CHANGES IN CANINE TRACHEAL SMOOTH MUSCLE CELL CALCIUM DURING RHYTHMICITY INDUCED BY METABOLIC DEPLETION

A thesis presented to the
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In partial fulfillment of the
Requirements for the Degree of
Master of Science

by

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ABSTRACT

Tracheal smooth muscle strips can be converted to mechanical and electrical rhythm by denervation, drugs or metabolic depletion.

In a metabolically depleted tissue, any chemical or electrical excitation that would normally produce a graded tonic contraction now evokes electrical membrane spikes with rhythmic contractions.

The rhythmically contracting canine trachealis exhibits a greater sensitivity to the calcium channel blocker D-600 and to the chelation of calcium by EGTA, than normal canine trachealis. The rhythmic tissue appears to be more dependent upon external calcium than normal. The inward calcium flux plays a greater role in tension development during metabolic depletion.

A cold induced contracture ,in a normal muscle, contracted by exposure to agonist, appears to be caused by mobilization of tighter internal stores of calcium because the effect persists after equilibration in a zero calcium Krebs solution or after exposure to the calcium channel blocker D-600.

Cold contracture cannot be produced in metabolically depleted tissue indicating that the tighter internal calcium is unavailable perhaps due to a limited glucose supply in this tissue.

Changes in cellular calcium as measured by isotopic calcium exchange indicated that rhythmically contracting muscle had greatest increase in calcium in the externally exchangeable membrane boundd calcium pool, suggesting its importance during metabolically induced rhythmicity.

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SECTION A

INTRODUCTION

This thesis will deal with the role of membrane associated calcium and its contribution to the ionic mechanism responsible for metabolically induced rhythmicity in canine tracheal smooth muscle. It will adress the question as to alterations in the size and mobility of the various calcium pools associated with the cell, play any role in the establishment and maintenance of rhythmicity?

Rhythms are found within practically all living processes; electrical pacemakers compose one subset of biological rhythms. Electrical pacemakers can be found in three types of animal tissue: cardiac muscle (Tsien and Carpenter, 1978), smooth muscle (Tomita, 1970; Connor etal, 1974), and neurons (Heyer and Lux, 1976; Gulrajani and Roberge, 1978). The pacemakers probably share common operating systems because nature tends to conserve function and mechanism (Rapp and Berridge, 1977).

Cardiac muscle in mammals is under an inherent myogenic drive with a neurogenic modulation (Pelleg etal, 1980). In many smooth muscle systems, the rhythmicity is myogenic because contraction still occurs after sodium channel blockade by tetrodotoxin or by denervation (Bose and Innes, 1974).

The pacemaker mechanisms may not just belong to a few specialized cells but rather represents a general phenomenon of most or all excitable tissue (Daniel and Sarna, 1978). The pacemaker mechanisms could be more prevalent, if not for a presumed loss during development.

Chamley (1979) observed cultured chick gizzard spontaneously contracting while adult gizzard was quiescent. Embryonic tissue such as embryonic skeletal muscle have rhythmic activity whereas the corresponding adult tissue is normally queiscent (DeHaan and DeFelice, 1978; Powell etal, 1979).

An electrically coherent conduction system for pacemakers to surrounding tissue is usually achieved with gap junctions which could be be characterized as low resistance bridges (Gabella, 1979; Tomita, 197Ø; Suzuki etal, 1976). In smooth muscle this system appears to be very complex leaving most cells with a potential for pacemaker activity even if this is only slow wave activity without action potentials or without bursting activity (Prosser, 1974; Barr etal, 1979).

Alternately, perhaps a few specialized smooth muscle cells are the pacemakers much like the specialization in heart 'nodes' and Purkinje fibers (Daniel and Sarna, 1978). The nervous system can modulate smooth muscle rhythmic activity leaving the possibility of nervous system rhythmicity and the smooth muscle only responding as a consequence of it (Holman, 197Ø).

Before discussing the specific phenomenon of metabolically induced rhythmicity in canine trachealis, classification of canine tracheal smooth muscle will be considered.

ULTRASTRUCTURE OF CANINE TRACHEAL SMOOTH MUSCLE

Tracheal smooth muscle belongs to a division of quiescent smooth muscle as opposed to spontaneously active smooth muscle (Suzuki etal, 1976; Widdicombe etal, 1963). Smooth muscle of a quiescent nature belongs to the multiunit classification of muscle and cannot produce electrical or mechanical spikes on direct stimulation (Kannan and Daniel, 1980). Single unit smooth muscle exhibits spontaneous mechanical activity, involving membrane spiking and action potentials (Prosser, 1974). Tracheal smooth muscle may belong to a diffuse category of multiunit smooth muscle proposed by Somlyo and Somlyo (1968) to emphasize that the differences between smooth muscles overlap. Although trachealis exhibits multiunit behaviour to agonist and electrical stimulation (Kirkpatrick etal, 1975; Kannan and Daniel, 1980), the spacing of innervation more typical of single unit smooth muscle (Kannan and Daniel, 1980; Cameron and Kirkpatrick, 1977).

Canine tracheal muscle cells have been measured to be 3.3$\pm$0.5 um S.D.(n=50) in diametre and more than 1000 um in length (Suzuki etal, 1976). Electronmicrographs of tracheal smooth muscle show occassional tight junctions (nexus) and gap junctions connecting cellular processes. In the long axis, the cell shape is elliptical aligning the cells parallel to each other and forming bundles separated by interstitial spaces (Chambley-Campbell etal, 1979; Prosser,

1974; Suzuki etal, 1976). Collagen fibres were noted between muscle cells and the interstitial space between the cell bundles were occupied by collagen and interstitial cells (Gabella, 1979). The muscle cells contained sparse sarcoplasmic reticulum, located below many caveolae on the plasma membrane (Gabella, 1979; Suzuki etal, 1976).

NEURONAL TISSUE WITHIN TRACHEAL SMOOTH MUSCLE

Nerve fibres among tracheal muscle cells were sparse and are represented all by unmyelinated: axon bundles and axonal varicosities (Suzuki etal, 1976; Kannan and Daniel, 1980; Cameron and Kirkpatrick, 1977). The axons were rarely represented in muscle bundles but were more commonly found in small groups (less than 10 per group) with various levels of sheathing by Schwan cells (Kannan and Daniel, 1980). The axonal varicosities typically were several micrometres from smooth muscle cells.

With the autonomic innervation to canine tracheal muscle being sparse, and no close contacts between nerve and muscle, the ratio of axons to tracheal smooth muscle was at a rather low ratio of 1:29 (Kannan and Daniel, 1980).

Cameron and Kirkpatrick (1977) working with bovine tracheal smooth muscle, noted that electron micrographs showed sparse innervation with one axon contacting ninety smooth muscle cells. The axons did not enter the muscle bundles, they lay only in the clefts between the bundles of cells. The axons were only 200nm distant from any smooth

muscle cell.

CELLULAR ELEMENTS OF NEURONAL TISSUE

Axonal varicosities in smooth muscle can be classified by the type of synaptic vesicles viewed by electron microscopy. Tracheal muscle contains axonal varicosities with small agranular vesicles, large granular vesicles and small dense- cored vesicles, composed in varying proportions (Suzuki etal, 1976; Kannan and Daniel, 1980). The small vesicles measured 40-70 nm and the large vesicles measured 80-100 nm in diameter. Small agranular vesicles predominate in axons seen (Kannan and Daniel, 1980; Suzuki etal, 1976). They were viewed in varicosities that were distal to the muscle and were considered to be cholinergic in nature. The small dense-cored vesicles observed in varicosities near blood vessels were thought to be adrenergic (Suzuki etal, 1976; Gabella, 1979).

Histochemical stains for acetylcholinesterase, stained between the muscle cells and on the muscle cell membranes. Noradrenaline was located in the perivascular region and in the vicinity of cartilage (Suzuki etal, 1976).

MECHANICAL COUPLING IN SMOOTH MUSCLE - AN OVERVIEW

The major movements of calcium in canine trachealis probably occur during EC coupling which is typical of a smooth muscle EC coupling (Perry and Grand, 1979; Somlyo

and Somlyo, 1968).

The resting cellular cytoplasmic levels of calcium are of the order of Ø.1 uM (Van Breemen etal, 198Ø), a concentration just below the threshold of myofilament activation. A large difference in calcium concentration occurs in the major phases on either side of the smooth muscle cell membrane. Normal levels in the resting cytoplasm are of the order of Ø.1 uM to Ø.Ø1 uM calcium whereas the extracellular phase has 1ØØØ uM (Bianchi, 1969). The extracellular compartment is very large and its content of calcium is resistant to change (Perry and Grand, 1979).

The major step toward contraction involves modification of myosin by posphorylation of the light P chain (Junod, 198Ø). Phosphorylation of the 2Ø,ØØØ dalton myosin light chain sub-unit is catalyzed by a calcium dependent myosin light chain kinase. The activated myosin can form an actin-myosin complex when exposed to actin (Junod, 198Ø). The phosphorylation is followed by an increase in actomyosin ATPase activity (Lanerolle and Stull, 198Ø; Paietta etal, 1978).

The phosphorylation can be related to the state of contraction. When spontaneously contracting rat myometrium is frozen in various states of contraction, the maximum incorporation of 32-P into the 2Ø,ØØØ Mr protein just precedes the maximum tension development, indicating regulation of spontaneous contraction (Janis etal, 198Ø).

The mediation of phosphorylation of the myosin light

chain is directed by calcium and/or cyclic nucleotides, depending upon the experimental preparation (Janis etal, 1980; Kaukel etal, 1978; Greengard, 1975). Rat myometrium did not show increased cyclic GMP levels during spontaneous contraction, indicating the increased phosphorylation was due to an elevated intracellular calcium activity (Janis etal, 1980). In other preparations of smooth muscle drug induced contraction can be correlated to increased phosphorylation by increasing intracellular levels of cyclic GMP. This effect is observed in guinea-pig trachea (Ohkubo etal, 1976), rabbit trachea (Kaukel etal, 1978), guinea-pig gallbladder (Anderson etal, 1977) and human artery (Hidaka etal, 1978).

Briefly, after an actomyosin ATPase system is activated by calcium, sliding of the two filaments can occur. The role of calmodulin is to act as a high affinity calcium acceptor regulatory protein leading to phosphorylation of myosin light chain and the activation of actomyosin ATPase (Junod, 1980; Ito etal, 1976; Perry and Grand, 1979).

The process is reversed by dephosphorylation of the P light chain of myosin by a mechanism probably involving decreasing calcium levels: either a cessation of the activating of phosphorylation or the activation of a phosphatase (Junod, 1980; Twarog, 1979; Janis etal, 1980).

NORMAL MEMBRANE POTENTIAL

Tracheal smooth muscle normally produces a graded depolarization in response to agonists that cause contraction. Kirkpatrick, (1976) and Cameron and Kirkpatrick, (1977) using bovine trachealis demonstrated that electrical stimulation of whole tissue only produced a small transient depolarization. The transient depolarization resulted in a twitch contraction. The twitch and depolarization could summate on repeated stimulation, however the summation never resulted in an action potential (Cameron and Kirkpatrick, 1977; Kirkpatrick etal, 1975; Suzuki etal, 1976; Kannan and Daniel, 1980).

Acetylcholine, histamine and serotonin are naturally occuring agonists capable of producing a contraction in trachealis (Kirkpatrick etal,1975). During contraction with histamine the membrane potential undergoes slow oscillations with correlated rhythmic activity (Kirkpatrick etal, 1975). The histamine contraction seems more dependent upon an external pool of calcium because zero-calcium bathing solution greatly inhibits the histamine contraction.

ABNORMAL MEMBRANE POTENTIALS WITH CHEMICAL MODIFICATIONS

Two agents used by investigators to induce arrhythmias in various excitable tissues and rhythmicity in smooth muscle preparations are tetraethylammonium (TEA) and

barium (Bulbring and Tomita, 1969; Droogramns etal, 1977; Casteels etal, 1977; Kirkpatrick etal, 1975; Walsh and Singer, 1980).

Barium was used as a calcium analogue (Bulbring and Tomita, 1969) to mimick the calcium current thought to be responsible for triggering membrane potential spikes(Bulbring and Tomita, 1969; Walsh and Singer, 1980). Walsh and Singer (1980) using stomach muscularis of the toad showed that barium can substitute for calcium as a current carrier in cells exhibiting calcium spikes. Calcium is also thought to trigger the potassium conductance in order for repolarization to occur (Walsh and Singer, 1980; Meech, 1978). Barium could not effect this trigger, therefore it acted as a blocker of the potassium channel, by sustaining a depolarization (Bulbring and Tomita, 1969; Walsh and Singer, 1980).

In the case of canine trachealis (Stephens etal, 1975), it is normally considered a quiescent multiunit smooth muscle. The leaky potassium current in this tissue normally prevents membrane potential oscillations (Kroeger and Stephens, 1975); however, alteration of the potassium current with TEA produces both a mechanical and electrical rhythmicity (Watanabe etal, 1979; Kroeger and Stephens, 1975; Kirkpatrick 1975(b)).

Bulbring and Tomita, (1969) working with guinea-pig taenia-coli caused electrical spike generation by the addition of barium to a calcium free solution, in the presence of adrenaline which repolarized the membrane

potential. The spiking created by barium in zero calcium is thought to replace the calcium spike activity. Spike amplitude is dependent upon barium concentration (Bulbring and Tomita, 1969).

Tetraethylammonium (TEA) has been used to induce mechanical and electrical rhythmicity in smooth muscle (Kroeger and Stephens, 1975). The classical mechanism of TEA action involves a reduction in potassium conductance, accounting for depolarizations and the start of excitation-contraction coupling in the preparation (Watanabe etal,1979; Meech,1979).

Spike potentials can be created with TEA in bovine trachealis (Kirkpatrick, 1975a) and spontaneous potentials occur in rabbit carotid artery after TEA (Mekata, 1971). Watanabe, (1979) showed that canine tracheal smooth muscle responded with several types of rhythmic contractions to TEA. They vary from large contractions of several minutes duration with fast twitches imposed on the tops of the contractions, to higher frequency contractions and high frequency twitches.

Suzuki etal (1976) demonstated that acetylcholine and histamine evoked a sustained contraction in canine trachealis. Pretreating the tissue with TEA however allowed the agonists to produce twitches associated with spike generation, superimposed on a smaller contraction.

Heyer and Lux, (1976) contend that TEA may prolong the calcium current and thus contribute to spiking activity in

snail.

Ouabain, a specific inhibitor of Na2+/K+ ATPase, can depolarize guinea pig taenia coli (Tomita etal, 1971) and facilitate repolarization in rabbit ear artery (Reiner, 1978) and cat spleen (Bose etal, 1972). Watanabe etal (1979) demonstrated that canine trachealis incubated with ouabain (1Ø uM) did not affect tension over one hour, indicating that contribution of an electrogenic sodium pump to the membrane potential is not important. However after rhythmicity was induced in trachealis by TEA, ouabain ceased further (mechanical and electrical) oscillation at the peak of a twitch, indicating the sodium pump was responsible for the relaxation phase of rhythmicity.

The mechanical rhythmicity in canine trachealis upon metabolic depletion can be abolished by the Ca- channel blocker, D-6ØØ or the Ca2+ chelator EGTA in concentrations that do not affect normal tonic muscle (Bose and Bose, 1977). Likewise in tracheal muscle made rhythmic by TEA treatment, D-6ØØ and EGTA allow the tissue to relax again, after hyperpolarization was blocked with ouabain treatment (Watanabe etal, 1979). This indicated that the Ca2+ permeability had increased or become more important during both types of rhythmicity phenomena in the same tissue.

PACEMAKER THEORY: AN OVERVIEW

A SURVEY OF BIOLOGICAL OSCILLATING SYSTEMS

The phenomena of rhythmic electrical pacemakers in tissues, overviewed previously, can be considered as a

subset of the universal behavior of biological oscillations. The universality of biological oscillating systems again holds the same tenets of pacemaker systems (Rapp and Berridge, 1977). A survey of biological oscillations shows its true diversity.

In vitro, enzyme studies have been done on oscillation of the oxidation of NADH using lactoperoxidase (Nakamura etal, 1969) and with phosphofructo-kinase (Frenkel, 1968). Oscillations in glycolysis and nucleotides have been studied in suspensions of ascites tumor cells (Ibsen and Schiller, 1967) where accumulating NAD+ is associated with oscillation and in cell free extracts of Saccharomyces carlsbergensis (Richter etal, 1975; Becker and Betz, 1972). Oscillations in Ca2+ and H+ in rat liver mitochondria demonstrate a feedback between Ca2+ uptake and Ca2+ efflux rate (Carafoli etal, 1965). Fukushima etal (1972) investigated oscillations in phosphorylation of protein in rabbit and brain microsomes. The periodic synthesis of cGMP and cAMP has been studied in slime mold, Dictyostelium discoideum (Wurster etal, 1977; Malchow etal, 1978). The role of fire-fly pacemakers has been studied in bioillumination (Hansen, 1978).

Rhythms in protein synthesis have been observed in sea- urchin embryo (Mano, 197Ø) and lactate dehydrogenase and glucose-6-phosphate dehydrogenase in hamster cell (Klevecz and Ruddle, 1968). Pure oscillations in the membrane potentials with periods between Ø.25-1Ø seconds

have been studied in mouse fibroblast, macrophage, and guinea-pig megakaryocyte (Nelson etal, 1972; Gallin etal, 1975; Miller etal, 1978). The endocrine cells also exhibit membrane oscillations as studied by Meissner (1976) on pancrectic islet and on rat adenohypophysis (Poulsen and Williams, 1976).

Due to the large physical size of individual neurons in invertebrates, many studies had demonstrated several with spontaneous oscillations. Alving (1968) has shown a pacemaker activity residing in the cell body of Aplysia ganglion cell. The cell body metabolism ultimately directed the observed membrane potential oscillations. Barker and Gainer (1975) demonstrated that neurons from Aplysia can still maintain an oscillation in the membrane potential even after the action potentials have been blocked with the sodium channel blocker, tetrodotoxin. Heyer and Lux, (1976) demonstrated a calcium current contributing to the rhythmicity of pacemaker neurons in the snail, Helix pomatia.

Oscillating behaviors in more complex neural networks also have been observed. Cunningham and Rylander (1961) observed spontaneous potentials in chick cerebellar organ culture for over five days. Of many neurophysiological studies, the olfactory bulb, like other cerebellar centres, exhibited an osillatory response to electrical stimulation (Freeman, 1972).

