DENERVATION INDUCED MECHANICAL RHYTHMICITY IN THE MULTIUNIT CANINE TRACHEAL SMOOTH MUSCLE

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T. M^{c}Kay

Department of Pharmacology and Therapeutics
Faculty of Medicine

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TERENCE McKAY

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Abstract

Cholinergic denervation, but not sympathetic denervation was shown to induce changes in the canine trachealis smooth muscle similar to that observed in the presence of tetraethylammonium or after metabolic depletion. Surgical inhibition of the motor supply resulted in oscillatory contractions of the muscle when stimulated with carbachol ($2X10^{-7}M$) or histamine ($10^{-7}M$) but not potassium chloride.

The phasic activity of the muscle continued in the presence of tetrodotoxin but was acutely sensitive to alterations in the calcium concentration of the bathing medium Mechanical oscillations were reduced and abolished in the presence of a calcium EGTA buffer or 0-Ca while the tonic component of the contraction was reduced only after larger doses were employed.

Differentiation of the mechanical tracing showed that the oscillatory behavior of the muscle was biphasic, consisting of short rapid oscilations superimposed on the slow mechanical contractions. Further investigation gave evidence which suggested that the two components might be due to different pools of calcium or that calcium might work in conjunction with chloride to produce the biphasic response.

The myogenic response observed upon quick stretch of the denervated tracheal muscle suggests that the surgical

treatment of the muscle sets up the conditions necessary for this phenomenon. Such a response is observed only after the denervated muscle has been stimulated with the appropriate agonist and was never observed in the innervated or unstimulated denervated preparation. It would appear that surgical denervation of the motor supply is responsible for a conversion from a multiunit type to a single unit type muscle.

Fluctuations in ATP have been implicated in the phasic activity of some muscles, (Westfall, 1975) and it has been suggested that variations in the ATP content may be, at least partially, responsible for observed alterations in ionic fluxes (Gradman and Slayman, 1975; Sperelakis and Schneider, 1976). Evidence of a decreased ATP content in the denervated tracheal muscle was found, however, the correlation between this decrease in ATP content and the phasic activity of the muscle can only be surmised.

Inhibition of the active pumping processes within the muscle, by cooling or through the administration of ouabain, had an inhibitory effect on the oscillatory nature of the denervated preparation. It is unlikely, however, that the electrogenic sodium pump is entirely responsible for the rhythmicity since the peak tension observed, after cooling or the administration of ouabain, does not reach the peak tension observed while the muscle is rhythmic.

Many of the changes observed in tracheal smooth muscle following motor but not inhibitory denervation are suggestive of a conversion from a multi to a single unit type muscle. These changes are similar to those seen in tracheal smooth muscle made rhythmic by substrate deprivation. The precise mechanism responsible for the oscillatory nature of this preparation remains to be elucidated.

Chapter I

INTRODUCTION AND HISTORICAL REVIEW

Fibrillatory or rhythmic behavior of muscle (striated or smooth muscle) is not a recently observed phenomenon. Such fibrillatory activity in striated muscle was first observed by Schiff in 1851 after motor denervation. He observed that the onset of rhythmic activity was variable, depending on the muscle and species studied and that this activity could be recorded for up to a year after denervation (Cannon, W.B. and Rosenblueth, A., 1949). Similar accounts of rhythmicity in striated muscle have been reported by Philipeaux and Vulpian, 1863, Heidenhain, 1883, Tower, 1939, Eccles, 1941, Purves, 1974, Thessleff, 1975, Camerino and Bryant, 1976 (Cannon, and Rosenblueth, 1949; Purves and Sakmann, 1974; Camerino and Bryant, 1976a, 1976b).

The intrinsic spontaneous activity of the smooth muscle of the alimentary canal has been observed for some time. Detailed descriptions of the mechanical and electrical behavior of the rhythmically active gut have been published in essays by Klee (1927), Dietlen (1913), Kaufmann and Kienbock (1911), Trendelenburg (1917), and more recently by Prosser (1974) and El-Sharkawy and Daniel (1975). The mechanical oscillatory nature of many vascular smooth muscle preparations is a well known phenomenon and is best described in fairly recent reviews by Somlyo and Somlyo (1968 and 1970).

