If normal saline was restored before the contraction dislodged the microelectrode, the V_m was seen to revert to its original level. Interestingly, when $[Mg]_e$ was elevated and $[Ca]_e$ reduced, the response of the V_m to the removal of Na_e changed from a depolarization to a transient hyperpolarization, although eventual contraction was not prevented (Fig. 16). It would seem, therefore, that under normal conditions, the depolarization was a direct or indirect result of Ca entry into the cell, and that this Ca entry, and associated depolarization, could be blocked by elevating $[Mg]_e$ and lowering $[Ca]_e$. Removal of Na_e inhibits, or possibly reverses the Na-Ca exchange mechanism (Baker, 1972), and increases the influx of Ca into the cell (Coraboeuf et al., 1981). However, whether or not an elevation of free Ca_i levels alone could be responsible for the observed depolarization cannot be determined on the basis of the available evidence. Nevertheless, under appropriate conditions the removal of Na does cause a

hyperpolarization of the HVMC membrane, an observation which is consistent with the proposal that this membrane has a slight resting permeability to Na.

Using the theoretical treatment applied by Moreton (1968) and Sattelle (1974) (see Results, section 4-A), the permeability ratio of Na to K ($p_{Na}/p_K = \alpha$) was found to vary from 0.01 to 0.14. The V_r (or V_m , assuming α does not change) could therefore be defined by a modified form of the Hodgkin-Katz equation, i.e.,

$$V_m (\text{mV}) = 58 \log . ([K]_e / [K]_i + \alpha [Na]_e / [K]_i) \quad (8)$$

This equation produces values for V_m which closely approximated those observed at $[K]_e$'s between 1mM and 40mM. In only four out of twenty successful experiments was the deviation between the observed values and those predicted by equation (8) as high as 10mV, and even then for only some points. Generally, the above deviation did not exceed 2-4mV (Table 8). However, it must be stressed that the HVMC's are small cells and it was very difficult to maintain successful impalement with microelectrodes for long periods of time without incurring membrane damage. Therefore, it is possible that the deviations between observed and predicted V_m 's may have been due to artificially low V_r 's from the start of the experiment. A greater sample size is required before firmer conclusions can be drawn.

d) The effect of Frescon on the resting potential

In the presence of 10mM Mg and 0.5mM Ca, Frescon had no effect on the V_r of the HVMC's during exposures of up to 60 min (Fig. 17). When Frescon was introduced in normal saline, the muscle cell membrane was initially hyperpolarized by about 2mV. After several minutes, however, V_m reverted to the original level (Fig. 18). These changes in V_r following Frescon treatment may have been due simply to changes in the physical state of the recording microelectrode alone, e.g. a contracture of the muscle cell may have distorted the microelectrode tip causing a change in the tip potential, although the consistency of the hyperpolarizing response of the HVMC membrane to Frescon suggests that a physiologically significant change cannot be ruled out. For example, it may be that Ca entry associated with Frescon action (see following section) caused a change in the cell membrane permeability to some other ion(s). It is known, for instance, that Ca entry in *Helix aspersa* neurons causes an increase in the efflux of K and subsequent hyperpolarization of the neurolemma (Meech & Standen, 1975). It may be that an identical phenomenon can be generated in *Lymnaea* HVMC's as well. In any case, Frescon does not alter the V_r of the HVMC's in a way that could be associated with prolonged contraction, i.e., the muscle membrane is not depolarized. Consequently, it was concluded that Frescon does not act by altering any of the parameters responsible for maintaining V_r (with the possible

exception of an increase in the outward movement of K, an event which, in any case, would not be associated with muscle contraction.

3) THE ACTION OF FRESCON ON CELLULAR CALCIUM REGULATION

Contraction in the *Lymnaea* HV was prevented when Frescon was introduced in low Ca-high Mg saline (Fig. 19). High levels of Mg_e are known to interfere with many Ca_e -dependent processes in different types of tissues by inhibiting Ca influx (Hodgkin & Keynes, 1957; Niedergierke & Harris, 1957; Gilbert, 1960; Alcock & MacIntyre, 1962; Douglas & Rubin, 1963). Therefore, given the failure of Frescon to depolarize the cell membrane, it may be postulated that this molluscicide somehow induces a large and irreversible increase in the sarcolemmal Ca permeability. However, since at least some molluscan cell membranes are permeable to Mg (Baker & Crawford, 1972), it may be that the Mg-dependent inhibition of Frescon action occurs at the level of the contractile apparatus, and that the action of this molluscicide is at an intracellular locus. Given this possibility, several hypotheses may be postulated to explain the action of Frescon as follows:

- 1) Frescon acts primarily to increase the Ca permeability of the sarcolemma to such an extent that the cellular sequestering and/or extrusion processes are overloaded, i.e.,

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the action of this molluscicide is at the level of the cell membrane.