Oscillations in mechanical and electrical activity have been observed in the three muscle groups: skeletal,

cardiac, and smooth free cells and in intact tissue.

With skeletal tissue, Purves etal (1974) noted spontaneous action potentials amounting to fibrillation of denervated rat diaphragm in organ culture that could proceed independent of tetrodotoxin. Ventricular muscle (Bolzer and Delahayes, 1973), atrial muscle (Brown, 1977) and Purkinje fibers (Tsien and Carpenter, 1978) all have shown spontaneous mechanical and electrical oscillation. Cultured chick heart cells also exhibit pacemaker activity (DeHaan and DeFelice, 1978).

Smooth muscle exhibits both slow oscillations and faster rhythms in various tissues. The muscles of the gastro-intestinal (GI) tract and several other smooth muscles can exhibit slow rhythms, e.g. stomach, small intestine, uterus, bladder, spleen and artery (Rosenberger etal, 1979; Connor etal, 1974; Ohba etal, 1977; Bose and Innes, 1974; El-Sharkawy etal, 1975(a); Daniel and Sarna, 1978; Mekata, 1979). Faster rhythms can be seen in trachealis, taenia-coli (Watanabe etal, 1979; Bose and Bose, 1977; Bulbring and Tomita, 1969; Barr etal, 1979, Mayer etal, 1972).

NEURON PACEMAKERS

Neuronal conduction systems on the whole can be characterized by chemical rather than electrical synapses. The pacemakers here tend not to synchronize due to the differing latencies of chemical synapses although some

synchronization of discharge occurs through electrical coupling (Gulrajani and Roberge, 1978).

The source of the pacemaker within a neuron has been localized by Alving (1968). By ligating the cell body from dendrites and axon the pacemaker was shown to reside in the cell body. Metabolic activities then could act as a source for the electrical rhythmicity (Meech, 1978).

The criteria that characterize pacemakers can be the same for neurons, smooth muscle and cardiac muscle and are attributable to biophysical membrane properties (Meech, 1978). The probability of discharge is proportional to the membrane potential and increases with depolarization (Somlyo and Somlyo, 1968). Pacemakers can have a resetable rhythm by an altered conductance after an action potential and prior to the next action potential which form the ionic basis for slow wave formation. In Purkinje pacemakers discharge results from an increasing Na2+ conductance whereas after discharge a time and voltage dependent decrease in potassium conductance occurs (Tsien and Carpenter, 1978).

RHYTHMIC POTENTIALS IN INTESTINAL MUSCLE

Organized motility in the gastrointestinal (GI) tract smooth muscle requires rhythmicity, synchronization and propagation. On dividing the muscle layers of the GI system, into longitudinal and circular layers, different contributions originate from them (Prosser, 1974). In cat the longitudinal layer contains the source for electrical

rhythm in slow waves whereas the circular layer shows spontaneous pacemaker potentials and propogating spikes but the rhythm is not steady or synchronized unless under longitudinal muscle control (El-Sharkawy and Daniel, 1975a; Connor etal, 1976). In other preparations (rabbit, dog, guinea-pig) the separation of the muscle layers proves difficult, resulting in the longitudinal layer, Auerbach's plexus and outer circular combination supplying more complex potential waveforms (Daniel and Sarna, 1978).

SLOW WAVES

The amplitude and rate of depolarization double when the longitudinal muscle layer is associated with circular muscle, at least in the case of the cat intestine (Daniel and Sarna, 1978). The doubling of depolarization amplitude and rate are carried out in the circular muscle after the low amplitude slow waves from the longitudinal layer have passively entered the circular layer where regenerative events increase the waves by changing the Ca2+ conductance. The potential then can spread back to the longitudinal muscle or propogate down the tract, as the longitudinal muscle is incapable of propogation completely by itself (Connor etal, 1974).

Several lines of evidence have accrued for a role of a fluctuating electrogenic Na2+ pump as a major contributor to the rhythmic slow waves in longitudinal smooth muscle (Daniel and Sarna, 1978; Connor etal, 1974).

The mechanism presumably resides in the pump quantally activating or increasing coupling of Na2+/K+ to repolarize as well as to commence depolarization by turning off or reversing coupling (Prosser, 1974; Connor etal, 1974).

Longitudinal muscle previously producing rhythmic slow waves will slightly depolarize after low dose ouabain into quiescence. Even hyperpolarizing the membrane back to resting potential, the slow waves do not resume (Connor etal, 1974). Additionally, K+ is required for slow waves because K- free Krebs bathing solution produces a depolarization, slow waves cease and cannot be reinstated upon repolarization.

The pump contributes to the membrane potential because the slow wave amplitude is greatest when external K+ is closest to pump contributing resting membrane potential. Reducing metabolism reduces slow wave amplitude (Connor etal, 1976; Watanabe etal, 1979). Radioactive Na2+ effluxes at the beginning of repolarization. Resistance changes cannot be seen with waves, only with spikes on waves (Connor, 1979). Hyperpolarization can decrease frequency and increase amplitude of slow waves. Depolarization increases frequency and decreases amplitude (Connor etal, 1974).

IONIC BASIS FOR RHYTHMICITY IN SMOOTH MUSCLE

The divergence in smooth muscle studies partially arises from the heterogeneity of the tissue itself.

Different preparations from different species give unique results. Divergence also lies in the background of the investigators and what aspects of rhythmicity they choose to study. Many electrophysiologists study smooth muscle rhythmicity solely as an electrical phenomena of the membrane, unrelated to the physical events of contraction and mechanical rhythmicity.

Two schools of thought can be broadly inferred from the smooth muscle rhythmicity literature. One school embodies the hypothesis of an active electrogenic Na2+ pump responsible for maintaining portions of oscillations (Prosser, 1974; Connor etal, 1974). The other school embraces the idea of passive ionic currents initiating rhythmicity (Mekata, 1979; Ohba etal, 1977; El-Sharkawy and Daniel, 1975). Given the complexity and diversity of smooth muscle, theories postulating hybridization of the two mechanisms can be found in the literature (El-Sharkawy and Daniel, 1975 (a)(b)(c)).

Conner et al (1974) studying the slow wave activity in longitudinal muscles of cat intestine, concluded that the oscillations in membrane potential are caused by oscillations in the activity of an electrogenic Na2+ pump. Evidence cited solely involved dimunition of slow waves by low temperature, anoxia, metabolic inhibitors and ouabain.

The evidence implicates an active energy dependent process for slow waves. Conductance changes were not evident during slow waves. Na2+/K+ ATPase was proposed as a candidate for the electrogenic ion transport system.

Where low temperatures would slow the kinetics of the system, anoxia and metabolic inhibitors reduce ATP levels available to the enzyme; and ouabain inhibits the Na2+/K+ ATPase (Brading etal, 1974).

The electrogenic transport system may function at a basal rate, pumping Na2+ out and K+ into the cell. After a time the transport stops or slows allowing inward leakage of Na2+ which depolarizes the membrane and starts the rising phase of the slow wave. An oscillatory prepotential only of two seconds duration and 5 mV amplitude, in quiescent preparations, was attributed to the electrogenic pump's running constantly and not stopping and starting as for slow waves. The pre-potentials could represent the quantal availability of ATP to the electrogenic pump which from a distance would appear as a constant running pump. The frequency of the prepotentials was much higher than the slow waves. The control of the pump could be through substrate depletion or inhibitor products (internal Na2+). Either mechanism would rely on hysteresis for activation and inactivation of the electrogenic pump, where low internal sodium activates the pump. For ATP depletion to act as a control for the pump, one must postulate high internal Na2+ to increase ATP production or high internal Na2+ to lower the threshold for activation of the pump. Working with isolated longitudinal strips of rabbit jejunal smooth muscle, El-Sharkawy and Daniel, (1975) argue for a control potential (slow waves) being initiated by Na2+

conductance changes then terminated by chloride pumps.

One variation of the electrogenic Na2+ pump hypothesis involves an increase in Na2+ permeability for depolarization and increased Na2+ pumping activity for repolarization. The other variation involves high ATP levels activating Na2+ permeability (depolarization) and high internal Na2+ and high ATP activating the Na2+ pump for repolarization. In the same experimental series, El-Sharkawy observed a notch of a few of millivolts in the plateau of the depolarization which seemed to separate the depolarization into two phases. The occurence of the notch could not be reconciled by any of the electrogenic Na2+ pump hypotheses. The presumed oscillating electrogenic Na2+ pump could be slowed or stopped by manipulations of low temperatures, hypoxia, poisons or inhibitors. However, the membrane potential defied the electrogenic pump theory by not depolarizing. Poisoning the pump with ouabain didnot allow the membrane to repolarize, however it never depolarized as was be expected. The notch on the plateau region of the slow wave (control potential) became more apparent at low temperatures. The control potential can be reduced by a 50% reduction in NaCl, a low external Cl- may contribute. The control potential may be caused by an increase in Na2+ influx. The potential is electrically excitable and can increase on hyperpolarization and decrease on depolarization. Proponents of the electrogenic pump argue for a controlling or contributing role to membrane potential, but the membrane potential should not

control the pump (independence) by turning it off (Prosser, 1974; Connor etal, 1974). Reducing external Ca2+ results in loss of control potentials and a depolarization. Returning Ca2+ promptly returns control potential activity before the repolarization of membrane potential starts. Loss of control activity could not be due to Ca2+ depolarization but may affect a triggering mechanism. For the initiation of control potentials, an inward Na2+ current may depend upon external Ca2+. A role of chloride in the second phase of the control potential depolarization may exist because chloride replacement with less permiant anions such as propionate lead to loss of the secondary depolarization of the control potential. Chloride replacement with more permiant anions (nitrate) only increase the frequency of control potentials with spiking on every potential. The secondary depolarization may be due to an increase in chloride permeability after the Na2+ current (Daniel etal, 1975). Tomita et al (1977) present evidence against a role of the sodium potassium pump in the direct control of slow wave potentials in circular muscle from guinea pig stomach. The slow waves from this preparation exhibit two components: (1) a voltage independent lower component and (2) superimposed upon it, a voltage dependent upper component. The top component is often associated with a spike component. Conductance changes can only be associated with the second and spike components. The slow inward component is unaffected by

hyperpolarization or depolarization. The first component however is temperature sensitive, suggesting an active transport mechanism. Reduction of external sodium concentration lengthens the slow wave duration and Na2+ below 1Ø mM causes rapid depolarization. The depolarization may be due to an increase in Ca2+ permeability because reduction of Ca2+ in Na2+ free solutions also reduces depolarization. Neither ouabain nor zero external K+ inhibit slow waves indicating the Na2+/K+ pump is not implicated. The duration of the first component depends upon external Ca2+ because K+ does not potentiate slow waves in Ca2+ free Krebs. Proposals for a) Na2+ competing with Ca2+ outside are put forth b) Na2+/Ca2+ exchange to move Ca2+ into the cell. Because a slight Na2+ reduction increases slow waves, this is compatible with the hypothesis that Na2+ and Ca2+ compete for the same site. Larger Na2+ reductions abolish slow waves, perhaps by rearranging the Ca2+ distribution.

Using rabbit aortic smooth muscle, Mekata (1979) studied evoked slow potentials. The slow potentials were evoked by noradrenaline which increased the membrane conductance, probably allowing a Na2+ current to enter the cell. A fall in K+ conductance along with an inward current may underlie the basis for oscillations in this preparation. Arguments against an electrogenic Na2+ pump include a temperature and metabolic inhibitor insensitivity of slow waves in arteries.

Various smooth muscles can exhibit a spiking activity

often associated with slow waves. Depending upon the preparation, a spike may preceed a slow wave or occur at the plateau of slow waves. Weigel et al (1979) demonstrated a dual role for calcium in the spiking activity of cat intestine circular smooth muscle. The upstroke of the spike appears to be caused by an inward Ca2+ current because the amplitude of upstroke decreases with decreasing calcium concentration. Sodium replacement with Li2+ and block of K+ conductance by TEA did not affect upstroke. Blocking Ca2+ conductance by manganese or verapamil blocked spiking (Bulbring and Tomita, 1972). Spike repolarization appears to be attributable to an outward K+ current because no repolarization occurs with TEA. The K+ current in this case appears to be activated by Ca2+ because replacement of Ca2+ with barium (which still conducts an inward depolarization current) can no longer activate an outward K+ current.

The source of the Ca2+ for spiking appears to be from membrane associated sites because replacement of Ca2+ with La3+ diminishes Ca2+ spiking. The La3+ replaces the bound Ca2+, occupying its storage site and prevents Ca2+ influx. The electron dense La3+ can be visualized on electron micrographs on the external lamina of the cell (Henkart and Hagiwara, 1976) and also due to great D-600 sensitivity (Bose and Bose, 1977; Watanabe etal, 1979).

METABOLIC PROCESSES DURING COOLING

In 1970 Scott, troubled by wide variations in the results in his experiments on porcine arterial wall respiration, designed experiments to explore artifacts in the tissue preparation. In doing so, he demonstrated a Pasteur effect in porcine arterial wall and a decrease in respiration and in glycolysis in tissues that were pre-cooled. Using oxygen uptake as an indicator of tissue respiration, the artery preparation failed to maintain a high oxygen uptake when prepared in glucose free medium at 37 C. By briefly lowering tissue temperature to 4C and then returning it to 37 C for 60 minutes the oxygen uptake could only recover to one-fifth of the uptake in tissue prepared at 37 C (Scott etal, 1970). Precooling halved the lactic acid production in medium containing glucose. A Pasteur effect can be seen in tissues maintained continually at 37 C in glucose-Krebs show a greater lactic acid production under anaerobic conditions than under aerobic conditions (Levy etal, 1973; Lehninger, 1970 (b)).

The reversibility of tissue metabolism after cooling has been held as a basic tenet in biology, amongst the few exceptions is the nitrogen fixing enzyme system in Clostridium pasteurianum (Lehninger, 1970 (a)). Membrane changes in mitochondria of arterial wall may account for a low respiration rate in pre-cooled tissue (Scott etal, 1970). The leakiness may extend to the plasma membrane to cause a possible lack of cytoplasmic cofactors and in

inactivation of glycolytic enzymes during cooling, as evident by a decreased rate of glycolysis in pre-cooled tissue (Casteels, etal, 1972).

Cold - induced plasma membrane changes can be further illustrated by comparing the aortic smooth muscle of the rat and hibernating ground squirrel both at 7 C. Even after 48 hours, ground squirrel aorta maintained sodium and potassium levels near normal (Kamm etal, 1979 (a)).The rat aorta lost potassium and gained sodium. More importantly, neither aorta changed in its calcium or magnesium content. The hibernating ground squirrels' adaptation for the maintenance of ionic gradients and membrane potential can probably be attributed to changes in the lipid content of the membrane (Kamm, etal, 1979 (b)). The exchange of sodium with a Q1Ø of 3.7 was not by passive diffusion. The changes in lipid may result in a greater lipid "fluidity" while cold around the Na-K+ pump and/or decrease in membrane permeability (Kamm etal, 1979 (b)). If for hibernators, the energy using processes stop before the energy supplying processes; this favors a functioning Na-K+ pump (Kamm etal, 1979 (b)).

Glucose seems to be required for the maintenance of arterial respiration (Shibata etal, 1967; Arnquist, 1978). Respiration wains in preparations lacking glucose after 9Ø minutes in tissues continually maintained at 37 C. Studies of pre-cooled arterial wall indicate that ATP production comes from equal portions of glycolysis and oxidative phosphorylation. The ratio of ATP appears to be 4Ø%

arterial glycolysis and 60% oxidative phosphorylation in tissues maintained continually at 37 C (Scott etal, 1970).

CYCLIC-GMP AND SMOOTH MUSCLE CONTRACTION

Cyclic GMP directed protein phosphorylation occurs in very few celllular systems investigated thus far. These include arthropod-lobster tail (Greengard, 1975; Beam etal, 1977; Posternak, 1978), smooth muscle (Andersson etal, 1977; Diamond and Hartle, 1976; Andjelkovic and Andjelkovic, 1979; Katsuki and Murad, 1977), plumonary tissue (Kaukel etal, 1978; Klass, 1978), cerebellum (Biggio etal, 1977; Schwabe etal, 1978) and heart tisse (Brooker, 1977; Nawrath, 1976).

Cellular calcium appears to determine smooth muscle tone and in several preparations, increasing contraction also can be related to an increase in cellular cGMP (Andersson and Hardman, 1975; Andersson etal, 1977; Diamond, 1977; Schultz etal, 1975). After the introduction of an agonist, the biochemical sequence of increased cellular calcium, increased cellular cGMP and increased contraction has not been agreed upon. The order and cause of the events of the sequence to each ther are also in dispute (Nilsson and Andersson, 1977; Diamond and Hartle, 1976; Andersson etal, 1977), raising the main question as to whether an alteration in cGMP metabolism is the primary effect of an agonist followed by changes in calcium distribution or is the reverse true or do intracellular

alterations of cGMP bear no relationship to calcium (Schultz and Hardman, 1975).

Several investigators attempting to determine changes of cGMP during smooth muscle contraction, noted equivocal results, depending upon the preparation and agonist used (Gruetter etal, 1979). Nilsson and Andersson, (1977) demonstrated that cGMP released calcium and inhibited calcium accumulation in preloaded plasma membrane fraction from rabbit colon. cGMP levels increased in the same rabbit colon preparation after contraction with carbachol. This supports the hypothesis that cGMP may function in positive feedback on the release of internal cellular calcium in rabbit colon (Nilsson and Andersson, 1977).

Schultz and Hardman, (1975) stated that the change in cGMP metabolism is secondary to primary alterations in calcium movement chiefly because removal of calcium from the bathing medium of rat ductus deferens and addition of agonists such as KCl or acetylcholine could not, as they would normally, elevate cellular cGMP. Removal of calcium lowered basal cGMP indicating calcium plays a role in regulating the cellular cGMP turnover rate.

Andersson etal (1977) working with tracheal smooth muscle, demonstrated an increase in cGMP upon contraction with carbachol or histamine. To answer the question as to whether cGMP increased, directly as a consequence of an agonist interaction and mediated a contraction, or whether cGMP increased only after contraction had started, Indometacin, a cyclooxygenase inhibitor was included in the

tracheal muscle bathing solution. Again, only after tension had developed, did cGMP increase and the increase in cGMP in response to acetylcholine was dependent upon calcium because omission of calcium allowed no change(Andersson etal, 1977).