The story of airway smooth muscle rhythmicity or peristaltic movement is certainly one of interest, especially

when one considers that tracheal smooth muscle is generally classified as a tonic muscle that rarely responds to a stimulus with phasic contractions. Early reports of tracheal and bronchial rhythmicity, in both whole animal and isolated tissue preparations, would tend to disagree with this general assumption. Rhythmic behavior, in tracheal and bronchial smooth muscle, was recorded by a plethysmographic technique in 1903 by Dixon et al. It is especially interesting to note that agents like pilocarpine and muscarine were observed to potentiate the oscillatory nature of this smooth muscle after vagotomy (Dixon and Brodie, 1903). Examination of the trachea and large bronchi, with the help of a bronchoscope, enabled Jackson in 1917 to see the rhythmic behavior of airway smooth muscle. He described in some detail the various oscillatory movements, the major type being a to-and-fro motion toward and from the bronchoscope as well as a number of lateral movements of the airway muscle. Much, if not all, of this rhythmic activity was attributed to extrinsic mechanical forces exerted on the bronchi and trachea via the lungs during inspiration and expiration and the cardiovascular system (Ellis, 1936). This is refuted by early X-ray studies by Bullowa (1920) and Reinburg (1925), who described the peristaltic activity in both man and dogs. Both investigators describe three types of movement in the trachea. The first is a lateral movement and the second is

a "bellows-like" expansion and contraction. These are attributed to purely mechanical forces from the heart and the inspiratory and expiratory movements of the lungs. The third type of movement i.e. peristaltic waves of low amplitude, were observed to travel up the trachea and reported to be independant of coughing, respiration and swallowing (Bullowa and Gottlieb, 1920; Reinberg, 1925). Such movements are claimed to be slower than those observed in the cough reflex yet too rapid for ciliary action. This peristaltic action has on occasion been shown to be quite pronounced, even to the point of being termed "tracheal vomiting" (Reinberg, 1925). A number of investigators have attributed this rhythmic behavior in the trachea to pathological conditions. Reinberg's observations were based on a patient diagnosed as having "paralysis of the recurrent nerves" (Reinberg, 1925), while Macklin claims that the peristaltic activity in the tracheas that he had observed were probably due to pathological conditions (Macklin, 1929). Along with these pathological conditions various changes in the physiological state of the preparation were observed to enhance the spontaneous contractions. Asphyxia, changes in temperature, bilateral vagotomy, histamine and cholinergic agents initiated or potentiated the rhythmic response (Ellis, 1936; Sollmann and Gilbert, 1937; Wish, 1952; Loofbourrow, et al, 1957).

Although little is known about the cause(s) or mechanism(s) of action underlying the peristaltic activity of the trachea, it has been suggested that the oscillatory behavior of the airway smooth muscle should not be too surprising, since it has a common derivation with the intestinal tract. It is also proposed that the motor innervation, by way of the vagus, and the presence of ganglia within the smooth muscle may possibly correspond to the ganglia of Auerbach and Meissner in the gut (Macklin, 1929; Sollmann and Gilbert, 1937). Although this is a pleasing theoretical possibility it fails to take into account the specialization of tissues during embryological development.

The functional significance of tracheal rhythmicity is still unknown. It is doubtful that it is of any benefit in expectorating substances from the airways since these oscillations are usually not powerful enough and the cough reflex appears to satisfy this function adequately (Widdicombe, 1963).

In 1948 Bozler subdivided smooth muscle preparations into two types, single unit and multiunit. Single unit muscles were those that had low electrical resistance between adjacent cells, propogated electrical activity within the tissue, spontaneous rhythmicity and myogenic contractions in response to stretch; while single unit muscles are sparsely innervated yet have a relatively large number of nexuses or tight-junctions (Westfall, et al, 1975). It has been pro-

posed that these nexuses represent low resistant pathways for the propagation of electrical activity (Somlyo and Somlyo, 1968a and 1970; Prosser, 1974; Westfall, et al, 1975). Singleunit muscles on the other hand are usually more densely innervated and reported to have fewer nexuses (Mekata, 1971; Westfall, et al, 1975). If each cell or group of cells has it's own innervation then there would be little need for the propagation of electrical activity. This may account for the graded response seen in multiunit muscle.

The classification of smooth muscle into spike generating (phasic) and gradedly responsive (tonic), is not based solely on electrophysiological studies. The potassium contractions and apparent membrane permeabilities to calcium (Ca^{++}) of the phasic muscles and the tonic muscles, are reported to be quite different (Somlyo and Somlyo, 1968a). Clearly, some smooth muscles would not conform completely to either one or the other category. It is reasonable to assume, therefore, that some smooth muscles may exhibit various shades of single or multiunit behavior (Somlyo and Somlyo, 1968a; Somlyo and Somlyo, 1968b).