- 2) Frescon acts to release intracellular Ca stores and, by inhibiting the relevant energy-dependent processes, prevents the re-uptake of Ca by these stores (e.g. the inhibition of the SR Ca-ATPase alone may be sufficient to release Ca_i stores; Endo, 1977), and
- 3) Frescon acts at the level of the contractile apparatus by inhibiting cross-bridge turnover (e.g. a paramyosin-like action), as well as to promote an increase in the sarcoplasmic levels of free Ca by one or both of the mechanisms described above.

The resolution of these hypotheses centres around the question of whether or not Frescon has an intracellular site of action.

A) The effect of calcium entry blockers on Frescon contracture

The heavy metals Ni, Co and Mn, and the rare earth La were all able to prevent, or almost completely reverse Frescon contracture in *Lymnaea* muscles (Figs. 20-23). As these agents are known to block Ca entry into cells (see Introduction, section 4), it is likely that Ca_e is an important requirement for Frescon action. It cannot be concluded that Frescon acts at the level of the sarcolemma on this basis alone, as there is no evidence that the heavy metals or La cannot penetrate into the interior of *Lymnaea* muscles. Consequently, it may be that in addition to promoting an increase in sarcolemmal Ca permeability, Frescon acts at a site inside the cell as well, and Ca_e may be necessary for the expression of this action. How-

ever, the following lines of evidence, taken together, argue strongly against an intracellular locus for Frescon action.

The Ca entry blocker D-600 not only inhibited Frescon contracture in the HV, it also prevented Frescon binding (Fig. 24, Table 9), although a much higher concentration of D-600 was required than that reported to block PDC channels in vertebrate muscles (e.g. see Kohlhardt et al., 1972). Its interference with this molluscicide was probably due to a non-specific inhibition, since the concentration of D-600 required to block Frescon action in the HV (2×10^{-4} M) has been shown to interfere with a variety of membrane-associated events (see Introduction, p. 44), although there is evidence that high concentrations of D-600 can block receptor-operated Ca (ROC) channels (Schumann et al., 1975). Nevertheless, its action *vis-à-vis* Frescon cannot be unequivocally attributed to a block of Ca entry on the basis of these results alone, although the ability of D-600 to inhibit Frescon binding argues in favour of a membrane site of action for this molluscicide. An alternative explanation could be that D-600 merely blocked a cell membrane permeability to Frescon. It was not possible to distinguish between these two possibilities on the basis of the techniques used to measure Frescon binding, although the former possibility seems more probable since it is unlikely that there are specific channels which can be utilized for an inward flux of Frescon and blocked by D-600.

(If Frescon acts to release intracellular Ca stores, its action should not be inhibited by the removal of Ca_e . However, when Frescon was introduced to either the HV or PRM in the absence of Ca_e , contracture was completely inhibited. On the other hand, contractions induced by caffeine, an agent which probably releases bound Ca from Ca_i stores (Weber, 1968), were not blocked by Ca-free saline (Figs. 25 & 26), indicating that these muscles have sufficient Ca_i stores to produce contractions. Consequently, the lack of an effect of Frescon in Ca-free saline cannot be attributed to insufficient Ca_i stores, suggesting that Frescon does not alter the function of Ca_i -sequestering mechanisms. Moreover, the profile of caffeine contraction was not altered by this molluscicide, i.e., the pre-treatment of a muscle with Frescon/0Ca did not alter the transient nature of caffeine/0Ca contraction (cf. Figs. 28 & 29). If Frescon caused, for example, an inhibition of cross-bridge turnover, the caffeine contraction should have been prolonged. As this was not the case, the molluscicide probably did not interfere with the contractile apparatus. Furthermore, that the caffeine contractions were able to relax at all in the presence of Frescon suggests that this molluscicide did not inhibit cell membrane-bound Ca extrusion mechanisms.

These arguments are based on the assumption that caffeine acts to release sarcoplasmic reticular Ca stores. However, the 'Ca-free' saline used in these experiments con-

tained a small amount of Ca^{2+} ($8 \times 10^{-6} \text{M}$). The Ca chelating agent EGTA caused irreversible damage to the muscles and could not be used. Consequently, the use of caffeine to underline a lack of an intracellular action for Frescon may be suspect. However, caffeine contractions were not blocked by the addition of 3.5mM Ni to the 'Ca-free' saline, even if the muscle preparation had been pre-treated for up to 15 min with 3.5Ni/0Ca saline (Fig. 29), whereas Frescon contraction was inhibited even by less severe ionic conditions (Fig. 22). With such a high Ni:Ca ratio, it is very doubtful that the action of caffeine can be explained by means other than the release of intracellular Ca. Furthermore, 0Ca saline reduced K contractures to a level which was less than 95% of that of caffeine contractions in the same saline (cf. Figs. 26 & 27). Therefore, unless caffeine increases the sarcolemmal Ca permeability to a much greater extent than high K_e , it is unlikely that Ca_e played a major role during the caffeine/0Ca contractions. Also, since the caffeine contractions were not reduced in magnitude or duration in the presence of Ni_e , whereas the action of Frescon was completely blocked (Fig. 29), the Ni-dependent inhibition of Frescon cannot be attributed to an action at an intracellular locus.