THE GUANYLATE CYCLASE ENZYME

Some of the difficulty in assigning a role to GMP has been its low level (1Ø nM) in tissues compared with the concentration of cAMP . The enzyme responsible for synthesis, guanylate cyclase can be found in both membrane associated insoluble form and a free cytomplasmic soluble form (Gruetter etal, 1979; Schultz etal, 1977(b)). The enzyme depends upon the divalent cation manganese for activity (Mittal etal, 1977(a)(b); Schultz etal, 1977(b)).

Several forms of phosphodiesterase can metabolize cGMP, one form can hydrolyze cGMP with a greater efficiency than cAMP, suggesting a GMP - specific enzyme and perhaps a specific function for cGMP in the tissue system where the phosphodiesterase resides (Posternak, 1978; Hardman etal, 1977). Although guanylate cyclase generally requires manganese as a cofactor for activity, superoxide dismutase can also activate guanylate cyclase to a lesser extent than manganese can (Mittal etal, 1977(b)). The dismutase's activation of guanylate cyclase in the soluble form from rat liver can be blocked by inhibitors of superoxide dismutase such as KCN or glutathione. Activation requires

both superoxide ion and H2Ø2 in the system, as catalase which utilizes hydrogen peroxide, reduces the activating ability of superoxide dismutase (Mittal etal, 1977(b)).

In cell - free systems, superoxide ions and hydrogen peroxide reacting together generate hydroxyl radicals that probably activate guanylate cyclase because scavenging the radicals from the system does not lead to activation. Depending upon oxidizing or reducing agents in the preparation, oxidizing agents will activate cyclase and consequently increase cGMP whereas reducing agents produce opposite effects. Pharmacologic alteration of redox or free radical production may account for changes in cellular cGMP under drug or hormone treatment (Mittal etal, 1977(a)(b)).

Guanylate cyclase can be activated by hydroxylamine, nitrite ion, nitroglycerin, nitroprusside (Schultz etal, 1977(a)), nitric oxide (Gruetter etal, 1979). Sodium azide activation on cyclase requires a protein factor such as catalase as well as oxygen. The activating agents under some conditions change to nitroso groups or nitric oxide capable of activating guanylate cyclase (Mittal etal, 1977(b)).

SMOOTH MUSCLE RELAXANTS AND RELATION TO cGMP

Not only can agonists which contract smooth muscle increase cGMP but also smooth muscle relaxants, such as sodium nitroprusside increase cGMP levels in ductus deferens (Schultz etal, 1977 (a) Gruetter etal, 1979).

Unlike the increase in cGMP after muscarinic stimulation to contraction, the nitroprusside -induced increase in cGMP is independent of extracellular calcium. Low extracellular calcium causes no contraction nor any increase of cGMP after acetylcholine. Smooth muscle relaxants and antihypertensives such as hydroxylamine, nitroglycerin and sodium nitrate can increase cGMP independent of extracellular calcium (Mittal etal, 1977(a); Schultz etal, 1977(a)).

The antihypertensives: diazoxide, hydralazine, minoxidil and the coronary artery dilaters dipyridamole, prenylamine, cinnarizine, lidoflazine, perhexiline, D-600, SKF525A, and chlorpromazine can increase cGMP independent of extracellular calcium. The drugs probably increase cGMP by a direct or indirect stimulation of guanylate cyclase and not by an inhibition of phosphodiesterase. The activity of phosphodiesterase, and nucleotide destruction is very dependent on external calcium for degradation of cGMP (Sprugel etal, 1977). Calcium channel blockers, D-600 and phenylamine still can increase cGMP.

An analog of cGMP, 8-bromocyclic GMP (10 uM) can reduce the agonist induced contraction of smooth muscle (Kukovetz etal, 1979). Whether the cGMP is increased by smooth muscle relaxants or smooth muscle agonists that have opposite effects on muscle tonus, the cGMP may act as an end signal to contraction or cellular excitability by inhibiting calcium influx (Schultz etal, 1977(a)).

POSSIBLE MOLECULAR ACTIONS OF cGMP IN SMOOTH MUSCLE

Greengard (1975) showed that a membrane fraction from guinea-pig ductus deferens contains membrane bound proteins susceptible to cGMP - dependent phosphorylation by endogenous kinases. More 32-P from labelled ATP was incorporated into these proteins after incubation with cGMP than without cGMP. Membrane proteins from guinea pig uterus and rabbit small intestine gave similar results, contending that the phosphorylated proteins compose the inner aspect of the muscarinic acetylcholine receptor or associated ion channels or calcium storage proteins (Greengard, 1975; Schultz etal, 1977; Beam etal, 1977; Hofmann, 1979; Lincoln and Corbin,1978).

Cyclic GMP apparently increases only after the increase of calcium after a mediator. Alternately, calcium through an acetylcholine receptor associated channel may be required to trigger the cGMP levels within the cell which could release the internal stores of calcium for contraction by dephosphorylation (Schultz and Hardman, 1975). This implies cGMP is involved in the contraction process only after intiation with calcium (Beam etal, 1977).

Cyclic GMP may function at the cellular level in terminating cellular excitability in some systems by its ability to activate GMP specific protein kinases (Lincoln and Corbin, 1978; Greengard, 1975; Kukovetz etal, 1979). Once the kinase has been activated, it can then proceed to

phosphorylate perhaps the inner aspect of the calcium channel, thus preventing further calcium entry. Teichberg etal (1977) demonstrated phosphorylated and dephosphorylated forms of the acetylcholine receptor and showed that the 48000 dalton subunit, a polypeptide ionophore, was phosphorylated.

SECTION B

METHODS

THE ANIMAL MODEL

Twenty-four hour fasted dogs of undetermined age, breed and sex were the source for tracheal smooth muscle. The canine trachealis model has been studied before and therefore a sizeable body of literature exists on its physiology, biochemistry and pharmacology. Canine trachealis provided an accessible supply of smooth muscle in large enough quantities for both biochemical and physiological studies. Other species could easily provide a larger source of tissue, such as bovine trachealis. Since this tissue is obtained from the abattoir, the viability would change with time.

A more rigid control of age, species and gender could be enforced if rats or guinea-pigs were used. The small tissue size from these animals would not permit simultaneous biochemical and tension analysis. Only a small number of samples can be taken from the trachea of small rodents. Tracheal sections are isolated and the change in intact tracheal diameter is related to force development. In canine trachea however, a single sample unit represents a single tracheal ring and therefore many samples can be taken from the trachea of a dog.

The trachealis was used because it represents a smooth muscle system of multiunit cells, oriented in the same direction. Canine trachealis can easily be cleaned of connective tissue. The experimental procedures can convert a quiescent multiunit smooth muscle so as to contract

rhythmically in a fashion similar to that of single unit smooth muscle.

DISSECTION

Dogs were fasted for 24 hours prior to the experimental day in order to deplete tissue glycogen stores, and were anaesthatized by intravenous injection of pentobarbital (35 mg/kg). The neck was shaved; the trachea quickly excised and placed into a beaker of normal Krebs. The preparation was immediately transferred to a gassed (95% Ø2 5% CO2) tray of 4 C normal Krebs where the dissection was performed with the trachea completely submerged in the solution to prevent anoxia. The mucous membranes and connective tissue were cleared from the trachea. The smooth muscle plane was cut free from the cartilage rings. The interior epithelial layer, along with the underlying fibrous mat of collagenous connective material was cleared from the plane of muscle. The plane was dissected width-wise into strips of about 25 x 2.5 mm. The thickness of the tissue plane approximated Ø.8 mm. Loops were tied to the ends of the tissue with cotton or polyester thread. The tissues were mounted in 1Ø ml tissue baths, warmed to 37 C by circulating water. The tissues were equilibrated in normal Krebs under one gram resting isometric tension for 3Ø minutes. The base-line tension for the duration of the experiment was one gram.

TREATMENTS

Throughout this study four methods of analysis were employed to determine Ca2+ movements and stores in canine trachealis. The Ca2+ dynamics were followed through a sequence of events from normal tissue to metabolic depletion and subsequent rhythmicity and finally to recovery. The analysis involved:

(a) whole tissue calcium measured by atomic absorption spectrophotometry

(b) intracellular calcium measurement

(c) tracer calcium uptake

(d) intracellular calcium uptake

(e) calcium compartmentalization by efflux

The tissues were placed into six treatment categories to follow the events of metabolic depletion.

(1) normal tissue at rest in Krebs

(2) normal tissue contracted with carbachol in Krebs

(3) depleted tissue at rest in glucose-free Krebs

(4) depleted tissue contracting with carbachol in glucose-free Krebs

(5) recovering tissue at rest in normal Krebs from glucose-free Krebs

(6) recovering tissue contacted in normal Krebs from glucose-free Krebs.

FIGURE 1:

Time course of the development of tension of tracheal smooth mucle strips with the agonist carbachol .2 uM . The lower figure adapted from Bose and Bose, 1977 shows a high speed trace of electrical and mechanical activity at the beginning of tension development. Eleven mM glucose was present in the Krebs solution.

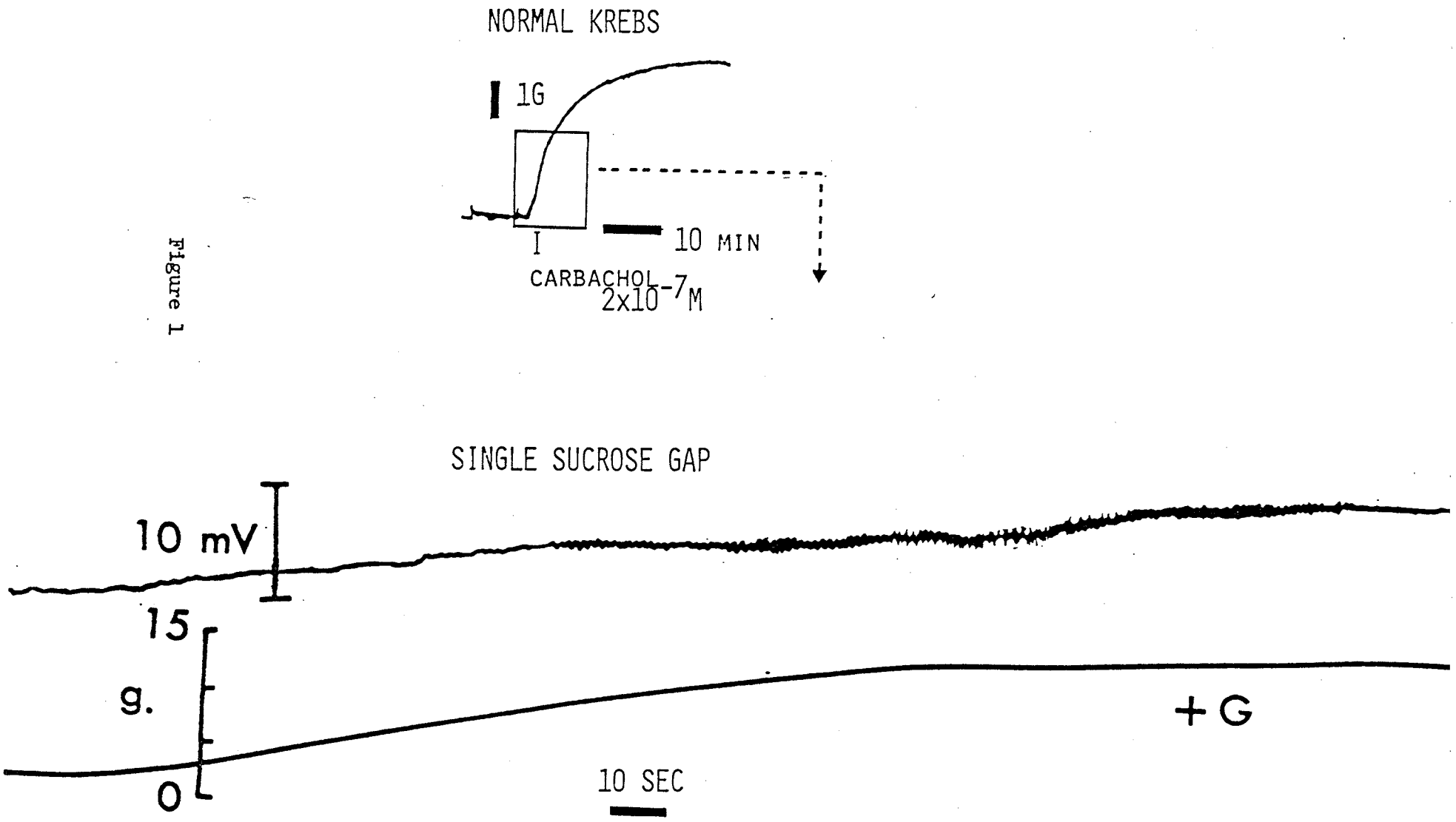

NORMAL KREBS
1G
10 MIN
CARBACHOL
2x10-7M
SINGLE SUCROSE GAP
10 mV
15
g.
0
+G
10 SEC

Figure 1

FIGURE 2:

The time course of the development of rhythmicity in a glucose free Krebs medium after the addition of carbachol Ø.2 uM. The lower figure adapted from Bose and Bose,(1977) shows a high speed trace of mechanical and electrical activity during one rhythmic contraction.

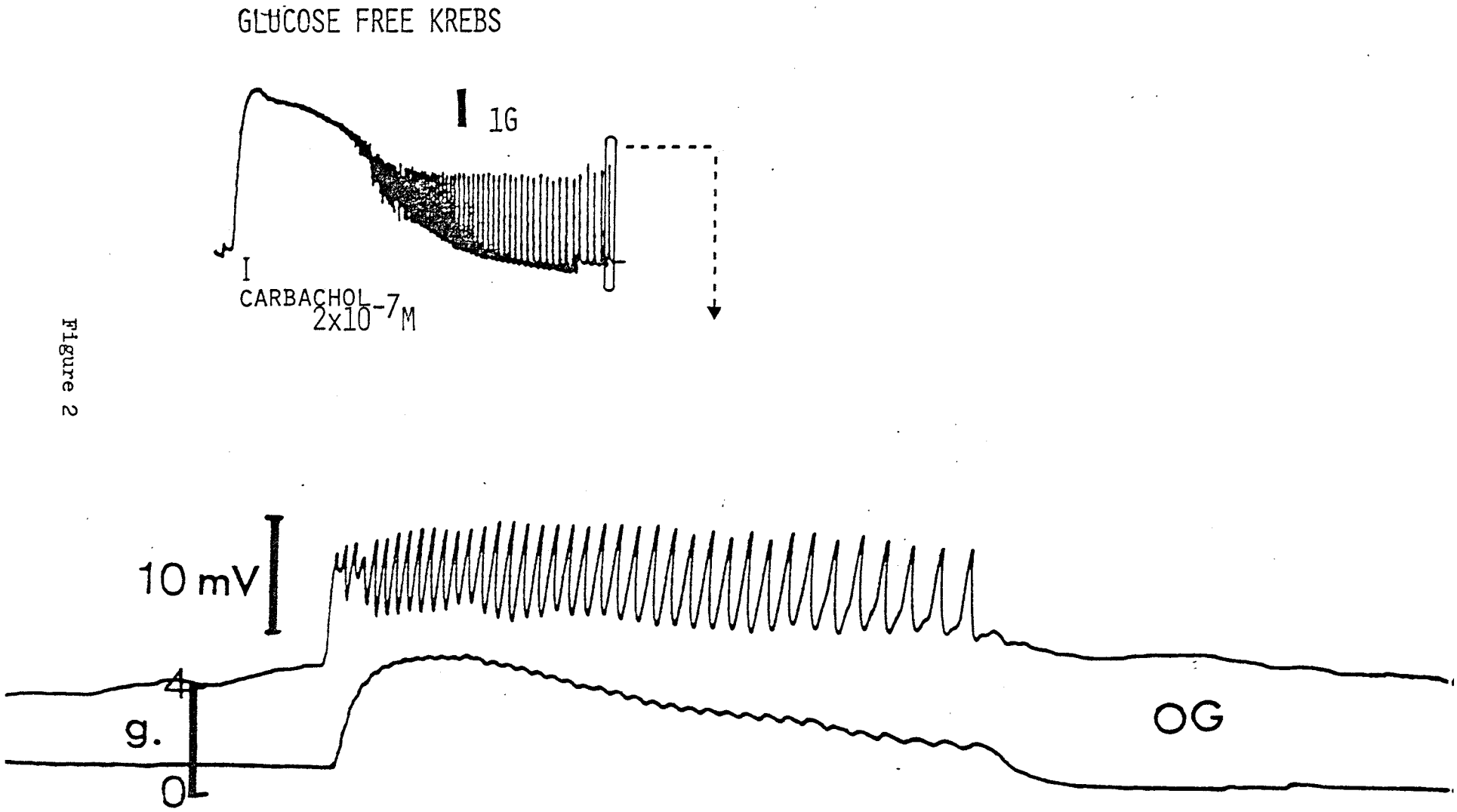


Figure 2

TISSUE EXTRACELLULAR SPACE

The first phase of the experiment involved assessing the changes in extracellular space during different phases of metabolic depletion and during different contractile states. The active tissues were equilibrated with 3-H-PEG-4000 for one hour, blotted, weighed and counted. The extracellular space was calculated according to the method of McIver and MacKnight (1974). After the tissue was equilibrated to the sought metabolic or contractile state, usually at one hour, 3-H-PEG-4000 was added to the bathing Krebs to yield a final concentration of 0.14 uCi/ml. At that time a treatment was started for one hour. After one hour the tissues were immobilized to preserve their tension, then blotted, and weighed. An aliquot of medium removed from the bath for counting usually had around 300 dpm/ul. The tissues were dissolved for 12 hours at 55C in 250ul NCS tissue solubilizer (Amersham/Searle) in plastic scintillation vials. 25ul 6N acetic acid were added to the vials to neutralize the contents of the digest along with 10mls tissue scintillation cocktail. Seventy five ul. of medium was added to 10 mls EGME scintillation cocktail for the total medium counts. Both vials were shaken and a tissue cocktail blank was prepared. The B-scintillation counter was set to a tritium channel and the samples were counted. The samples were expressed as cpm/mg for tissue and cpm/ul for medium. Each was converted to dpm.