Ultrastructure

Microscopic examination of smooth muscle cells reveals a distinct membrane, about 80Å thick, surrounding each cell

with no evidence of direct cytoplasmic continuity between adjacent cells. Each cell has a central elongated, ellipsoid nucleus, often containing one or two nucleoli (Somlyo and Somlyo, 1968a). In areas of close apposition or nexus the fused membrane of adjacent cells lack a basement membrane. These intercellular connections provide a means of direct transfer of information from one member of a given cell population to another of the same or to a different cell population. Since electrical transmission would be impossible over large intercellular distances due to short circuiting by the extracellular fluid (ECF), the nexuses would provide a low resistance pathway for conducting action potentials (AP), (Somlyo and Somlyo, 1968a; Prosser, 1974; Daniel, 1978). Available evidence indicates that there is a good positive correlation between the presence of nexuses and conducted action potentials. In addition to these nexal regions there are also peg and socket type of interdigitations and gap-junctions found along the membrane. These may serve to greatly increase the surface area of the cell and possibly aid in cell to cell communication (Somlyo and Somlyo, 1968a; Bose and Innes, 1974).

It has been reported that these structures are found in far greater numbers in single unit muscles (Somlyo and Somlyo, 1968a) and may be only sparsely distributed or even lacking in some multiunit muscles (Mekata, 1971).

Small vesicles are found in large numbers along the inner side of the smooth muscle membrane. These small bodies are known to accumulate Ca^{++} in concentrations equal to that of the ECF and, therefore, several times greater than that of the myoplasm. They appear to be rich in ATPase and may be involved in the relaxation process by means of removing Ca from the myoplasm, (Somlyo and Somlyo, 1968a; Prosser, 1974). Because these vesicles greatly increase the surface area of the membrane they could provide binding sites for both the release of calcium into and the reuptake of calcium from the cytoplasm (Van Breemen, et al, 1972). It has been suggested that these vesicles may be smooth or rough endoplasmic reticulum or even pinocytic vesicles detached from the muscle membrane (Somlyo and Somlyo, 1968a). Because they are not fixed structures, but communicate freely with the ECF, it is possible that they are concerned with the maintenance of intracellular calcium levels. The mitochondria in vascular smooth muscles are often in close apposition to the surface vesicles, and it has been suggested that cations accumulated by mitochondria may be extruded into the extracellular space through the surface vesicle-mitochondrial contacts (Somlyo, et al, 1974; Somlyo and Somlyo, 1976).

In addition to the round vesicles communicating with the ECF there is also a "closed vascular-tubular system", at least in some of the spike generating smooth muscles.

Some of these closed tubules and vesicles appear to make contact with the open pinocytotic vesicles. It has been suggested that they function analogously to the triads of skeletal muscle and are involved in intracellular translocation of calcium (Somlyo and Somlyo, 1970). It should be mentioned too, that the number of these vesicles can be easily overestimated. During histological preparation of the tissue, sections may be taken transversely through membrane invaginations or sockets, thus giving false estimates of the number and location of these vesicles.

The sarcoplasmic reticulum (SR), is a system of tubules present in most, and probably all, smooth muscles. The amount of SR is relatively small in comparison with that found in striated muscles. Tubules of the sarcoplasmic reticulum lie very near the inner cell membrane, separated by a gap of only a few nanometers (Somlyo and Somlyo, 1976). It has been suggested that these are sites where action potentials may release calcium activating twitch contractions (Somlyo and Somlyo, 1971). The volume of sarcoplasmic reticulum differs in different types of smooth muscle and it's extent correlates with the ability of a given smooth muscle to contract in the absence of extracellular calcium. Even in calcium free solutions drugs can elicit relatively large contractions in the smooth muscle of large elastic arteries in which the SR occupies approximately 5-7.5% of the cyto-

plasmic volume. Portal-anterior mesenteric vein and taenia coli smooth muscle contain an SR of only 1-2% of the cytoplasmic volume and such muscles generally fail to contract in calcium-free medium (Somlyo and Somlyo, 1970; Devine, et al, 1972; Popescu, et al, 1974; Somlyo and Somlyo, 1976).