A further observation which strengthens the argument of separate sites for Frescon and caffeine actions is that a caffeine contraction can be superimposed over a Frescon contraction. This would not be expected if Frescon released caf-

feine sensitive Ca stores. Moreover, the depletion of these stores with repeated applications of caffeine did not inhibit; or even reduce the magnitude of Frescon contracture (Fig. 27), again indicating that these stores are not essential for Frescon action.

An incidental observation during the experiments with Ca entry blockers was that these agents reduced the resting tension of the HV, but not the PRM (e.g. Figs. 21 & 24). A possible explanation for this could be a reduction of 'resting' Ca influx into the HVMC's. Molluscan hearts respond to stretching or perfusion with spontaneous contractions (Hill & Welsh, 1966), suggesting that they possess some form of 'stretch receptors' which are coupled to Ca channels (cf. Prescott & Brightman, 1976). Blocking these channels (or removing extracellular Ca) would presumably lead to an inhibition of spontaneous contractions and a reduction in resting tension. The PRM, on the other hand, presumably has a very low resting muscle tone as the Ca entry blockers (or the removal of Ca_e) did not reduce the resting tension of this muscle (e.g. Fig. 29).

B) The action of the adenyl cyclase inhibitor RMI 12330A

Often, the treatment of a Frescon-contracted PRM with caffeine resulted in a transient relaxation which followed the caffeine-induced enhancement of tension (Fig. 27). Since caffeine is known to inhibit phosphodiesterase, it is possible

that the observed relaxation was brought about by an increase in the sarcoplasmic levels of cAMP (cf. Theobald et al., 1978). This suggests the possibility that Frescon may act by interfering with the synthesis of cAMP, perhaps via the membrane-bound adenylyl cyclase. However, the action of the adenylyl cyclase inhibitor RMI 12330A (which also induced contracture) differed from that of Frescon in two important respects, viz., 1) the action of RMI 12330A was not inhibited by Ca-free saline whereas that of Frescon was, and, 2) the contracture induced by the adenylyl cyclase inhibitor was transient compared with the sustained contracture induced by Frescon (Fig. 30). Although these results certainly are not conclusive, especially since RMI 12330A may have prominent non-specific effects, e.g. the inhibition of mitochondrial respiration (Grupp et al., 1980), they do not support a possible derangement of cAMP metabolism during Frescon action.

4) CALCIUM IONOPHORE A23187: A COMPARISON WITH FRESCON ACTION

If Frescon induces an increase in sarcolemmal Ca permeability, its action should be mimicked by known Ca ionophores. The response of the HV and the PRM to the divalent cation ionophore A23187 was almost identical to the response to Frescon. However, unlike Frescon, this ionophore appears to have an intracellular as well as a sarcolemmal site of action, as A23187 contractures were not prevented by the

removal of Ca_e , although their magnitude was reduced by about 60% (Fig. 33). Even caffeine- Ca_s -depletion did not completely eliminate A23187-induced contractures, although they were reduced to about one-third the magnitude of those induced in 0Ca saline alone. A23187 treatment of caffeine- Ca_s -depleted muscles also partly restored the response to caffeine (Fig. 35). This partial restoration of caffeine sensitivity suggests that the ionophore may have released intracellular Ca from stores other than the SR (perhaps the mitochondria) which was then taken up by the SR, and/or A23187 may have released more tightly bound SR Ca which was previously inaccessible to the action of caffeine. Alternatively, there may have been enough residual Ca in the 'Ca-free' saline to account for this observation, since K contractures (which presumably utilize Ca_e) could be evoked in PRM's which had been exposed to 0Ca saline for up to 30 min, although the magnitude of these contractures was reduced by about 95% compared to K contractures in normal saline, and the time course was reduced to 3-6 min (Fig. 37). These K contractures were completely prevented either by 1mM La or by caffeine- Ca_s -depletion (Figs. 38 & 41). In the former case, the inhibition was probably at the level of the sarcolemma, whereas the latter treatment, having considerably reduced SR Ca stores, probably established a potent 'Ca sink' which could rapidly take up the small amount of Ca which entered the cell during high K-0Ca treatment, thereby preventing the initiation of contrac-