Percent extracellular space was calculated by:

$$\frac{\%\ \text{extracellular space by 3-H-PEG}}{} = \frac{[\text{dpm/mg wet tissue weight}]}{[\text{dpm/ul bathing medium}]}$$

EFFLUX OF 3-H-PEG-4000

Efflux of extracellular space was measured to verify that the PEG under all conditions of metabolic depletion occupied only one compartment using a modification from McIver etal. (1974). The experiment involved using trachealis tissue strips under 4 conditions:

(1) in normal Krebs at rest

(2) in normal Krebs contracting under the agonist action of carbachol.

(3) in glucose-free Krebs at rest

(4) in glucose-free Krebs rhythmically contracting under the influence of carbachol.

The tissues were equilibrated in their respective Krebs solutions for twenty minutes. Carbachol was then added to verify tissue viability by means of a contraction and then washed away.

Carbachol was then added as treatment for tissues #2, #3 and #4. After the tissues in glucose-free Krebs (#3, #4) exhibited a stable rhythmicity (after depletion of glycogen stores); bath #3 (glucose-free Krebs at rest was washed) and approximately 0.12 uCi/ml 3-H-PEG-4000 was added to all the baths. The PEG was permitted to equilibrate for one hour, after which the tissues were immobilized with dental wax by means of adhering the top

thread to the stainless steel rods. The tissue rods were immobolized specifically for efflux along side hollow stainless steel tubes along side of the main stem of the tissue rods by heat shrinkable plastic tubing. Each hollow stainless steel rod was fitted with plastic tubing attached to a regulated air supply of 95% O2 and 5% CO2 (Linde trade mark). The tissues were dipped into 37 C oxygenated Krebs of identical composition to the bathing medium except 3-H-PEG-4000 was omitted. The tissues remained in the bath for 2-3 seconds to wash off any adhering isotope. All four tissues were simultaneously incubated in test-tubes containing Krebs of the same composition with or without the PEG. The test tubes contained 4 mls and were warmed in a thermally regulated aluminium block at 37 C and oxygenated as described earlier. The tissues were simultaneously and serially transferred to the next row of test tubes in an aluminium block. The times of the incubations in the block were:

(a) 30 seconds apart for 10 tubes (b) then every minute for 10 tubes (c) every 5 minutes for 15 tubes (d) then every 10 minutes for 5 tubes. Each tissue stepped through 40 serial incubations during each respective treatment to maintain the same metabolic and mechanical state as recorded from the active tissues in the baths before the experiment. After the efflux series the tissues were placed into plastic scintillation vials and digested as described earlier . A one ml aliquot was removed from each test tube of the efflux series and placed in a plastic

scintillation vial. The vials were gently dried to reduce the volume of sample but not caramelize the glucose in the Krebs. After drying the vials were cooled to room temperature and the electrolytes and isotope of the sample were redissolved in 100 ul of distilled water. To each vial 10 ml of EGME scintillation cocktail was added. The vials were counted in an identical fashion for tritium as in the extracellular space determination using 3-H-PEG-4000. The counts per minute were converted to dpm by the computer programme DPM.BAS. The result was corrected for volume and expressed as dpm/vial. All the dpm's/vial were summed with the counts left in the tissue at zero time in the efflux curve. The experiment had 40 time periods plus the counts remaining in the tissue which represents an infinite time period (41 contributing dpm's). The percent remaining radio- activity can be calculated for any time period (i) by:

$$\frac{\text{Sum of Total DPM - Sum of 1st DPM to (i)th DPM}}{\text{Sum of Total DPM}} \times 100$$

A plot of the log of percent remaining radioactivity vs. time gives an exponential decay curve. The curves were analyzed by a curve peeling technique in PEEL.BAS [See APPENDIX] into efflux parameters of compartment size, expressed as percent remaining activity, compartment rate, expressed as percent remaining per minute, and compartment half-time expressed in minutes.

WHOLE TISSUE CALCIUM ESTIMATION

As with all calcium estimation experiments, six treatments were performed to outline metabolic depletion. The treatments were (1) NK-REST (2) NK-CARB (3) GF-REST (4) GF-CARB (5) GF-REST-NK (6) GF-CARB-NK. The experimental protocol was stated before for checks of tissue viability. Once the desired metabolic state of the tissue had been achieved, the treatments were started for one hour under a stable metabolic state. The tissues were then immobilized with dental wax on a stainless steel mounting rod to maintain the same tension. Tissue calcium under two conditions were determined (a) whole tissue calcium and (b) internal calcium after lanthanum (La3+) displacement. If internal calcium were to be measured the experimental procedure differed only in the extra step of placing the tissues in iced Ø.Ø5M La3+ Tris Solution for one hour. The La3+ ions in this procedure displaced any externally bound Ca2+ and sealed the membrane to prevent the intracellular Ca2+ form fluxing outwards (Godfraind, 1976). The tissues were cut free from the mounting rods and blotted twice between #1 Whatman paper for 5 seconds with a lead weight. The tissues were individually weighed and frozen in acid washed plastic capped vials. A fresh acid solution was prepared before hydrolysis of the tissue. A one:one solution of perchloric acid and nitric acid was mixed in a fume hood to about 1Ø ml total volume. The plastic vials containing the tissues were opened and 1ØØ ul

of acid solution was added. The vials were recapped and placed in a 37 C water bath for four days to aid hydrolysis. After that time two ml of Ø.ØØ4M La3+ diluent solution was added to each vial, making a total volume of 2.1 mls. Standard concentration calcium solutions were prepared with CaCl2 in La3+ diluent to concentrations of Ø.Ø5, Ø.1, Ø.15, Ø.2, and Ø.3 umoles/ml. The standard blank was prepared with only La3+ diluent.

The 4Ø-Ca2+ was estimated by atomic absorption spectrophotometry on a Perkin Elmer machine for one complete study and a Jarrel-Ash AA Model 85Ø for another study. Both machines were calibrated according to manufacturer's protocol for 4Ø-Ca2+ estimation. Any extra absorbance due to acid in the samples was graphically corrected by using an acidified blank. A randomized complete block analysis of variance with Duncan's new multiple range test of significance was used to analyze the data.

CALCIUM EXCHANGE EXPERIMENTS

The experiments to assess the exchange of isotopic calcium were similar in design to that of loading 3-H-PEG into tissue. Six treatment groups were assigned in each exchange experiment:

(1) resting tissue in NK

(2) contracted tissue in NK

(3) resting tissue in GF

(4) rhythmic tissue in GF

(5) recovered resting tissue in NK

(6) recovered contracting tissue in NK

The tissues were checked for viability and brought to the desired metabolic state in about two hours. After the tissues were metabolic depleted, Ø.5 uCi/ml of 45-Ca2+ were added to each bath. One hour was allowed for equilibration with isotopic calcium. The tissues were immobilized with dental wax against the tissue rods and removed from the baths. The method changed slightly if only internal exchangeable calcium was to be assessed.

For measurement of internal calcium, the tissues were placed in iced Ø.Ø5M La3+ Tris solution for five minutes. The tissues were manipulated similarly to whole tissue Ca2+ exchange estimation by shaking, blotting dry between #1 Whatman filter paper and weighing. The tissues were dissolved in plastic scintillation vials with 25Ø ul NCS tissue solubilizer (Amersham/Searle) for 5 hours in a 5Ø C water bath. 25 ul of 6N HoAc were added to neutralize the

NCS and 1Ø mls of tissue scintillation cocktail were added. The total counts in the bath were determined by adding 75 ul of radioactive medium from each bath into scintillation vials with 1Ø mls EGME scintillation cocktail [See REAGENTS section]. The Beta scintillation counter was calibrated for an optimum channel width for detection of 45-Ca2+. The counts in cpm were converted to dpm by computer. The amount of calcium was expressed as mMoles Ca2+/(kg. wet wt.) and calculated according to Godfraind (1976) by the formula:

$$\frac{\text{Ca2+ mMoles}}{\text{Kg wet wt.}} = \frac{\text{DPM/mg(tissue)}}{\text{DPM/ul(medium)}} \times \text{Ca2+ mMoles/litre(medium)}$$

COLD INDUCED CONTRACTURE EXPERIMENTS

A small series of experiments was performed to determine if the phenomenon of cold contracture occurs during metabolically induced rhythmicity. The tissues were mounted in test-tubes that served as organ baths with aeration and tension measurement. The test-tubes were fitted into holes in a thermostatically controlled aluminium block. The block acted as an even controlled source of heat for the embedded organ baths. By controlling a heating element that heated the aluminium block, the temperature of the baths could be easily varied.

REAGENTS

For anaesthesia a pentobarbital injection of the following stock solution: 2Ø ml ethanol, 1Ø ml propylene glycol, 6 g pentobarbital used; all up to 1ØØ ml with saline. The stock solution yielded 6Ø mg pentobarbital/ml.

The bathing media used throughout the experiments were two variations of a Krebs-Henseleit physiological solution, referred to as normal-Krebs and glucose-free Krebs. pH7.4

Normal Krebs	mM Reagent	Glucose-free Krebs
118 mM	NaCl	118 mM
4.7	KCl	4.7
1.4	KH2PO4	1.4
25	NaHCO3	25
1.2	MgSO4	1.2
2.5	CaCl2	2.5
11	Glucose	Ø

It was deemed the total molar difference in glucose-free Krebs to normal Krebs was not sufficiently great to create hypo-osmotic swelling and other damaging effects. On investigation, glucose-free Krebs was formulated with a physiologically inert replacement for glucose, equimolar sucrose. Experimentally, no difference was noted between the two glucose-free Krebs solutions(R. Bose, spoken communication).

The lanthanum solution for 45-Ca2+ exchange.

NaCl	122 mM
KCl	5.9
MgCl2	1.25
[glucose	11]
LaCl3	5Ø
Tris Maleate	15

Tissue scintillation cocktail:

Diphenyloxazole (PPO) 6 g. in 1 litre of scintillation grade

toluene

EGME scintillation cocktail:

Diphenyloxazole (PPO) 8 g.

p-bis-2(5-phenyloxazolyl) benzene (POPOP) Ø.1 g.

ethyleneglycol monoethyl ether (EGME) 5ØØ mls.,

in 2 litres of scintillation grade toluene

SECTION C

RESULTS

EXTRACELLULAR SPACE AS DETERMINED BY 3H-PEG-4000

Some of the problems encountered in measuring tissue Ca2+, inpart involve accounting for external sources of Ca2+ which contribute to the total. One external source of calcium is the extracellular space (ECS) of the tissue. This region presumably contains calcium in the aqueous (ionized) form in the same concentration as the bathing medium. The Ca2+ in the Krebs solution was 2.5 mM.

ECS was measured in the tissue during four metabolic conditions: 1) normal resting; 2) normal contracted; 3) depleted resting; 4) depleted contracting. It was assumed the normal resting tissue would closely approximate recovered resting tissue for ECS and normal contracting would be similar to recovered contracting tissue.

TREATMENT	PERCENT ECS
NK - REST	36.1 $\pm$ 4.1%
GF - REST	40.3 $\pm$ 8.6%
GF - REST - NK	36.1 $\pm$ 4.1%
NK - CARB	39.0 $\pm$ 7.1%
GF - CARB	33.0 $\pm$ 3.7%
GF - CARB - NK	39.0 $\pm$ 7.1%

n= 6

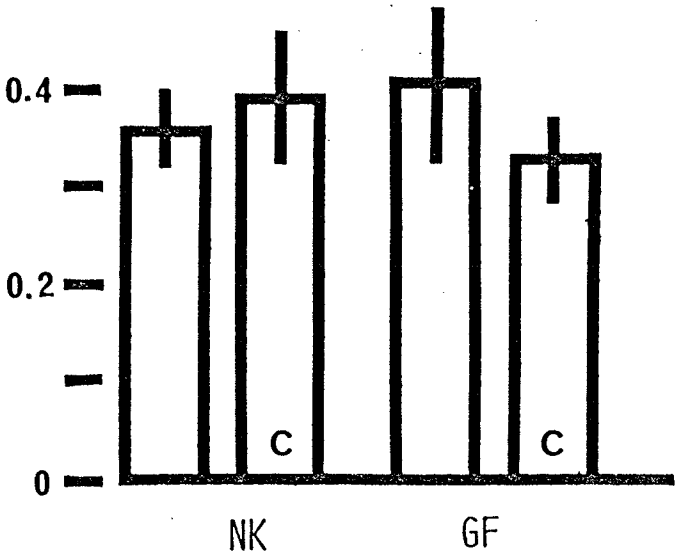
No significant difference between treatments was noted in the ECS as determined by 3H-PEG-4000 as shown above and in Figure 3. The amount of aqueous Ca2+ located in the extracellular space did not change in metabolic depletion in resting muscles, tonically contracting or

rhythmically contracting muscles.

FIGURE 3:

Summary of tissue extracellular space as determined by 3H-PEG-4000 throughout various treatments. The ordinate axis is graded in decimal fraction of extracellular space.

EXTRACELLULAR SPACE
(BY ^{3}H-PEG 4000)



C- CARBACHOL $2x10^{-7}M$
NK- NORMAL KREBS
GF- GLUCOSE FREE KREBS

Figure 3

The bathing medium contained 2.5 nanomoles Ca2+ /(ul). On average 37.1% of the muscle was extracellular space. Each mg equivalent of tissue contains Ø.93 nanomoles Ca2+ in the extracellular space.

EFFLUX OF PEG FROM THE EXTRACELLULAR SPACE

The efflux of tritiated PEG was measured during different time periods from trachealis in four states of contracture and depletion as stated in METHODS. The efflux was expressed as % PEG remaining in the tissue at time (t) from the start of efflux. Three efflux parametres were assessed

(1) compartment size of PEG space expressed as % PEG remaining

(2) compartment rate of decrease expressed as % per min.

(3) compartment half time expressed in minutes.

The three efflux parametres were obtained from a curve peeling programme, PEEL.BAS (See Appendix). The resolved parameters of each compartment were then averaged and pooled for analysis of variance. The treatment to treatment differences were calculated with Duncan's new multiple range test. The purpose of the 3H-PEG-4ØØØ efflux was to determine if it left the tissue presumably from one or more compartment. The curve peeling technique on this efflux study only resolved one compartment for all tissues in various states of contracture and in metabolic depletion.

The average compartment size was 29.42% and not significantly different within any treatment. The average compartment rate was (-Ø.Ø234)%/min and did not significantly differ from treatments. The compartment half-time did not differ between treatments and was on average 29.669 minutes. The length of the half-time was expected to be much shorter and could be due to hidden fast first order component missed in the efflux analysis. This could have been caused by a few seconds of prewash time required to remove any adhering isotope from the tissue. Efflux from the extracellular space could have been expected to be in order of a minute and not several minutes. With this hypothesis the first component could have been missed. The more complex analysis yielded multicomponent curves that presented difficulties in randomized complete block of analysis because of the different number of compartments from experiments.

No significant differences existed between the compartment size, rate and half time. The compartment analysis may have failed to resolve a small short lived compartment at the beginning of efflux as the half-times for all the tissue treatments could represent an interaction of the PEG with tracheal tissue's massive intercellular matrix (Gabella, 1979).

An example of an exponential decay curve composed of three compartments is:

$$y(i) = A\exp(-\text{alpha } X(i)+B\exp(-\text{beta } X(i)+C\exp(-\text{gamma } X(i))$$

where:

y(i) = dpm in tissue

X(i) = time period

A, B, C = compartment sizes

alpha, beta, gamma = compartment decay rates

TABLE 1

COMPARTMENTAL ANALYSIS OF 3H-PEG-4000 EFFLUX

DIFFERENCE	MEAN	POOLED	SIGNIFICANT ERROR
SIZE (in % 3H remaining) N=5			
NK-REST	67.824 ±7.028	±4.562	N.S.
NK-CARB	78.364 ±5.666	±4.562	N.S.
GF-REST	78.936 ±2.451	±4.562	N.S.
GF-CARB	81.196 ±0.917	±4.562	N.S.
RATE (In % per minute) N=5			
NK-REST	-.02416 ±.00151	±.0008	N.S.
NK-CARB	-.02352 ±.00063	±.0008	N.S.
GF-REST	-.02262 ±.00088	±.0008	N.S.
GF-CARB	-.02324 ±.00059	±.0008	N.S.
HALF TIME (In minutes) N=5			
NK-REST	29.066 ±1.654	±.9686	N.S.
NK-CARB	29.542 ±0.805	±.9686	N.S.
GF-REST	30.874 ±1.357	±.9686	N.S.
GF-CARB	29.194 ±0.444	±.9686	N.S.

45-CALCIUM EXCHANGE STUDIES

Addition of isotopic calcium to the bathing media provided a method for the measurement of calcium uptake into tissues. The method of Godfraind (1976) was used and correction of extracellular space according to McIver etal,(1974) was applied.

The calcium exchange technique was conducted with treatment groups similar to the 40-Ca2+ estimation experiments. The results can be divided into three categories:

(1) total exchange calcium corrected for extracellular space

(2) internal exchangeable calcium after lanthanum (La3+) treatment

(3) exchangeable calcium displaced by La3+; the membrane exchangeable calcium and glycocalyx calcium

Total calcium for the various stages of metabolic depletion ranged from 6 to 12 mMoles/(kg. wet tissue weight) after one hour incubation. Correction for the calcium present in the extracelluar space still left us with these high values. Only one significant difference exists between metabolic groups, as shown in Figure-4 and Table -2. The rhythmically contracting metabolically depleted tissue has significantly more cellular calcium than its resting control. The data were analyzed by grouped analysis of variance and the significance determined by the least-significant difference test. The tissue in the GF-carb treatment group showed a significant

increase (13.9 mM/Kg) in comparison with other treatments when rhythmic. All other treatments are not significantly different from each other.

Exposure to La3+ results in displacement of extracellular calcium. The La3+ binds to glycocalyx . This in effect displaces any non-intracellular calcium (Godfraind, 1976). The results after La treatment indicate only sources of intracellular calcium. The exchangeable calcium remaining after the displacment of extracellular was less than 2 mmoles/(kg. wet wt.). All treatments between metabolic groups are not significantly different from each other as determined by Duncan's multiple range test. Changes in internal calcium levels after carbachol treatment was consistently lower but the difference was not statistcally significant. See Figure-5 and Table -2.

The difference between the levels of measured 45-Ca2+ in the two procedures indicates the size of external stores of calcium, such as in the glycocalyx. The difference seen in the uptake experiment reflects an increase in calcium binding and storage in the glycocalyx during rhythmic contractions as a result of the GF-carb treatment. The calcium displaced by La3+ (membrane associated Ca2+) as shown in Figure 6 is the exchangeable Ca2+ and significantly increases in metabolically depleted rhythmic tissue as compared to other treatments.

A trend for this increase can be accounted by a similar increase in total Ca2+. A trend toward an increase

of calcium in the contracting tissue in metabolic group exists.

TABLE 2

45-CA2+ EXCHANGE method for CALCIUM (mM/KG WET TISSUE WT.)

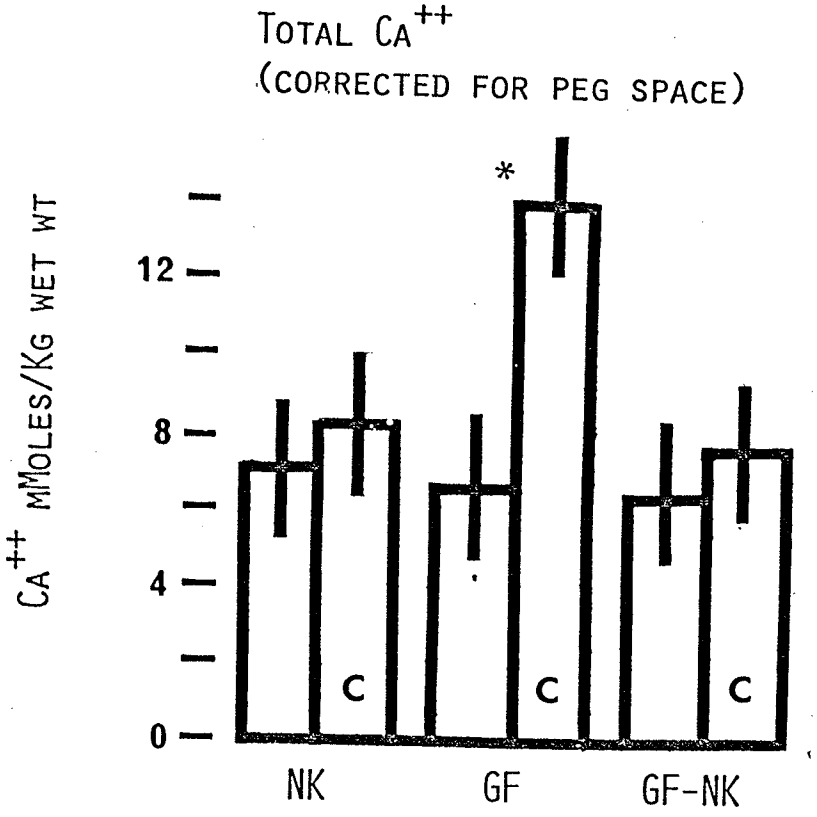
TREATMENT	TOTAL TISSUE	INTERNAL	GLYCOCALYX
NK-REST	7.020 ±1.822	1.416 ±0.275	5.604 ±1.843
NK-CARB	8.184 ±1.822	0.905 ±0.245	7.279 ±1.843
GF-REST	6.549 ±1.822	1.172 ±0.167	5.379 ±1.843
GF-CARB	13.903*a ±1.822	1.150 ±0.135	12.753*b ±1.843
GF-REST-NK	6.345 ±1.822	1.123 ±0.231	5.225 ±1.843
GF-CARB-NK	7.679 ±1.822	0.800 ±0.035	6.875 ±1.843
	N=6	N=6	N=6

*a GF-CARB significantly different (S.D.) $p<0.05$
*b GF-CARB significantly different (S.D.) $p<0.05$
Pooled standard error

FIGURE 4:

Summary of 45-Ca2+ uptake in tracheal smooth muscle strips. Total tissue content of 45-Ca2+ uptake correcting for any contribution of the tissue extracellular space. The histogram compares tissue in resting, contracted and rhythmic states.

$^{45}Ca^{++}$ Cellular Exchange



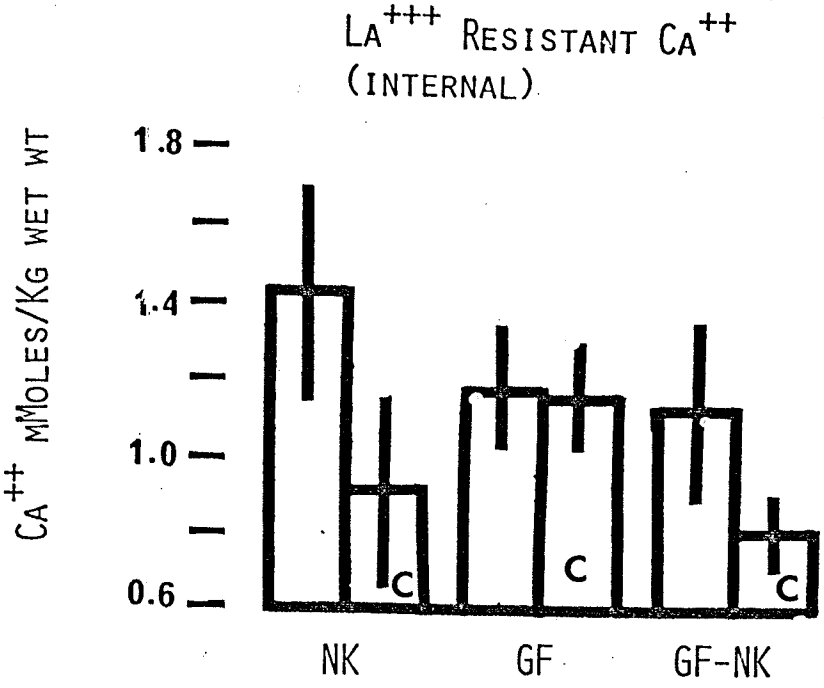
C- carbachol $2x10^{-7}M$
NK- normal Krebs
GF- glucose free Krebs

Figure 4

FIGURE 5:

Summary of the 45-Ca^{2+} uptake in tracheal smooth muscle strips under La^{3+} treatment. The La^{3+} resistant 45-Ca^{2+} is presumed to represent the intracellular (internal) Ca^{2+}. The histogram compares tissue in resting, contracted and rhythmic states.

$^{45}Ca^{++}$ Cellular Exchange



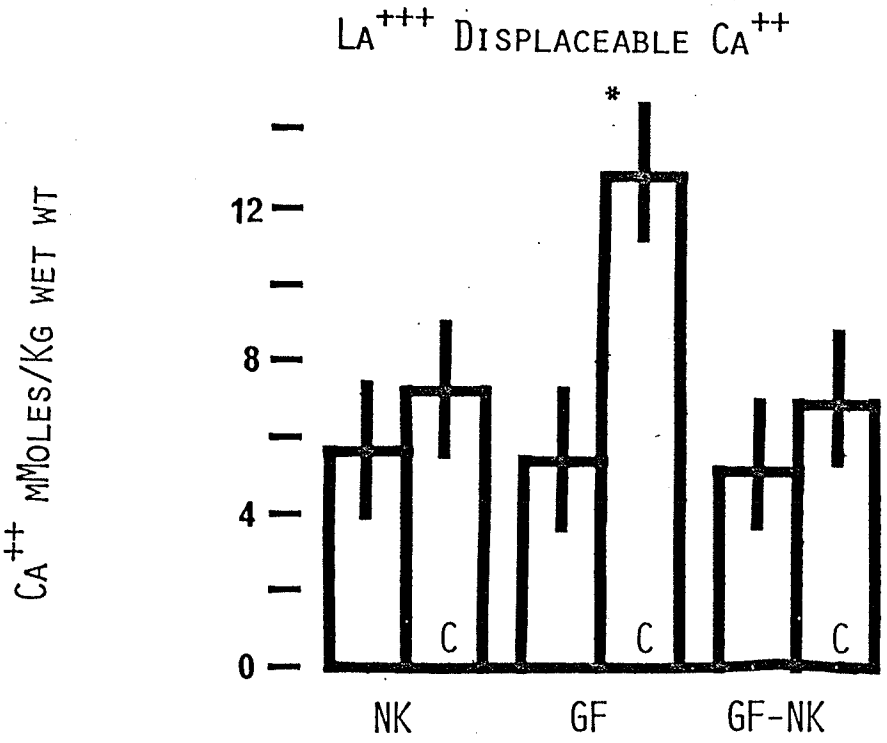
C- carbachol $2x10^{-7}M$
NK- normal krebs
GF- glucose free krebs

Figure 5

FIGURE 6:

Summary of the 45-Ca^{2+} displaced from tracheal smooth muscle strips after La^{3+} treatment. The La^{3+} displaceable 45-Ca^{2+} is presumed to represent glycocalyx calcium. The histogram compares tissue in resting, contracted, and rhythmic states.

$^{45}Ca^{++}$ Cellular Exchange



C- carbachol $2x10^{-7}M$
NK- normal krebs
GF- glucose free krebs

Figure 6

significance. The exchangeable calcium pool of rhythmic tissue may play a role in rhythmicity and in that tissues shows greater susceptibility to Ca2+ blockers (Bose etal,1977; Watanabe, 1977). These calcium sites can restore themselves to their former state upon returning glucose to the system and repleting the metabolic state by addition of glucose.

SPECIFIC ACTIVITY OF 45-Ca2+/40-Ca2+

In assessing the cellular Ca2+ by exchange methods the equilibrium of the tightly associated internal Ca2+ and lossely bound external Ca2+ was considered. Any change in these pools with depletion may not be entirely due to changes in size but also in exchangeability. One must consider the changing tightness in internal Ca2+ and changing looseness in external pools.

The specific activity of 45-Ca2+/40-Ca2+ was calculated. The fractional exchangeability or 45-Ca2+/40-Ca2+ shows that the 45-Ca2+ contrbutes more in determining the Ca2+ in rhythmic tissue.

The internal Ca2+ in figure [8a'] has the lowest 45-Ca2+ exchange by a specific activity under 1. The tighter internal stores. The 45-Ca2+ method probably underestimates internal Ca2+ and then consequently overestimates membranes associated Ca2+.

The 45-Ca2+/40-Ca2+ ratio displaced by La3+ shows that the 45-Ca2+ exchange is increased for all active tissues and largest for rhythmic tissue. The

exchangeability of the membrane Ca2+ increases more than the pool size in the

TABLE 4

SPECIFIC ACTIVITY 45CA2+/40CA2+

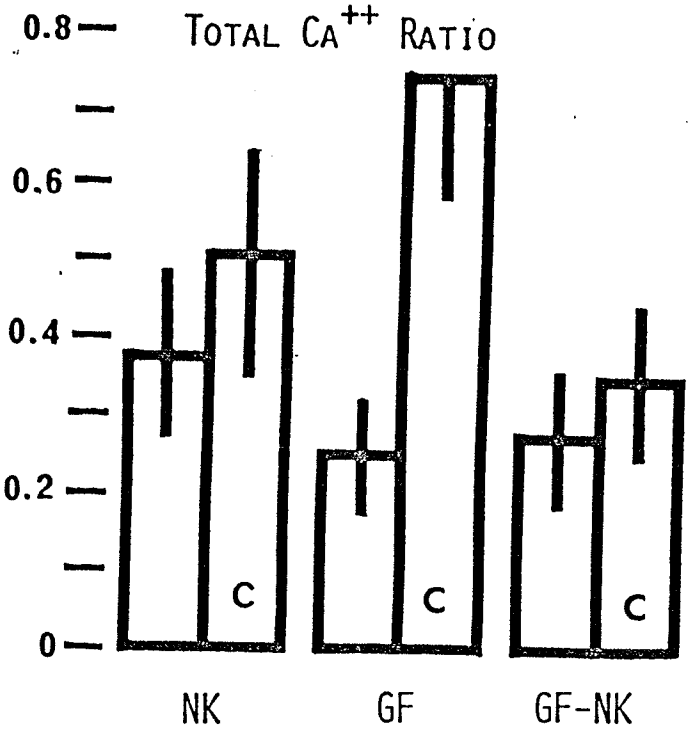
TREATMENT	TOTAL	INTERNAL	GLYCOCALYX
NK-REST	Ø.368 ±.1Ø8	Ø.157 ±.Ø4Ø	Ø.559 ±.249
NK-CARB	Ø.513 ±.143	Ø.Ø84 ±.Ø26	1.4Ø8 ±.897
GF-REST	Ø.257 ±.Ø77	Ø.Ø94 ±.Ø17	Ø.414 ±.171
GF-CARB	Ø.755 ±.147	Ø.Ø84 ±.Ø13	2.658 ±1.778
GF-REST-NK	Ø.281 ±.Ø87	Ø.1Ø8 ±.Ø27	Ø.426 ±Ø.183
GF-CARB-NK	Ø.354 ±.Ø95	Ø.Ø58 ±.ØØ7	Ø.869 ±Ø.4Ø4

N=6

FIGURE 7:

Summary of the specific activity of 45-Ca^{2+}/40-Ca^{2+} to determine changes in exchangeability of total tissue Ca^{2+} with metabolic depletion. The ordinate is represented as an arbitrary decimal ratio of exchangeability.

RATIO $^{45}CA^{++}$ ACTIVITY/$^{40}CA^{++}$

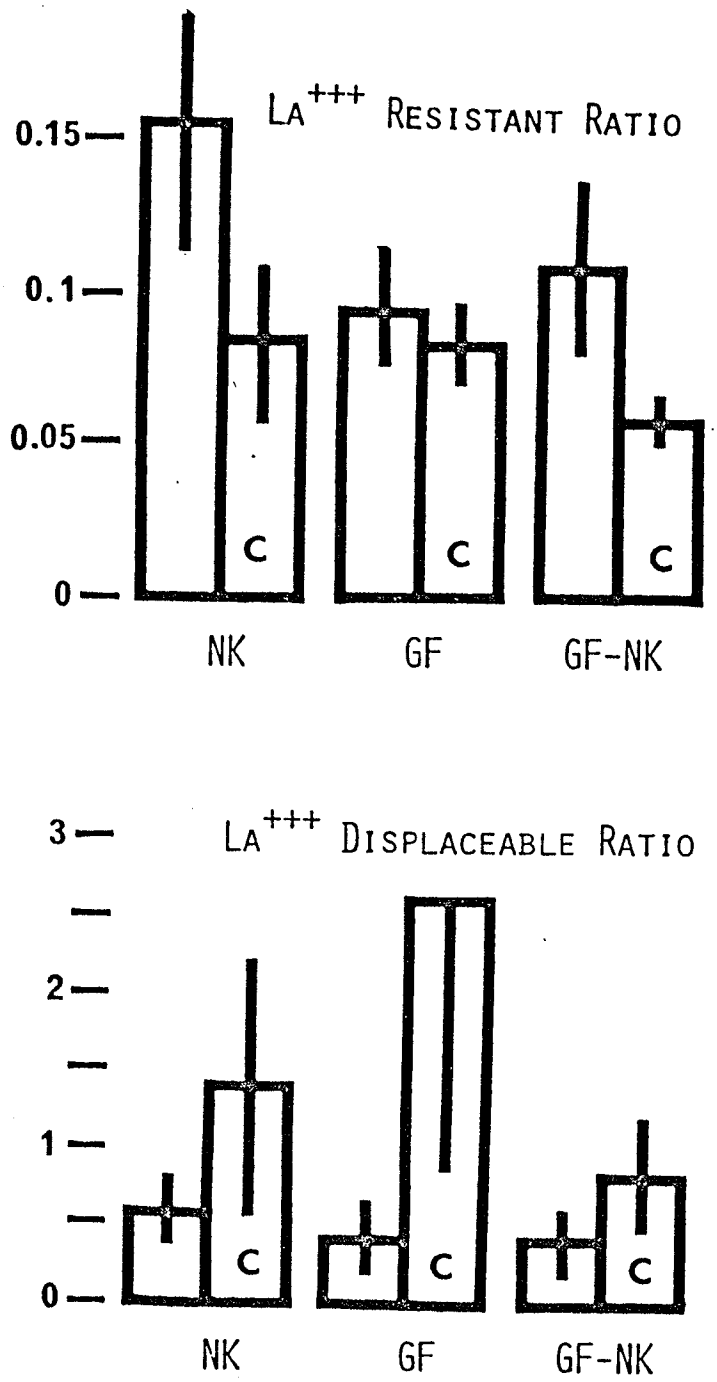


C- CARBACHOL $2x10^{-7}M$
NK- NORMAL KREBS
GF- GLUCOSE FREE KREBS

Figure 7

FIGURES 8a, 8b:

Summary of the ratio of specific activity of 45-CA2+/40-CA2+ to determine changes in exchangeability of La3+ resistant and La3+ displaceable calcium. The ordinate is represented as an arbitrary decimal ratio of exchangeability.



C- CARBACHOL $2x10^{-7}M$
NK- NORMAL KREBS
GF- GLUCOSE FREE KREBS

Figure 8

active contracting state.

Contracting tissues uses more membrane Ca2+ to give a small 40-Ca2+ pool size yet the remaining membrane pool vigorously exchanges with the aqueous Ca2+ to give an apparently large 45-Ca2+ membrane pool. The rhythmic tissue has the largest ratio indicating it exchanges its membrane Ca2+ the most vigorously, thus creating a large Ca2+ turnover in the membrane pool.

TISSUE 40CA++ DURING METABOLIC DEPLETION

Cold calcium content of trachealis was estimated in two types of experiments. Treatments followed by lanthanum La3+ and treatments without any La3+ were performed. The total 40Ca2+ tissue content was obtained by omitting La3+ from the final treatment scheme.

Throughout the treatments total tissue calcium was monitored by atomic absorption spectrophotometry as outlined in the METHODS section. The data were subjected to grouped analysis of variance with Duncan's new multiple range test for significance.

First a total tissue Ca2+ obtained by omitting the La3+ treatment; secondly a La3+ resistant Ca2+, which probably resembles internal Ca2+, was obtained by including La3+ in the experiment. La3+ displaceable Ca2+ was calculated from the difference between total Ca2+ and internal Ca2+. The La3+ displaceable Ca2+ probably represents an external membrane associated pool of calcium

(the glycocalyx). The Ca2+ concentration was expressed as mMoles Ca2+/(kg wet weight).

All values for 40Ca2+ estimation of trachealis were larger than tissue Ca2+ values from other preparations, such as guinea pig taenia coli, rat uterus (Bauer etal, 1965; Hurwitz etal, 1969). The larger Ca2+ levels could be due to a collagenous matrix found between the smooth muscle plane and the epithelial sheet of the intima of the trachea. When these planes are separated much of the collagenous matrix can be associated with the smooth muscle layer. Collagenous connective tissue can bind Ca2+ and contribute to its total content (Bozler,1963; Villamil etal, 1973). Removal of all traces of connective tissue without extensive damage to the muscle was not practically feasible. For comparisons the tissues were grouped into three metabolic states: normal, metabolically depleted and recovered from depletion. Each metabolic group had its own stimulated category (by the addition of carbachol) and this was also true for recovery after metabolic depletion. The metabolic groups were designated NK, GF, and GF-NK.