tion. However, the A23187/0Ca contracture in a caffeine- Ca_s -depleted muscle was about twice the magnitude and much more prolonged than the 30K/0Ca contracture in untreated muscles (cf. Figs. 35 & 37). Therefore, either A23187 is much more effective in promoting an increase in sarcolemmal Ca permeability than high K_e , or the amount of residual Ca in 'Ca-free' saline was insufficient to entirely account for A23187/0Ca action in a caffeine- Ca_s -depleted muscle. Nevertheless, the observation that caffeine- Ca_s -depletion greatly reduced the magnitude of the A23187/0Ca contractures compared to the magnitude induced in untreated muscles (cf. Figs. 33 & 35) is consistent with an intracellular site of action for this ionophore. However, as the exposure of a caffeine- Ca_s -depleted muscle to A23187/0Ca partly restored caffeine sensitivity (Fig. 35), it would appear that the SR can take up Ca even in the presence of this ionophore. This suggests that the SR Ca pumps were not inhibited (or, at most, only weakly inhibited) by A23187. This observation warrants further investigation.

The action of A23187/0Ca was completely and irreversibly inhibited by 1mM Ni, even if this heavy metal ion was applied after the ionophore presumably had already entered the sarcolemma and thence the cell (Fig. 33). This suggests either that Ni can penetrate the sarcolemma, or that it is transported into the cell by the ionophore. It was not possible to distinguish between these two possibilities. However, it is not likely that the binding of Ni and Ca to this iono-

phore is competitive, since the restoration of normal saline alone to an A23187/Ni-treated muscle did not restore contracture (Fig. 32), suggesting that Ni was irreversibly bound to the ionophore. It is not known if an exchange between Ni and an intracellular divalent cation could occur under these conditions. However, it is possible that A23187 did transport Ni into the cell whence this cation may have interfered with the contractile apparatus or some other mechanism(s) regulating Ca fluxes, since when A23187/normal saline was restored to the A23187/Ni-treated muscle, the magnitude of the contracture was reduced by about 50% compared to that before Ni treatment (Fig. 32). In contrast, caffeine contractions were not affected by Ni_e (Fig. 29), suggesting that this ion cannot enter the muscle cell without the aid of an ionophore. Alternatively, it may be that the first A23187 treatment simply reduced the Ca_i stores, making less Ca_i available for the second application. However, this second possibility is less likely since the post-Ni application of the ionophore was in normal saline, i.e., Ca_e, not Ca_i, should have been the governing factor.

The entry of Ni into the ionophore-treated cell is further supported by a comparison with the action of A23187/La treatment. The latter element reduced the ionophore-induced contracture almost exactly to the extent achieved by 0Ca alone. The subsequent addition of Ni, however, reduced the contracture to the resting level (Fig. 34). These results

suggest that La does not penetrate the cell (which is further evidence that La does not interfere with Frescon action at an intracellular site).

Another difference between Frescon action and that of A23187 in the HV is that the ionophore was not affected by D-600 (Fig. 36), even at the relatively high concentration of 4×10^{-4} M. Since D-600 is thought to interfere with already existing Ca channels, whereas A23187 bypasses them, this result is not surprising. In view of these results, it is very improbable that Frescon and A23187 act by similar mechanisms. However, it cannot be concluded that Frescon does not act as an ionophore. Whereas A23187 translocates Ca via a carrier-type mechanism (Szabo, 1981), Frescon might act as a channel-type ionophore. Nevertheless, the evidence presented thus far indicates that the action of Frescon is restricted to the level of the sarcolemma. The remaining question, therefore, is: does Frescon act by creating new Ca channels, or by forcing open already existing ROC and/or PDC channels?

A partial answer to this question may come from an analysis of the response of the *Helix* PRM to Frescon. The transient nature of this response is not consistent with the creation of new channels by Frescon unless one postulates that these new channels can somehow be inactivated, which seems unlikely. Moreover, the transient contractions can be repeatedly evoked by electrical stimulation, and eventually are transformed into a sustained, irreversible contracture

(Fig. 12). This type of response would be expected if Frescon acts to prolong the 'open state' of PDC channels. It would also appear that the Ca channels are more susceptible while in the open state since repetitive electrical stimulation is necessary to induce a sustained contracture. Although this response was different from that of the *Lymnaea* PRM where a sustained contracture was generated very shortly after Frescon treatment, it is possible that the action of this molluscicide may also be explained in a similar manner for *Lymnaea* muscles, i.e., an alteration of function of existing PDC and/or ROC channels.

5) THE CALCIUM CHANNEL HYPOTHESIS OF FRESCON ACTION

A) The 'two-channel' hypothesis

If Frescon acts on existing Ca channels, its action might be mimicked by compounds for which a similar action has been proposed. The anthelmintic praziquantel (PQ) is thought to act by increasing the permeability of the cell membrane to Ca (Fetterer et al., 1980). This compound does not appear to act as an ionophore (Chubb et al., 1978), and has been implicated in the alteration of both PDC (mammalian) and ROC (invertebrate) channel function (Pax et al., 1978; Chubb et al., 1978). PQ produced contractures in the *Lymnaea* PRM which were very similar in time course and profile to

those induced by Frescon, although, in contrast to Frescon action, PQ contractures were completely reversed by washing with normal saline (Fig. 39). Moreover, it was found that PQ probably acts at intracellular sites as well as at the level of the cell membrane, since removal of Ca_e did not prevent PQ contractures even in the presence of La, although La was shown to inhibit the sarcolemmal component of PQ action (Figs. 41 & 42). The duration of PQ contracture was, however, much shorter in the absence than in the presence of Ca (Fig. 39), resembling a caffeine-like action. In fact, caffeine- Ca_s -depletion completely inhibited the action of PQ in Ca-free saline (Fig. 40), suggesting that both PQ and caffeine act by releasing Ca from the same intracellular stores - stores which apparently are not affected by Frescon. However, notwithstanding these differences between the actions of PQ and Frescon, their actions at the level of the cell membrane could be similar.

It has already been mentioned that D-600 did not block Frescon action at concentrations in the range which have been reported to block PDC channels (e.g. see Fleckenstein, 1971). Therefore, either D-600 is not effective against *Lymnaea* PDC channels, or Frescon does not affect PDC channels but does affect ROC channels which may be inhibited by a relatively high concentration of D-600 (Schumann et al., 1975). The anaesthetic sodium pentobarbital (NAPB) has also been shown to inhibit PDC channels in some cell types,

although, at concentrations above 1mM, its action is probably non-specific (Blaustein & Ector, 1975). Frescon contractures were not blocked by 5×10^{-3} M NAPB, although they were reduced in magnitude (as evidenced by a rapid increase in muscle tension upon restoration of normal saline) and the rate of tension development was retarded (Fig. 43), showing that NAPB has at least a partial inhibitory effect on Frescon action. However, NAPB almost completely inhibited K contractures (Fig. 43), suggesting that it is an effective blocker of Ca entry through PDC channels. Since NAPB did not totally block Frescon action, it would appear that the action of this molluscicide includes some mechanism which does not involve PDC channels, i.e., Frescon may primarily affect ROC channels. On the other hand, as Frescon contracture was partially inhibited by NAPB, it may be proposed either that Frescon acts only to alter ROC channel function and NAPB also partially blocks these channels, or that Frescon acts only on PDC channels and NAPB is not entirely effective in blocking this action, or that Frescon acts on both ROC and PDC channels, and NAPB blocks only the PDC channel component of Frescon action.

That Frescon might affect PDC channels is suggested by the response of the *Helix* PRM to this molluscicide in that its action was expressed through electrical stimulation which, presumably, opened PDC channels. Apparently, in *Helix*, Frescon contractures become sustained only if the PDC channels

are opened repeatedly by other means. Such a 'use-dependent' property has been ascribed to the action of D-600 on PDC channels (Ehara & Kaufmann, 1978; McDonald et al., 1980) and to the blocking action of certain local anaesthetics in the Na channel (Khodorov, 1981). Whether or not a similar action for Frescon occurred in *Lymnaea* muscles could not be determined as it would have been masked by the spontaneous, rapidly developed contracture which may have been due to an action on primarily ROC channels. This suggests that the ROC channels in *Helix* are either absent or are not susceptible to Frescon because of structurally different receptors from those in *Lymnaea*.

Evidence against a primary Frescon action on PDC channels in *Lymnaea* comes from the observation that Frescon contractures can be superimposed on K⁺ contractures in muscle cells which had been depolarized with high K_e, although the rate of development of the Frescon contracture was substantially reduced under these circumstances (Fig. 46). The converse has also been demonstrated, i.e., K contractures can be superimposed on Frescon contractures (Fig. 44). This is especially evident in the case of the HV (Fig. 45). If the molluscicide had opened all or most of the PDC channels (which is not unreasonable to assume given the magnitude of the Frescon contractures), then it would be expected that the subsequent exposure of the muscles to high K_e would have little or no further effect on tension. It is not likely that

Frescon inhibits the PDC channel inactivation mechanism or the active extrusion or sequestration of Ca , as K contractures superimposed over Frescon contractures were still transient. This was easier to observe in the case of the HV where the magnitude of the Frescon contracture diminished to about one-third of the maximum tension (cf. Figs. 44 & 45).

However, the possibility that Frescon does have some effect on PDC channels cannot be ruled out. The reduction in the rate of development of Frescon contractures by K depolarization might be explained by proposing that the PDC channels, which normally may have been available to Frescon, had been opened and inactivated by high K_e . Consequently, fewer channels (presumably only ROC channels) would be available for Frescon action. As a result, Ca_i levels might not increase as rapidly as if PDC channels had also been available, and the rate of contracture would be attenuated. The above results suggest that even if PDC channels are involved during the action of Frescon, their contribution to the total Frescon contracture is probably less than that of ROC channels.