The total tissue 40Ca2+, corrected for the extra cellular space as determined by the PEG experiment, ranged from 6 to 10 mMoles (kg wet weight). The treatment scheme started with resting tissue in normal Krebs. As carbachol was added to the bathing solution the tissue contracted tonically. The next treatment event entails removal of glucose for metabolic depletion to proceed. For this the solution was changed to a glucose-free Krebs (GF)

containing carbachol. Rhythmicity started when tissue glycogen store are depleted. This corresponded to a GF-carb treatment, which again shows a further decreasing trend but nonsignificant. Each contracted tissue had non-significantly smaller calcium pool than its metabolically paired tissue in the resting state. The resting depleted tissue had more tissue Ca2+ than metabolically normal tissue. This phenomena of higher tissue Ca2+ was partially reversible on return to normal metabolism as shown by a decrease in tissue Ca2+, in GF-NK series toward the metabolically normal Ca2+ levels.

The rhythmic tissue (GF-carb) from this point followed two different treatment paths. One was the removal of carbachol from the bath, which causes the muscle to enter a quiescent state from its former rhythmic state (GF-carb). Now under the "GF-rest" treatment, the whole tissue calcium rises to the highest level (10.145 mM/Kg). Only metabolically depleted resting tissue had calcium pools was significantly larger than contracted metabolically normal tissue. This indicates a metabolically depleted tissue in the resting state can accumulate significantly more calcium than resting normal tissue and contracted normal tissue. Perhaps the tissue in this energy poor situation cannot maintain a calcium homostasis (Casteels etal, 1972). The levels in GF-rest are surprisingly higher than those in GF-carb. One

TABLE 3

40-Ca2+ ESTIMATION

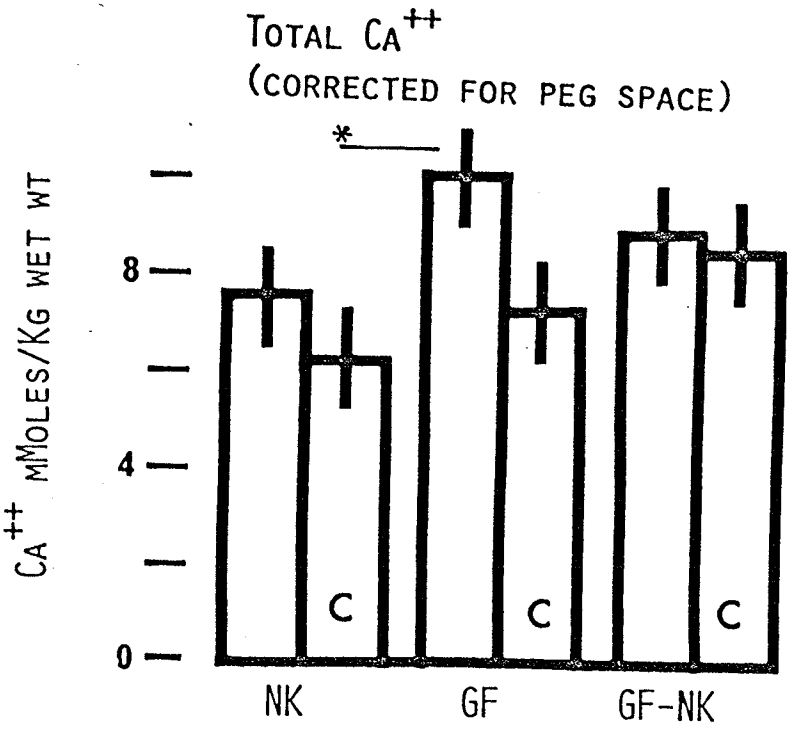
	TOTAL TISSUE CA2+	INTERNAL CA2+	GLYCOCALYX CA2+ (mM/Kg)
NK-REST (pooled S.E.)	7.591 ± 1.058	3.602 ± 0.725	3.991 ± 1.513
NK-CARB	6.335 ± 1.058	4.277 ± 0.725	2.0573 ± 1.513
GF-REST	10.145 *a ± 1.058	4.985 ± 0.725	5.164 *d ± 1.513
GF-CARB	7.317 ± 1.058	5.477 *b ± 0.725	1.841 ± 1.513
GF-REST-NK	8.988 ± 1.058	4.126 ± 0.725	4.864 *e ± 1.513
GF-CARB-NK	8.622 ± 1.058	5.478 *c ± 0.725	3.147 ± 1.513
	N=13	N=7	N=7

*a GF-rest to NK-carb are significantly different (S.D.) at $p<0.05$
*b GF-carb to NK-rest are S.D. $p<0.05$
*c GF-carb to NK to NK-rest are S.D. at $p<0.05$
*d GF-rest to NK-carb and GF-carb are S.D. at $p<0.05$
*e GF-rest NK to GF-carb are S.D. at $p<0.05$

FIGURE 9:

Summary of the 40-Ca^{2+} estimation in whole tissue as determined by atomic absorption spectrophotometry. Total tissue determination of Ca^{2+} excludes any contribution of the tissue extracellular space. The histogram compares tissue in resting, contracted and rhythmic states.

$^{40}Ca^{++}$ Estimation



C- carbachol $2x10^{-7}M$
NK- normal krebs
GF- glucose free krebs

Figure 9

would expect the converse. The rhythmically active tissue (GF-carb) should be at a double disadvantage regarding calcium homeostasis, with little energy for calcium regulation and an increased demand for contractile and excitatory calcium. Yet levels in GF-carb are lower (7.317 mM/Kg) than GF-rest (10.145 mM/Kg). The overall tissue levels can be lower but the turnover of calcium in this preparation can be very high to maintain the contractile apparatus for its continuous oscillating behavior. In effect, one could have a preparation where more calcium actually passes through the cell.

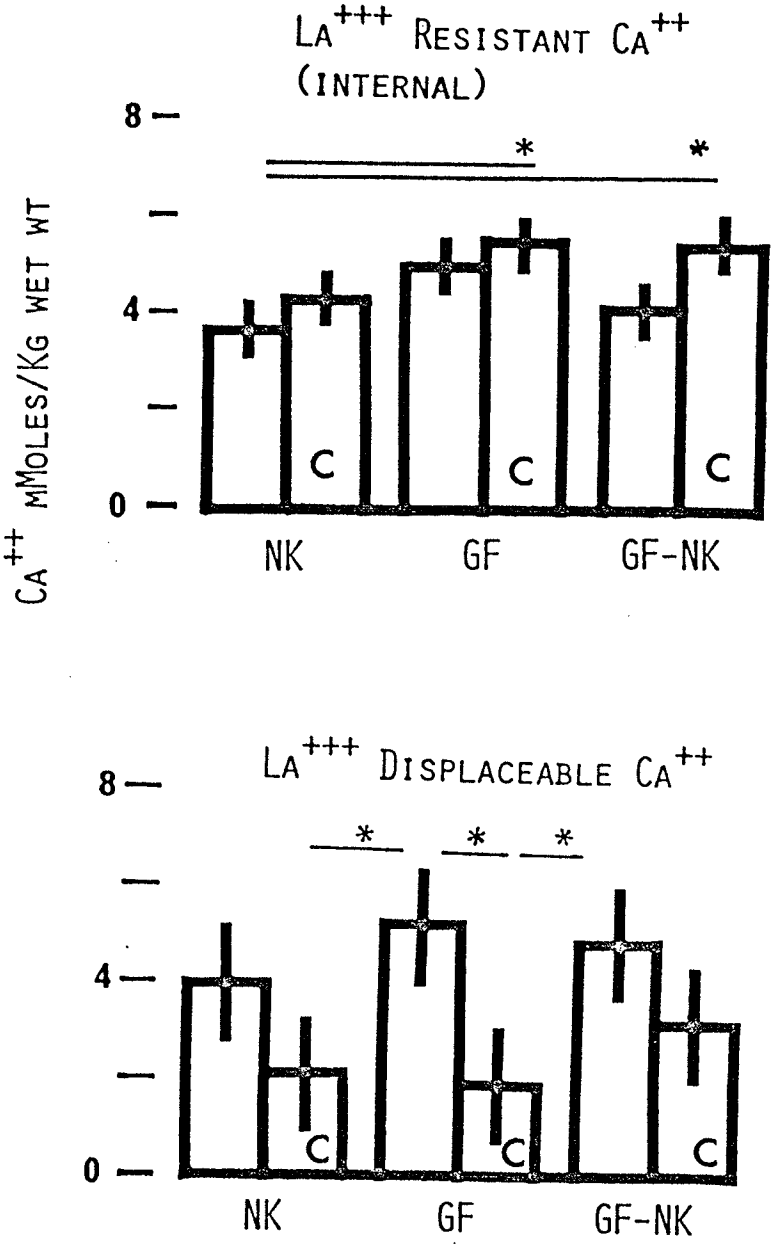
After the GF-carb treatment (with its rhythmicity), the next course of treatment was to allow tissues to metabolically recover by returning glucose to the bathing medium. In either case, returning glucose in the presence or absence of carbachol still maintained the calcium levels high. No significant difference in calcium was noted between GF-rest and GF-rest-NK and between GF-carb and GF-carb-NK. A lack of change under these treatments may be due to the two following reasons. It may take more than one hour for metabolicrecovery; although the tissues immediately contract tonically on the addition of glucose [see Figure 11'], underlying damage (changes) to membranes and calcium stores may take hours to repair. The eucaryotic plasma membrane takes about 4 hours to turnover. The membrane changes during metabolic depletion may be quite extensive (Casteels etal, 1973, 1972).

FIGURES 1Øa, 1Øb:

Summary of the 4Ø-Ca2+ estimation by atomic absorption spectro photometry in tissue under La3+ treatment. The La3+ resistant 4Ø-Ca2+ is presumed to represent in the intracellular (internal) Ca2+.

Summary of the 4Ø-Ca2+ displaced from tracheal smooth muscle stips after La3+ treatment. The La3+ displaceable calcium is presumed to represent glycocalyx calcium. The histogram compare tissue in resting, contracted and rhythmic states.

$^{40}Ca^{++}$ ESTIMATION



C- CARBACHOL $2x10^{-7}M$
NK- NORMAL KREBS
GF- GLUCOSE FREE KREBS

Figure 10

In glucose free media, after the tissue has exhausted its supply of glycogen, the only energy source is the oxidative phosphorylation of lipids and fatty acids (Stephens etal, 1970). These constituents could come from the plasma membrane. To maintain itself energetically, the cell would selectively disassemble its membrane of certain lipid components. Alteration of membrane architecture substantially alters function, half-times, affinity, fluidity and capacity of membrane associated structures: enzymes, pores, channels, receptors (Fink etal, 1976; Huang etal, 1979; Vanhoutte, 1976). An explanation for the oscillatory behaviour seen in metabolic depletion may come from the selective reduction of lipid from the plasma membrane.

The Ca2+ remaining after La3+ treatment (internal Ca2+) was not different for the different metabolic groups although a trend for increased calcium was noted for the carbachol stimulated tissue in each metabolic group. Again a trend for increasing Ca2+ upon metabolic depletion was noted in the resting state with recovery of internal Ca2+ levels upon recovery of metabolic depletion. In the carbachol stimulated tissues the internal Ca2+ rose upon metabolic depletion yet the levels did not return upon reversal of depletion. The increase of Ca2+ upon metabolic depletion in rhythmic tissues and tonically contracted, recovered tissue was significantly larger than normal resting tissue according to a Duncan's test of significance at the 0.05 level.

From the difference between total tissue Ca2+ and

FIGURE 11:

The upper trace shows that the addition of glucose to a tonically contracted preparation has no effect on tension. The lower trace shows the addition of glucose to a rhythmically contracting trachealis in glucose-free Krebs results in rhythmicity being replaced by tonic contraction.

RECOVERY FROM RHYTHMICITY USING GLUCOSE

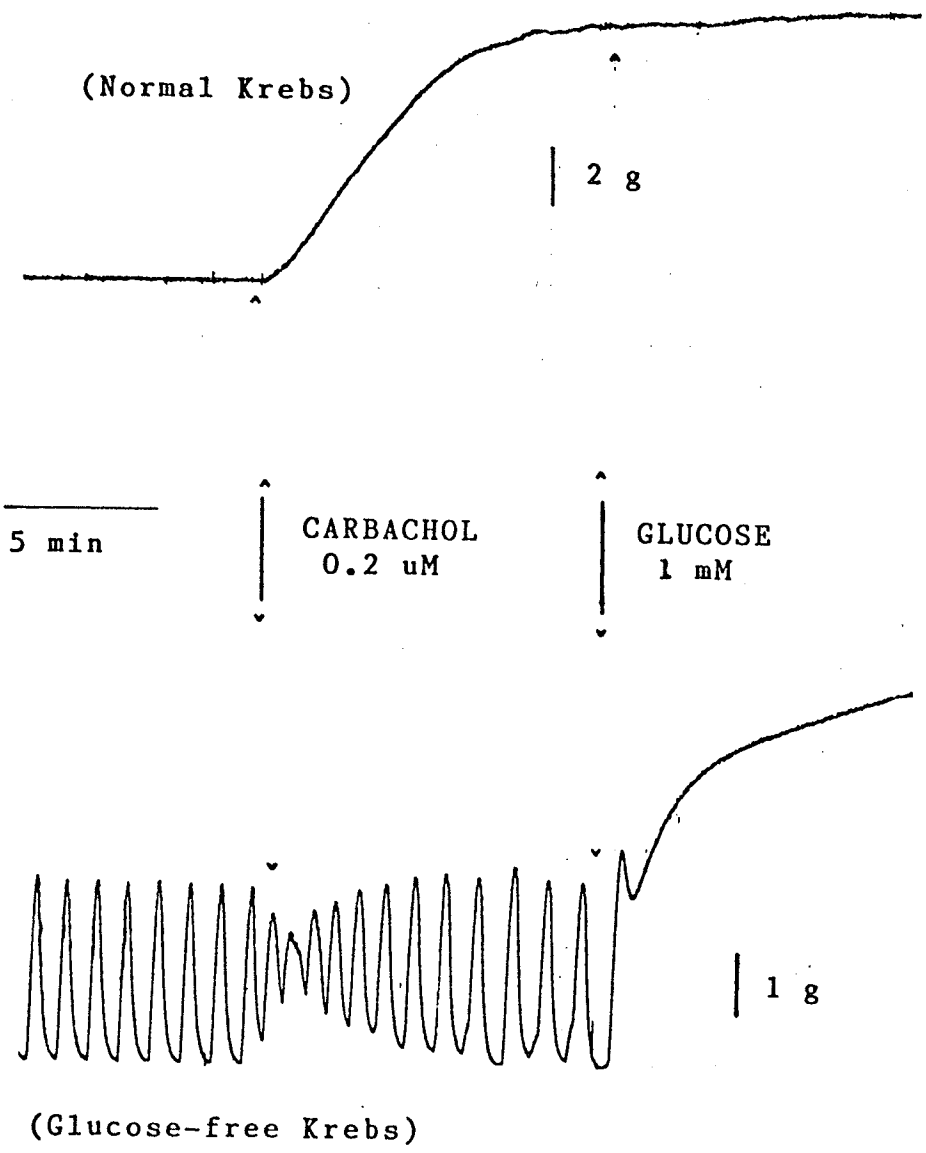


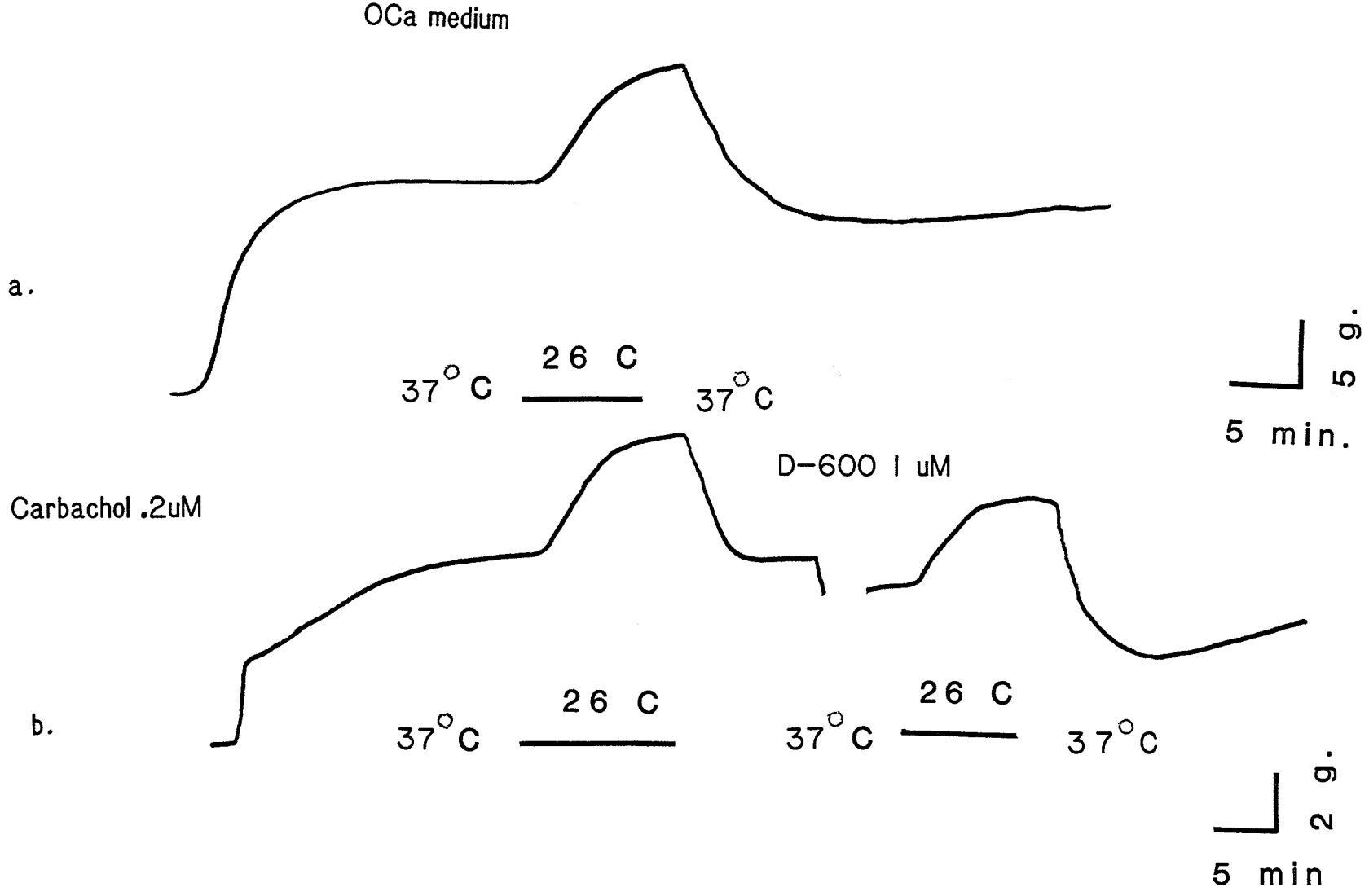
Figure 11

internal Ca^{2+} one can calculate the Ca^{2+} displaced by La^{3+} treatment or the Ca^{2+} associated with the membrane. The values range from 2 to 5 mMoles Ca^{2+} /(kg wet tissue weight). The La^{3+} displacable Ca^{2+} values did not change in the resting state during metabolic depletion and upon recovery. In the carbachol stimulated tissues Ca^{2+} levels did not change with respect to each other during metabolic depletion. The significant changes that took place were within metabolic groups. The membrane 40Ca^{2+} levels were smaller in the carbachol stimulated state than in the resting state for all phases of metabolic depletion. This may represent an external pool of Ca^{2+} that is loosely associated to the membrane Ca^{2+} such that this pool may be mobilized not only for rhythmic contraction but also tonic contraction.