A further, although weaker, argument against a significant involvement of PDC channels during Frescon action in *Lymnaea* is that the membrane potential of the HVMC's was not depolarized by this molluscicide. The opening of PDC channels is usually associated with a measurable Ca current during membrane depolarization (Baker et al., 1971; Kostyuk & Krishtal, 1977; Tillotson, 1979; Hagiwara & Byerly, 1981). However, it

is not known how much Ca must enter an HVMC before it can be detected by standard electrophysiological techniques, nor is it known how much Ca must enter to initiate a contracture of the magnitude induced by Frescon. Presumably, membrane Ca permeability would also have to be rapidly increased for an associated current to be measurable with microelectrode recording. A slow Ca entry may not generate a measurable current as there are many intracellular Ca buffers which would quickly bind free Ca. Certainly, Frescon contractures, when compared with the time course of 'Ca spikes' are very slow indeed,

Although the evidence is not conclusive, it does favour a primary site for Frescon action at ROC — rather than PDC — channels. Superimposition of K contractures over Frescon contractures and the observation that NAPB only partially inhibited the action of Frescon support this hypothesis. However, the implication of ROC channels in Frescon action is largely inferential. In fact, there is neither evidence for nor against the existence of ROC channels in either the PRM or HV of *Lymnaea*, although the ubiquity of these channels in a diversity of tissue types, including many molluscan genera (Kehoe & Marder, 1976; Price & Greenberg, 1980), is in favour of their existence in *Lymnaea* also.

The molecular structure of Ca channels is virtually unknown. The properties of Na and K channels are much better understood than those of Ca channels (Hagiwara, 1980). The

former channels are thought to be multisubunit protein aggregates which span the membrane. The pore of these 'voltage-sensitive' channels is thought to consist of a narrow 'outer selectivity filter' and a wider, relatively non-selective inner mouth which incorporates a voltage-sensitive 'gate' (Armstrong, 1975; Meves, 1978; Khodorov, 1981). The general form of this model may likewise apply to Ca channels (Hille, 1978). Presumably, in ROC channels, the 'gate' would somehow be coupled to the channel's 'receptor' rather than to a 'voltage sensor'. How this coupling is accomplished is not known, but it may be that Frescon, by irreversibly binding to the 'receptor', keeps the channel in a constantly open state. Alternatively, Frescon may diffuse more deeply into the membrane and somehow force open the 'gate' without involving the receptor directly.

B) *The 'one-channel' hypothesis*

Although pharmacological evidence favours the existence of at least two classes of Ca channels in many types of excitable tissues, the evidence for this is not conclusive (Bolton, 1979). Therefore, the possibility that only one type of channel exists must also be considered when evaluating the action of Frescon. If such a Ca channel existed in *Lymnaea* then it would have to have (or appear to have) the combined properties of both ROC and PDC channels, i.e., it should be activated either by a 'receptor-agonist' inter-

action and/or by a transmembrane potential change. By the above model, this would mean that the Ca channel would have a voltage-sensitive 'gate' which would also be coupled to a 'receptor'. In view of the results with Frescon and high K_e , such a gating configuration would appear to be less likely than the existence of two separate Ca channels. For example, unless the 'potential-dependent' and 'agonist-activated' components cause different increases in Ca permeability through this channel, it would be difficult to explain the superimposition of a Frescon contracture on a K contracture, and vice-versa. Furthermore, it is difficult to see how inactivation would apply to a single channel model. Unless inactivation is entirely dependent on membrane potential (i.e., on a depolarization of the sarcolemma) rather than on Ca-dependent Ca inactivation (see Introduction, p. 37), it would be difficult to explain why K contractures, where the Ca channels were probably inactivated (cf. Baker et al., 1973b), were relatively transient (Fig. 44) compared with the sustained contractures generated by Frescon (Fig. 9), where the Ca channels were presumably not inactivated. The different nature of these two responses favours the existence of at least two separate types of Ca channels in the muscle cells of *Lymnaea*.

C) The 'barrel stave' hypothesis

An alternative model for ionic channels based on the aggregation of mobile channel-forming molecules ('staves') into a 'barrel-like' structure (Mueller, 1975; Lecar et al., 1975) has also been considered. This model, which may explain the nature of such compounds as alamethicin, etc., depends on a large dipole moment at one end of the aggregate which moves through the membrane in response to changes in membrane potential. However, since Frescon does not alter V_r , it is difficult to see how the molluscicide could force the formation of these channels without a favourable electrical gradient. This hypothesis is also unsatisfactory as an explanation for Frescon action in that there is no evidence of such channel-forming molecules occurring naturally in excitable membranes. Furthermore, as Frescon action would be expected to be fairly universal with such a model, the difference in response of the *Helix* and *Lymnaea* PRM's to Frescon cannot be readily explained, nor does this model explain the lack of Frescon sensitivity in the *Helix* HV. It would appear that either of the 'receptor-type' channel models have the important advantage over the 'barrel stave' model in that the relative specificity of Frescon is easier to explain. Ca channels are not very conservative from species to species (Hagiwara, 1980). Consequently, certain molluscan muscles may have evolved 'receptors' at the ROC channels which turn out to be especially sensitive to Frescon, whereas the 'receptors' of

Frescon-immune muscles are insensitive or non-existent. The great variety of Ca channels with respect to 'agonist' specificity strengthens this possibility (Bolton, 1979).

D) *Advantages of the 'two-channel' model*

The two-channel model more easily explains the different actions of NAPB on Frescon-induced and high K_e -induced contractures in the PRM of *Lymnaea*. For example, if a high concentration of D-600 (but not NAPB) blocked ROC channels (the presumed targets for Frescon), then its blocking action against Frescon binding could be explained. On the other hand, if NAPB blocked primarily PDC channels (which may be less sensitive to Frescon), then Frescon might still act on ROC channels, thereby initiating contracture in the presence of the anaesthetic. In either of the one-channel models described above, however, it would be difficult to explain the much greater inhibition of K contractures than Frescon contractures by NAPB. If only a single channel exists, then it would seem that both Frescon and high K_e contractures should be equally inhibited, unless Frescon (but not high K_e) displaces NAPB from its site of action. This possibility, however, introduces the added complication of explaining why D-600 (which, like NAPB, is water-soluble and, therefore, also much less lipophilic than Frescon) was not displaced from the membrane by the molluscicide. In fact, D-600 seems to prevent the binding of Frescon to its site of action

(Table 9). These questions cannot be answered until more is known about the mechanism of action at the molecular level for either D-600 or NAPB.

6) OTHER POSSIBLE ACTIONS FOR FRESCON

Although the evidence strongly favours a sarcolemmal site for Frescon action, the proposition that it alters Ca channel function is not conclusive. Also, it has not been unequivocally proven that Frescon does not enter the cell, although the indications are that if it does so, it has no immediate effect on molluscan muscle function. However, Frescon may act to alter membrane-related processes other than the function of Ca channels. For example, the removal of extracellular Na causes a contracture which is very similar to that caused by Frescon (Fig. 48). It would appear that the 0Na contracture results from the inhibition or reversal of the Na-Ca exchange mechanism, thereby elevating $[Ca]_i$ (Baker, 1972). It is, however, unlikely that Frescon has a similar action. Firstly, the exposure of HVMC's to 0Na resulted in a depolarization of the membrane potential (Fig. 16), in contrast to the action of Frescon. Secondly, 0Na contractions could be superimposed over those of Frescon, suggesting that the molluscicide does not inhibit the Na-Ca exchange process, although it is possible that 0Na saline is more efficient in the inhibition of this process than Frescon. This,

however, is unlikely as the magnitude of the plateau phase of Frescon-induced contracture was greater than the equivalent phase of 0Na contracture (see Fig. 48). Thirdly, caffeine contractures were not altered by Frescon. Caffeine has been reported to stimulate the Na-Ca exchange process (Jundt et al., 1975), which probably accounts for the transient nature of caffeine/ 0Ca contractions. If Frescon acted to inhibit this process, then it would be expected that the caffeine contractions would be prolonged.

A second possibility is that Frescon acts to inhibit cell membrane-bound ATPase. Tyler & Henley (1981) reported that the isolated SR Ca-ATPase of rat skeletal muscle is inhibited by Frescon. The present evidence argues against such a possibility in *Lymnaea* since such an inhibition would be expected to alter the action of caffeine, or even cause contracture in Ca-free saline by releasing SR Ca stores (Endo, 1977). It has been shown that the SR is able to retain its Ca stores during the action of Frescon (Fig. 27). Furthermore, rats are very insensitive to Frescon (Shell, 1974), making it difficult to reconcile such an action with the low toxicity of Frescon to this animal. Although Frescon can apparently inhibit the Ca-ATPase in fragmented cell preparations, it is very unlikely that this can explain Frescon action *in vivo*. However, a possible effect of Frescon on sarcolemmal Ca-ATPase cannot be excluded, although, once again, it would be difficult to explain the high specificity of Frescon through

such an action. Nevertheless, experiments (e.g. possibly using heptachlor epoxide (shown by Yamaguchi et al., 1979. to inhibit Ca-ATPase)) are being designed to explore this possibility.