FIGURE 12 a:
Record of a tonically contracting tracheal smooth muscle in response to 0.2 μM Carbachol in a normal Krebs medium with no added calcium. The contracture produced by cooling is still present.

FIGURE 12 b:
Trace of a tonically contracting tracheal smooth muscle. Cold contracture is observed in a muscle before and after adding 1 μM D-600, a Ca channel blocker, suggesting release of calcium from internal stores during cold contracture.

FIGURE-12


OCa medium
a.
37° C
26 C
37° C
5 g.
5 min.
Carbachol .2uM
D-600 1 uM
b.
37° C
26 C
37° C
26 C
37° C
2 g.
5 min

COLD INDUCED CONTRACTURE

A canine tracheal smooth muscle preparation tonically contracted with carbachol (Ø.2 uM) can produce an additional contracture when cooled to 15C. The cold induced contracture appears to be caused by mobilization of tighter internal stores of calcium. The external calcium does not seem to be important in cold contracture because tissue equilibrated in zero-calcium Krebs can still maintain a cold contracture and blocking the Ca2+ channels with D-6ØØ does not affect cold contracture (Figure- 12').

Metabolically depleted tissue rhythmically contracting in zero glucose cannot produce a contracture on cooling. Rhythmicity is also lost on cooling. Rewarming the tissue promptly returns rhythmicity. This further supports the hypothesis that the internal Ca2+ is not used in rhythmicity but that the membrane Ca2+ may be involved.

The metabollically depleted tissue lacks glycolysis as an energy source and only relies on the oxidation of fatty acids for energy (Bose etal, 1977).

Selectively eliminating oxidative phosphorylation in a tonically contracting preparation can be accomplished by equilibrating the preparation with 95% N2/5% CO2 to create an anoxic condition. Even under anoxia and with a regular glucose supply, a tonically contracting tissue can maintain a cold contracture. The loss of oxidative phosphorylation perse does not prevent cold induced contracture. The energy supply from glycolysis seems adequate for a cold contracture

(see Figure 13c').

FIGURE 13a:

Record of tonic mechanical contraction of canine trachealis during cold induced contracture. The tracing shows a tissue under normal contracture induced by Ø.2uM carbachol, further cooling of the Krebs medium produces an additional contracture of approximately 2 g. tension.

FIGURE 13b:

Record of metabolically induced rhythmic contraction during cooling of the Krebs medium. The tracing shows a tissue loosing amplitude and frequency of rhythmicity upon cooling result in the loss of all oscillations at 24C. Rewarming the Krebs promptly returns rhythmicity.

FIGURE 13c:

Record of cold induced contracture during anoxia in normally contracting tissue. The tracing shows introducing nitrogen gas as a replacemnt of oxygen causes a loss in tension. Upon cooling the anoxia tissue exhibits a cold induced contracture that can be reversed on rewarming.

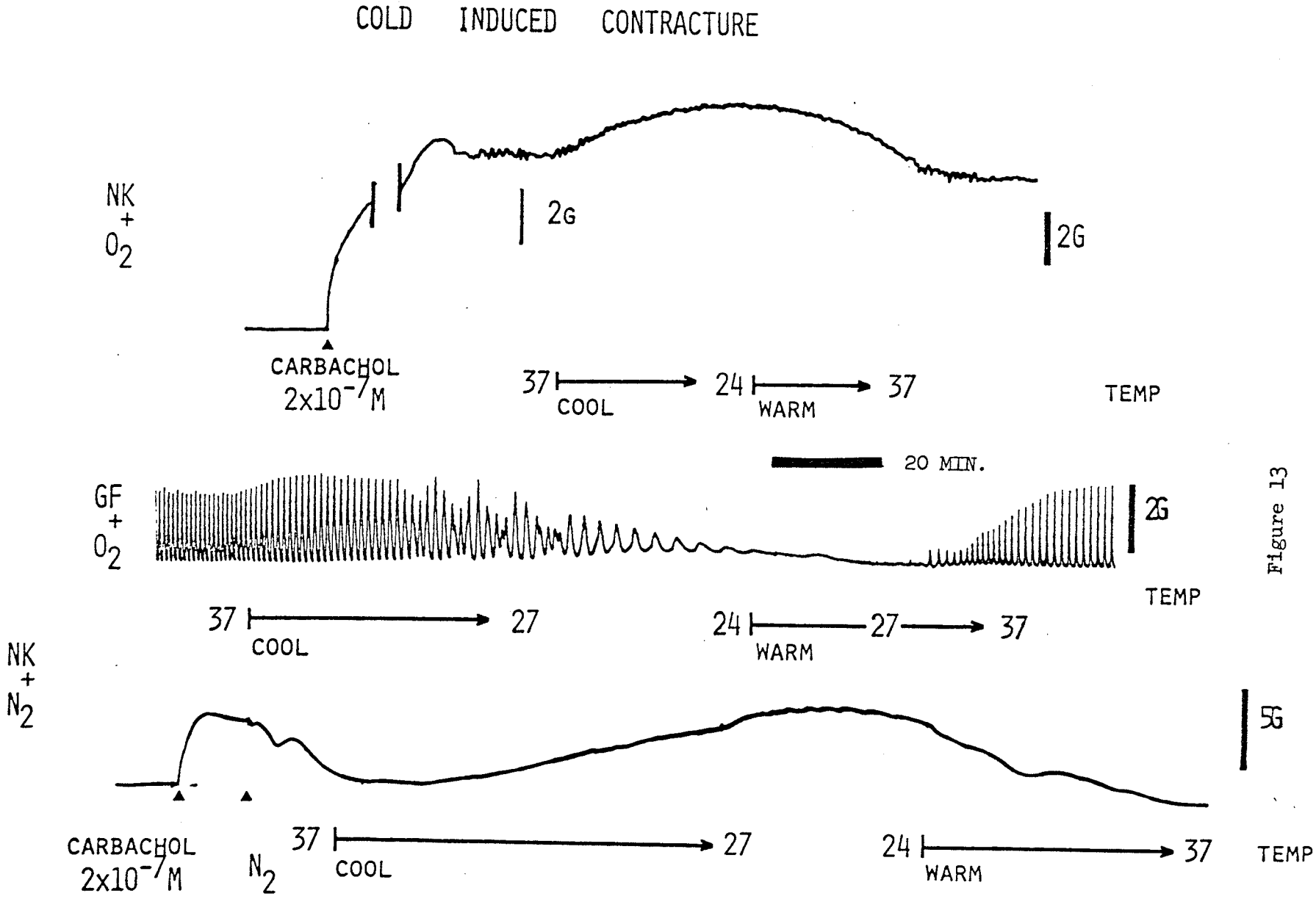
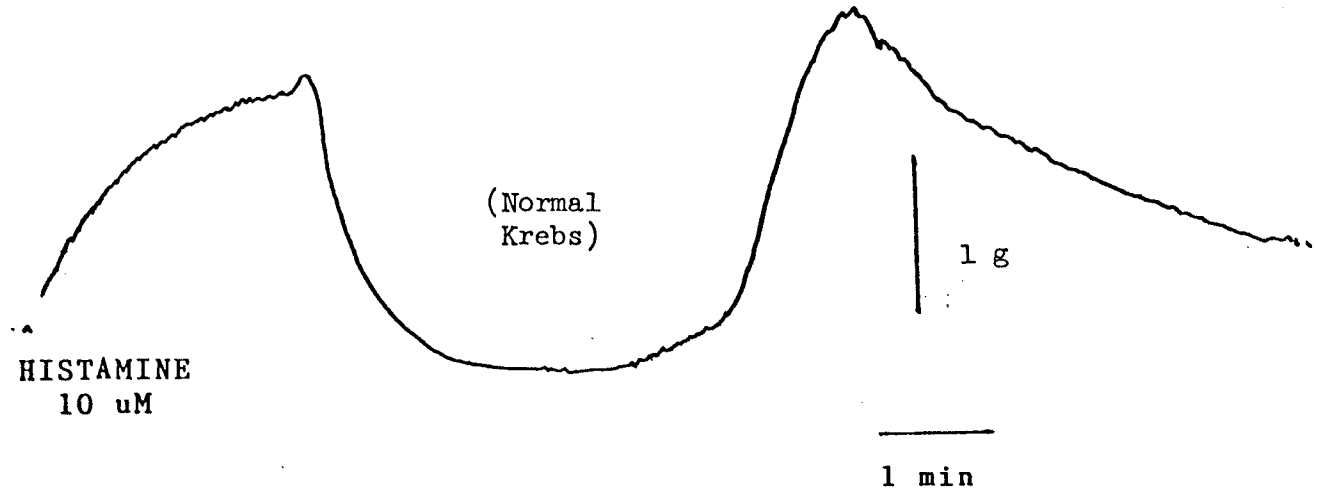

COLD INDUCED CONTRACTURE
NK + O_2
2G
2G
CARBACHOL $2x10^{-7}$M
37
COOL
24
WARM
37
TEMP
20 MIN.
GF + O_2
2G
TEMP
37
COOL
27
24
27
WARM
37
NK + N_2
5G
CARBACHOL $2x10^{-7}$M
N_2
37
COOL
27
24
WARM
37
TEMP

Figure 13

FIGURE 14:
Cooling causes a loss in tension in preparations contracted with histamine (1Ø uM) in both normal Krebs and glucose-free Krebs.

EFFECT OF COOLING ON HISTAMINE CONTRACTURE



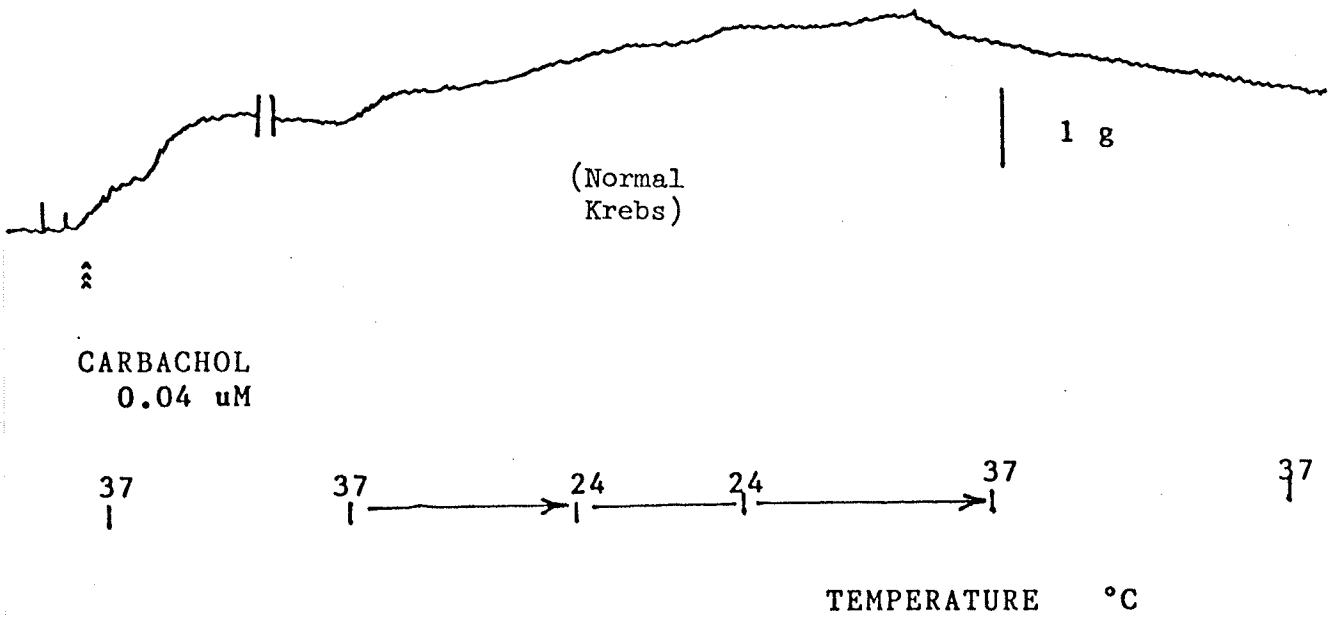


Figure 14

The rhythmic depleted tissue cannot mobilize tighter internal stores of Ca2+ for cold contracture, whereas tonic tissue has the energy supply and/or ability to mobilize internal Ca2+ for the contracture. Glucose may be essential for cold contracture, in that depleted tissue lacks glucose and glucose utilizing biochemical paths for cell calcium homeostasis.

Histamine can cause a contracture in canine trachealis independent of the glucose concentration in the medium. Figure 14' shows tissue contracted with histamine (1Ø uM) in normal Krebs looses its tension upon cooling from 37 to 24C. The tension can be regained on rewarming.

Histamine induced contracture derives most of its Ca2+ from the outer membrane via Ca2+ channels. A similar profile can be observed in a partially depleted tissue in glucose free Krebs. Rhythmic activity starts on the tonic plateau of contraction, cooling diminishes tension and rewarming regains the tension as well as some phasic activity. The loss of tension in both preparations on re-equilibration may be caused by the rate of rewarming. This suggests glucose is not responsible for maintenance of external calcium stores. The maintenance of the internally bound stores of Ca2+ in trachealis may require ATP from glycolytic pathways for Ca2+ homeostasis because addition of a small amount of glucose to depleted rhythmically contracting trachealis immediately returns the tissue to its tonically contracting state [see Figure 11'], and restores cold contracture.

SECTION D

DISCUSSION

DISCUSSION:

The discussion and interpretation of the experiments were covered in the RESULTS section. The DISCUSSION section will be devoted to possible causes and probable events in metabolically induced rhythmicity.

CLASS AND JUNCTIONS

The rigid classification of tracheal smooth muscle as multiunit smooth muscle becomes inadequate when considering canine trachealis. Some criteria involved in a classification of this sort take into account contraction initiated by transmitters and a high density of innervation and a few gap junctions (Suzuki etal, 1976). By having few nexuses or gap junctions, tissue would be less electrically coupled. This leaves excitation by innervation to be the only avenue for contraction.

Unfortunately and uniquely in canine tracheal muscle, several supporting criteria are not the norm. The ratio of nerve axons to muscle cells were 1:29; a low ratio indicating less importance of nerve stimulation. No close contact between nerve and muscle were noted (Kannan etal, 198Ø). Gap junctions were noted in the tissue, allowing current to spread over many cell lengths. The number of gap junctions could be increased by exposure of the tissue to TEA allowing the muscle to become less quiescent and more spontaneously active (Kannan etal, 198Ø; Kirkpatrick, 1975). With slight innervation, and gap junctions present and a long space constant, tracheal smooth muscle cells are

electrically coupled and capable of excitation without nerves under the proper conditions (Kannan etal, 1980).

ATP COMPARTMENTALIZATION

When a cell becomes metabolically compromised, both ATP production systems can be effected. The cytosol ATP system has a reserve and may play a role in membrane maintenance, as in the case of smooth muscle. The mitochondrial ATP system provides energy for the ATP consuming processes such as tonic contraction (Sorball etal, 1978).

In our trachealis model, anoxia produces a slower shallow rhythm, perhaps because the membranes is being maintained to some extent by cytosolic ATP production via glycolysis. The spare ATP can travel via diffusion to deeper energy dependent processes such as contractile elements, when oxidative phosphorylation in the mitochondria had ceased due to hypoxia.

Exposing trachealis to zero glucose and thus terminating glycolysis, leaves only fatty acid-oxidative phosphorylation in the mitochondria to provide energy. Hence outward diffusion of ATP from these structures, past the deep consuming machinery toward the membrane occurs. In metabolic depletion, perhaps the concentration of ATP for membrane maintenance is inadequate, resulting in an easily activated membrane, with a faster spontaneous rhythm.

This low energy supply or pulsatile energy delivey from the mitochondria allows the membrane to fall into a state of selective disrepair. This would allow a shorter period of phosphorylation of proteins and an increased excitability of

the membrane.

The mitochondria in metabolic depletion, provide energy from oxidation of fatty acids. Ultimately, membrane elements may contribute to this fatty acid pool for ketogenesis. The membrane of smooth muscle cells is very heterogenous and may experience a selective breakdown which dually provides substrate for energy production and could increase excitability. Low ATP levels in the vicinity of the membrane could allow for an easier calcium spike generation into a cell.

Why does the readmission of glucose result in immediate tonic contraction from the rhythmically depleted tissue?