Banna & Plummer (1978) and Plummer & Banna (1979) reported that Frescon inhibits intracellular enzymatic functions and causes a disruption of lysosomes in the heart of the terrestrial snail *Archachatina marginata*, and suggested an intracellular site for Frescon action. In view of the results of the present investigations, these reported changes in the metabolic processes of the cell were probably a response to Ca overload. Excessive levels of Ca_i are known to interfere with a variety of metabolic processes as a result of a chronic high-energy phosphate deficiency (Fleckenstein, 1971). Therefore, care must be taken when basing an interpretation of a possible Frescon action on any energy-dependent process.

7) SUMMARY AND CONCLUSIONS

The present experiments suggest that the most likely explanation for Frescon action on snail musculature is that this molluscicide induces an increase in the Ca permeability of the sarcolemma, and that the magnitude of this increase is sufficient to overload the cellular mechanisms which are responsible for maintaining low levels of free sarcoplasmic Ca.

Frescon probably does not act to interfere with muscle contraction at the level of the contractile apparatus, nor does it release intracellular Ca stores. It is also unlikely that this molluscicide directly inhibits cellular Ca regulation mechanisms at intracellular sites. These conclusions are based on the following observations:

- 1) Frescon contractures are prevented by the Ca entry blockers Ni, Co, Mn and La, and by low Ca-high Mg saline.
- 2) D-600 blocks Frescon contractures (albeit at a relatively high concentration) and probably prevents Frescon binding. Since the site of action for D-600 is likely to be at the level of the cell membrane (see Introduction, p. 45), this suggests a membrane locus for Frescon action also.
- 3) Ca-free saline completely inhibited the action of Frescon but not the actions of caffeine, A23187 or PQ. These latter agents probably release intracellular Ca stores, which suggests that Frescon does not.
- 4) Frescon probably does not act at the level of the contractile filaments, since it does not alter the profile of caffeine/0Ca contractions.

The second, more tentative, conclusion that Frescon acts to alter Ca channel (primarily ROC) function is based on the following observations:

- 1) Frescon contractures were not blocked by low levels of D-600, which have been shown to block PDC channels in many diverse tissue types (see Introduction, p. 42). However, higher concentrations of D-600, which have been reported to block ROC channels (Schumann et al., 1975),

prevented Frescon contractures.

- 2) NAPB almost completely inhibited K contractures (and, by inference, PDC channel function), but only partially inhibited the action of Frescon.
- 3) K contractures were superimposable over those of Frescon and vice-versa, suggesting that Frescon does not primarily act to alter PDC channel function.
- 4) It is unlikely that Frescon acts to inhibit the Na-Ca exchange mechanism as the response of the HVMC's was different during the actions of Frescon and ONa saline, with only the latter treatment producing a depolarization of the cell membrane. Moreover, ONa contractures were superimposable on those of Frescon.
- 5) The action of the adenylyl cyclase inhibitor RMI 12330A was not similar to that of Frescon, suggesting that the reduction of CAMP levels through the inhibition of membrane-bound adenylyl cyclase is not associated with Frescon action.

Although it was not possible to directly demonstrate that Frescon does indeed alter ROC channel function, this is the most likely explanation given the available evidence. The possibility of a one-channel model to explain the action of Frescon cannot be excluded on the basis of the present evidence, but the two-channel model is more convincing.

Finally, it is very likely that the action of Frescon on isolated *Lymnaea* muscles can satisfactorily explain its molluscicidal activity. One of the first symptoms of Frescon poisoning is a loss of muscular co-ordination in the *Lymnaea* foot, and Frescon has been shown to have a direct effect on this organ. Furthermore, the rapid action of Frescon on iso-

lated snail muscles is more than sufficient to account for the time course of the appearance of poisoning symptoms in the whole animal (cf. Brezden & Gardner, 1980a).

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APPENDIX: THE FORCE TRANSDUCER AMPLIFIER, OFFSET CIRCUIT
AND FORCE TRANSDUCER CALIBRATION CURVE

The force transducer amplifier (Fig. 50) was designed for high gain amplification of the signal from the force transducer. The driving power for the transducer was provided by 4 Mallory RM-12R mercury batteries (5.4V total). The output of the transducer was routed through an offset circuit to allow arbitrary 'zeroing' of the output signal (Fig. 49).

The amplified signal from the transducer was calibrated to provide 3mV output per mg of weight applied at the input (amplification = approximately 5000X). The calibration curve

Figure 49. The offset and driver circuit for the force transducer.

Figure 50. Schematic diagram of the force transducer amplifier.

A1, A2 = LM310A; A4 = LM301; A5-A8 = OP-07

All resistors 1/4W, 1% unless otherwise specified

(Fig. 51) was derived by loading the transducer with known weights. Since the Gould Statham UC-2 is rated at less than 0.15% deviation from linearity between 0 and $\pm 30\text{g}$, the calibration curve is primarily an indicator of the linearity of the amplifier.

Figure 51. The calibration curve for the Gould Statham UC-2 force transducer and amplifier.

END

0	5	1	0	9	1	8	4
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FIN