The possibility may exist for a coupled membrane protein complex involving: the muscarinic receptor, Ca2+ channels, internal Ca2+ sites, glucose transport-hexokinase and guanylate cyclase. Yeast cells contain glycolytic oscillators where glucose transport is coupled to the hexose kinase (Becker etal, 1972). The system requires ATP and phosphorylates the glucose to glucose-6-phosphate. The glucose-6-P can feedback inhibit the transport. In some mammalian systems, glucose may be phosphorylated similarly upon transport into a cell. The hexokinase is the only glycolytic enzyme that can be found associated with a membrane particulate fraction. The glucose-6-phoshate could participate in energy production or in fast re-phosphorylation of internal sites to release bound calcium for tonic contraction. When glucose transport is not functioning, then perhaps it is on a 'standby' basis and

along with other factors may alter the protein complex by locking calcium in a bound state. The readdition of glucose can cause a change in membrane configuration that ultimately allows for a release of internal calcium. The structural requirements for transport do not have to be met for this because 3-O-methyl glucose does not produce tonicity in a rhythmic preparation (Bose and Bose, 1977). The requirements for catalysis by hexokinase may have to be met.

Can the rhythm ultimately be ATP concentration dependent? Probably not because the frequency does not vary throughout the experiment and the initial baseline starting frequency is generally the one that remains throughout the experiment.

PHOSPHORYLATION ON CELL CONTROL

Rubin etal (1975) cite protein phosphorylation mechanism as a good overall process for cell control. The rapid reversibility of the process is insured by phosphoprotein phosphatases that can regulate the phosphate turnover in bioregulatory phosphorylation reactions. Protein kinases and phosphate-acceptor proteins constitute portions of subcellular organelles and the plasma membrane. Phosphorylation of particulate proteins may be associated with specific functional modifications in systems of transport (Horl etal, 1978), permeability (Teichberg etal, 1977) and secretory processes (Michaelson etal, 1979).

During metabolic depletion, the labilization of the

cells' plasma membrane as a result of a switch to free fatty acids for energy metabolism, may additionally alter the proper operation of the membrane bound guanylate cyclase. The contribution of the guanylate cyclase as a positive feedback on the release of calcium (Andersson etal, 1977) may be affected and thus contribute to the rhythmicity phenomena.

Stewart etal (1979) while studying the muscarinic coupling of secretion of the salt gland in the duck, noted that sodium transport ultimately depended upon a ouabain sensitive Na2+/K+ ATPase. Cholinergic agonists activated sodium transport in the gland as well as increased cGMP. This action was directly dependent on external calcium in the medium. Cyclic GMP and guanylate cyclase stimulators, hydroxylamine and sodium azide, also activated the ouabain sensitive Na2+/K+ ATPase independently of any external calcium. The authors suggest that cGMP acts as a tertiary link in stimulus secretion coupling and presents the possibility of processes that can alter cGMP can subsequently alter the sodium pump. Similar observations were made by Shi etal (1980) on the ouabain sensitive Na2+/K+ ATPase activity in rat submandibular gland. Calcium was reguired for muscarinic agonist activation of the enzyme whereas cGMP activation proceeded independently of external calcium. Shi etal (1980) have proposed that cyclic GMP could activate the sodium pump, and the oscillation in Ca2+ may well reflect oscillating levels of cyclic GMP.

The absolute dependence of rhythmicity on an external

calcium supply in the trachealis model implies that a possible defect in the mechanisms of release and rebinding of internally bound calcium may be at fault.

If the refilling of internal stores of calcium is more energy dependent than the external calcium sites, the cell will not waste the low ATP in phosphorylating internal sites for the rebinding of calcium. It appears that the glycolytic ATP is involved in refilling of internal stores.

Calcium through an acetylcholine receptor-associated calcium channel may be required to trigger the cyclic GMP levels within the cell and release the internally bound stores of calcium for contraction (Schultz etal, 1975). The bound guanylate cyclase may not be able to produce cGMP with trigger calcium entry because of conformation changes resulting from changes in the membrane structure (Casteels etal, 1973), or due to a lack of ATP reaching the membrane. This could result in the cell receiving only erratically released calcium or, more likely, the external calcium through the ion-pores for contraction processes.

SECTION E

CONCLUSIONS

CONCLUSIONS:

The contractile behaviour of canine trachealis can be changed from its normally quiescent state to a rhythmic state by denervation or drugs. Also, tracheal smooth muscle can be converted to a mechanically and electrically rhythmic preparation by metabolic depletion. In a glucose - free bathing medium, the trachealis metabolically depletes its own tissue glycogen reserves after two hours. After depletion, any chemical or electrical excitation that would normally produce a graded tonic contraction now evokes electrical membrane spikes with rhythmic contractions.

During one rhythmic contraction, a single depolarization is actually composed of superimposed spikes of no greater than 20 millivolts amplitude and probably represents changes in membrane permiability to calcium. The tension of one phasic contraction during rhythmicity is composed of many small contractures.

External calcium is important for rhythmicity to exist. The chelation of calcium by EGTA only produces a partial loss of tension in normal tonically contracting tissue, whereas the rhythmic tissue is very sensitive to low amounts of EGTA, the amplitude of rhythmic contraction decreases with a lower external calcium from EGTA treatment. The rhythmically contracting canine trachealis exhibits great sensitivity to the calcium channel blocker D-600, by abolishing rhythmic mechanical activity at a thousand fold lower dose than that which would affect tension in normal

canine trachealis. The rhythmic tissue appears to be more dependent upon external calcium than normal. The inward calcium flux plays a greater role in tension in metabolic depletion by being more sensitive to block than in normal tissue.

Cooling the normal muscle by 15C can induce a contracture after exposure to agonist. This cold induced contracture appears to be caused by mobilization of tightly bound internal stores of calcium because the effect is still elicited after equilibration in a zero calcium Krebs solution or after addition of the calcium channel blocker D-600.

Cold contracture cannot be produced in metabolically depleted tissue indicating that the tighter internal calcium is unavailable perhaps due to a limited glucose supply in this tissue. This is supported by the observation that normal tissue undergoing anoxia, can maintain a cold contracture. The loss of oxidative phosphorylation from anoxia does not in itself prevent cold contraction.

Cellular exchange with isotopic calcium showed that rhythmically contracting muscle had a great increase in total tissue calcium, and much of this increase was in the externally exchangeable membrane bound calcium pool. Thus the extracellular pool of calcium appears to become more important during metabolically induced rhythmicity.

SECTION F

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SECTION G

APPENDIX

CURVE PEELING PROGRAMME

```
10   REM *** PEEL.BAS ***      VERSION 67      APRIL 28, 1977
20   PRINT 'DOES A COMPARTMENT ANALYSIS BY PEELING'
30   PRINT 'NO INITIAL ESTIMATES ARE USED'
40   REM --- THE MAXIMUM NUMBER OF DATA POINTS IS
50   DIM X(100), Y(100)
55   PRINT 'IS DATA ON FILE';
56   INPUT A$
57   IF POS(A$,'Y',1)>0 HEN GOSUB 4000
58   GO TO 460
60   REM --- INPUT A NEW DATA SET FROM THE KEYBOARD
70   PRINT "COMMENT LINE:"
80   INPUT H$
90   PRINT "HOW MANY DATA POINTS?";
100  INPUT N
110  PRINT "IS X-DATA EQUALLY SPACED (Y OR N)?";
120  INPUT A$
130  IF A$="Y" GO TO 230
140  IF A$="N" GO TO 170
150  GO TO 110
160  REM --- @ I.PUT BOTH X AND Y
170  FOR I=1 TO N
180  PRINT STR$(I);")";
190  INPUT X(I),Y(I)
200  NEXT I
210  GO TO 310
220  REM --- GENERATES X'S - INPUTS Y'S
230  PRINT "FIRST X AND INCREMENT?";
240  INPUT F1,I1
250  FOR I=1 TO N
260  X(I)=F1+(I-1)*I1
270  PRINT STR$(I);")";X(I);
280  INPUT Y(I)
290  NEXT I
300  REM --- ERROR RECOVERY
310  PRINT "ANY ENTRY ERRORS (Y OR N)?";
320  INPUT A$
330  IF A$="Y" GO TO 360
340  IF A$="N" GO TO 390
350  GO TO 310
360  PRINT "ENTRY NO., X AND Y VALUE?";
370  INPUT B1,X(L1),Y(L1)
380  GO TO 310
390  PRINT 'DO YOU WANT TO FILE DATE'
392  INPUT A$
395  IF POS(A$,'Y',1)=0 GO TO 460
400  REM --- WRITE NEW DATA TO FILE
405  PRINT 'ENTER FILENAME FOR DATA';
406  INPUT F$
407  OPEN F$ FOR OUTPUT AS FILE #1
410  PRINT #1,H$
420  PRINT #1,H
```

```
430  FOR I=1 TO H
440  PRINT #1,Y(I)
442  PRINT #1,Y(I)
450  NEXT I
455  CLOSE #1
460  PRINT 'DO YOU WANT A COPY OF RESULTS ON PRINTER';
462  INPUT A$
470  IF POS(A$,'Y',1)=Ø GO TO 5ØØ
480  OPEN 'LP:LP.DMP' FOR OUTPUT AS FILE #1
490  A9=1
5ØØ  M=Ø
5Ø2  B=Ø
5Ø3  NE=Ø
5Ø4  I=N+1
510  J=T
520  J=J-1\IF J<1 GO TO 91Ø
530  IF Y(J)<=Ø GO TO 52Ø
540 I=J\K=1
550 I=I-1\IF I<1 GO TO 59Ø
560 IF Y(I)<=Ø GO TO 55Ø
570 K=K+1\IF K=4 GO TO 61Ø
580 GO TO 55Ø
590 I=1\IF K=3 GO TO 61Ø
595 N=K
598 GO TO 624
610 N=Ø\X1=Ø\U2=Ø\Y1=Ø\V2=Ø\U9=Ø\S9=Ø\S9=Ø\Z=Ø
620 FOR P=I TO J\GOSUB 2ØØØ\NEXT P
622 N=N-S9
624 IF N>2 GO TO 63Ø
625 PRINT 'LESS THAN 3 DATA POINTS IN COMP.';N3+1
626 IF R=Ø GO TO 91Ø\T=T5\J=1\RETURN
630 X2=U2-1*X1/N\Y2=V2-Y1*Y1/N\X9=U9-X1*Y1/N
635 K=K9/X2\A=Y1/N-K*X1/N\D5=N-2\S5=Y2-X9*X9/X2
640 IF R=Ø GO TO 655\RETURN
655 IF S5>Ø GO TO 67Ø\S5=1.ØØØØØE-Ø5
670 IF I<2 GO TO 755
672 P=I-1
672 IF Y(P)<=Ø GO TO 674
673 T9=1\GOSUB 21ØØ\T9=Ø\GO TO 68Ø
674 P=P-1\IF P1 GO TO 676\GO TO 672
676 IF Z<1 GO TO 755\IF Z<3 GO TO 72Ø\GO TO 755
680 Z=Z+1
685 S=SQR(ABS((S5/D5)*(1+1/N+(U-X1/N)^2/X2)))
690 C=A+K*U
700 IF Z<3 GO TO 7Ø5\C=C+S
7Ø5 IF V<=C GO TO 72Ø
710 IF Z<4 GO TO 674\GO TO 755
720 I=I-1\IF Y(I)<=Ø GO TO 72Ø\P=I\N=N+S9\GOSUB 2ØØØ
755 GOSUB 19ØØ\GOSUB 3ØØØ
760 IF N3<1 GO TO 81Ø\T5=(K(N3)-K)/SQR(V1+V3)
770 IF M=Ø GO TO 85Ø\IF T>T5 GO TO 83Ø\GO TO 85Ø
780 N3=1\A(1)=Y1\N\K(1)=Ø\S5=Y2\GO TO 86Ø
810 T5=(Ø-K)/SQR(S5/D5/X2)
820 IF T>T5 GO TO 78Ø\GO TO 85Ø
830 M=Ø\J=J(N3)\N3=N3-1
```

```
840 GO TO 610
850 N3=N3+1\A(N3)=A\K(N3)=K
860 M=1\I(N3)=I\J(N3)=J\D5(N3)=D5\S5(N3)=S5\X2(N3)=X2
870 X8(N3)=X1/N\F(N3)=1
880 IF R=0 GO TO 510\RETURN
910 FOR A8=0 TO A9\PRINT #A8,\PRINT #A8,TAB(22);'THE COMPARTMENTS'
911 PRINT #A8,\PRINT #A8,H$\PRINT #A8
915 PRINT #A8,'COMP';TAB(15);'INTERCEPT';TAB(36);'-SE+'
920 FOR C=1 TO N3\I=N3-C+1\GOSUB 1700\NEXT C
925 PRINT #A8,\PRINT #A8,TAB(15);'SLOPE'
930 FORCC=1 TO N3\I=N3-C+1\PRINT #A8,C\GOSUB 1720\NEXT C
935 PRINT #A8\PRINT #A8,TAB(18);'T1/2';TAB(56);'BOUNDRIES'
940 FOR C=1 TO N3\I=N3-C+1\PRINT #A8,C\GOSUB 1735\GOSBB 1770\NEXT C
950 S5=0\N==0\FOR I=1 TO J(1)\GOSUB 970\NEXT I
960 S5=S5/N-N3*2)PRINT #A8\PRINT #A8,'DEV';SQR(S5)\GO TO 1205
970 Y=0\FOR C=1 TO N3\Y=Y+EXP (A(C)+K(C)*X(I))\NEXT C
980 IF Y(I)<=0 GO TO 990\S5=S5+LOG(Y/Y(I))^2/N=N+1
990 RETURN
1205 GOSUB 1300\NEXT A8
1206 IF A9=1 THEN CLOSE #1
1210 STOP
1300 A9$='PLOT'\IF A8=1 THEN A9$='PRINTER PLOT'
1305 PRINT\PRINT A9$;' (Y OR N) ';\INPUT X$
1310 IF A8=1 THEN PRINT #A8,CHR$(12)
1320 IF X$='Y' GO TO 1330\RETURN
1330 Y1=Y(1)
1332 FOR I=2 TO N
1334 IF Y(I)>=Y1 GO TO 1340\IF Y(I)<=0 GO TO 1340\Y1=Y(I)
1340 NEXT I
1350 Y1=LOG(Y1)\Y9=LOG(Y(1))\Y2=(Y9-Y1)\63\I=1
1360 FOR J=1 TO 70\PRINT #A8,'-';\NEXT J\PRINT #A8
1370 FOR X=0 TO X(N)+X(N)/50 STEP X(N)/50/PRINT#A8,'|';/GOSUB1400
1380 NEXT X\RETURN
1400 Y=0\FOR C=1 TO N3\Y=Y+EXP(A(C)+K(C)*X)\NEXT C\Y=LOG(Y)
1405 IF Y>=Y1 GO TO 1410\Y=Y1
1410 IF I>N GO TO 1490\IF X(I)>X GO TO 1490\Y3=LOG(Y(I))
1415 IF Y3>=Y1 GO TO 1420\Y3=Y1
1420 IF Y>Y3 GO TO 1430\GO TO 1440
1430 PRINT #A8,TAB(Y-Y1)/Y2);'*';TAB((Y3-Y1)/Y2);'X';\GO TO 1450
1440 PRINT #A8,TAB((Y3-Y1)/Y2);'X';((Y-Y1)/Y2);'*';
1450 PRINT #A8,'---';I\I=I+1\RETURN
1490 PRINT #A8,TAB((Y=Y1)/Y2);'*'\RETURN
1700 D=D5(I)\GOSUB 3000
1705 PRINT #A8,C
1708 S=SQR((S5(I)/D5(I))*(1/(D5(I)+2)+X8(I)^2/X2(I)))
1710 PRINT #A8,EXP(A(I)),'(';EXP(A(I)-S*5);'<>';EXP(A(I)+S*T);')'
1715 RETURN
1720 S=SQR((S5(I)/D5(I)/D5(I))/X2(I))\U=K(I)-S*T\V=K(I)+S*T
1725 PRINT #A8,K(I),'(';U;'<>';V;')'\RETURN
1735 S=SQR((S5(I)/D5(I))/X2(I))\U=K(I)-S*T\V=K(I)+S*T
1740 IF K(I)=0 GO TO 1760
1745 PRINT #A8,.693/ABS(K(I)),'(';.693/ABS(U);'<>';.693/ABS(V);',
1750 RETURN
1760 PRINT #A8,'INFINITY','(';.693/ABS(U),'<> INFINITY)',\RETURN
1770 PRINT #A8,'[';I(I);'-';J(I);']'\RETURN
```

```
1800 I=I(T8)\GOSUB 610
1820 GOSUB 755\R=0\RETURN
1900 IF F(N3)>0 GO TO 1910\V1=S5/D5/X2\V3=0\D=D5\RETURN
1910 V1=S5/D5/X2\V3=S5(N3)/D5(N3)/X2(N3)
1920 D=(V1+V3)^2/(1*V1/D5+V3*V3/D5(N3))\RETURN
2000 N=N+1\IF Y(P)>0 GO TO 2005\S9=S9+1\RETURN
2005 GOSUB 2100\X1=X1+U\U2=U2+U*U
2010 Y1=Y1+V\V2=V2+V*V\U9=U9+U*V
2020 RETURN
2100 U=X(P)\V=Y(P)
2105 FOR C=1 TO N3
2106 V=V-EXP(A(C)+K(C)*U)
2107 NEXT C
2110 IF V>0 GO TO 2130
2112 IF T9=1 GO TO 2127
2115 S9=S9+1
2116 PRINT 'AUTO-REJECT #';P
2127 U=0\V=0\RETURN
2130 V=LOG(V)\RETURN
3000 REM --- T - TABLE
3010 REM --- 1-30 DF, P=.05, 2-SIDED
3020 IF D<30 GO TO 3050
3030 T=2.042
3040 GO TO 3090
3050 RESTORE
3060 FOR Q9=1 TO D
3070 READ T
3080 NEXT Q9
3090 RETURN
3100 DATA 12.706,4.303,3.182,2.776,2.571,2.447,2.365
3110 DATA 2.306,2.262,.2.228,2.201,2.179,2.16,2.145,2.131
3120 DATA 2.12,2.11,2.101,2.093,2.086,2.08,2.08,2.074,2.069
3130 DATA 2.064,2.06,2.056,2.052,2.048,2.045
4000 PRINT 'ENTER FILENAME FOR DATA'
4002 INPUT F$
4010 OPEN F$ FOR INPUT AS FILE #1
4020 INPUT #1,H$
4030 INPUT #1,N
4040 FOR I=1 TO N
4042 INPUT #1,X(I)
4044 INPUT #1,Y(I)
4046 NEXT I
4050 CLOSE #1
4060 RETURN
```