The contractile mechanism in smooth muscle appears to be based on the sliding of filaments in a manner fundamentally similar to that of striated muscle (Somlyo and Somlyo, 1970; Somlyo and Somlyo, 1976). Although it has been difficult to isolate and identify these filaments, recent work by Somlyo and Somlyo, 1976, has shown that a thin actin, a thick myosin and an intermediate filament are present in most if not all smooth muscles. The myosin filaments, which are in parallel and arranged in contractile units, are longer than those in striated muscle. This may be why smooth muscle can develop tension at least equivalent to that developed by skeletal muscle, in spite of the relatively low concentrations of myosin in smooth muscle. The thin (actin) filaments are observed to be inserted into the cell membrane and in the dense bodies which are believed to act as anchor points for these filaments. The average ratio of thin to thick filaments is approximately 16:1. In the best organized examples of cross sections the thin filaments form a rosette surrounding a central thick filament (Somlyo and Somlyo, 1976). A third type of filament, frequently asso-

ciated with the dense bodies, has been described in a variety of smooth muscles. These structures are composed of neither actin nor myosin and their function is basically unknown (Somlyo, et al, 1971; Cooke and Chase, 1971; Somlyo, et al, 1973). In abnormal smooth muscle fibers these intermediate filaments may replace large proportions of the myofilament latice (Somlyo, et al, 1973).

Dense bodies are dark-staining spindle-shaped areas which vary in length from 4000 to 9000 Å and in width from 2000 to 5000 Å. They are often found close to or adhering to the cytoplasmic side of the membrane or dispersed within the membrane (Somlyo and Somlyo, 1968a; Somlyo and Somlyo, 1976). A large proportion of these structures appear to have a function similar to that of the Z-line of striated muscle (Somlyo and Somlyo, 1976).

Tracheal Smooth Muscle

The primary function of the smooth muscle of the lung is to control the distribution of inspired gas entering the lung. Smooth muscle is distributed from the trachea downwards as far as the respiratory bronchioles but it's attachments vary, so that the effects of muscle contraction are different in the largest airways from the effects in the smallest ones. The mass of muscle, in proportion to the diameter of the airways, increases as one goes distally

from the larynx to the lungs (Kamburoff, 1976).

In the trachea and main bronchi, which have complete cartilagenous rings, the muscle is arranged circularly being attached to the outer aspects of the tips of the cartilage. Contraction of this part of the muscle can, therefore, dramatically reduce the diameter of the trachea and main bronchi. Changes in tracheal and bronchial diameter occur during the respiratory cycle, dilating during inspiration and contracting during expiration.

The muscle attachment to the cartilage disappears at the level of the hilum of the lung and from then onwards due to the direction of the muscle contraction, produces not only narrowing of the bronchi but also longitudinal shortening (Kamburoff, 1976).

Tracheal Innervation

The vagus nerves, in the dog, carry both sympathetic and parasympathetic fibers and are ultimately responsible for both the afferent and efferent impulses to and from the trachea. The recurrent laryngeal nerves which arise from the vagi, supply the parasympathetic or motor control, while collaterals from the sympathetic trunk travelling with the vagi provide the inhibitory control over the tracheal smooth muscle.

There are parasympathetic ganglia throughout the

tracheo-broncheal tree as far as the alveolar ducts, but their density in the small airways are reduced. For this reason it was believed that the small airways were relatively unresponsive to vagal stimulation. More recent evidence would suggest, however, that this is not the case (Woolcock, 1969).

Studies in both animals and man have shown that stimulation of irritant receptors in the airways by physical or pharmacological agents results in a reflex broncho-constriction. This reflex is blocked by atropine, vagal cooling or vagotomy which would indicate that it is mediated by the parasympathetic nervous system (Gold, 1972).

In early life alpha receptors, (a-receptors), are found in abundance in the dog tracheal smooth muscle and their stimulation results in contraction. Beta adrenergic receptors, (B-receptors), predominate with time and stimulation of these results in relaxation of the tracheal smooth muscle. Cholinergic receptors produce good contraction throughout life and do not appear to change with time. Adrenergic stimulants produce little or no contraction in later life because of the predominance of B-receptors, however, if an alpha stimulant is added with a cholinergic agonist an additive effect may be seen (Suzuki, 1976; Pandya, 1976).

There is some evidence in favour of a non-adrenergic,

non-cholinergic inhibitory response to nerve stimulation in the trachea which may be due to stimulation of "purinergic" nerves in which ATP has been suggested to be the likely transmitter (Farmer and Farrar, 1976). However, in Suzuki's study of the dog trachea he finds no reason to suggest any important role by such "purinergic" nerves (Suzuki, et al, 1976).

Ionic Basis of the Smooth Muscle Resting and Action Potential

The ionic currents in the membranes of smooth muscles are dependant on the electrochemical potential gradients and the ease with which the ions may cross the cell membrane. The nerve and muscle cell membrane is much more permeable to potassium (K^+) than to sodium (Na^+), and it is this property which is the determinant of the membrane potential (Hodgkin, 1951; Hodgkin, 1958). The same principles hold true in smooth muscle, with the essential difference that calcium and chloride (Cl^-) appear to play a much more prominant role in smooth muscle. The equilibrium potential for a given ion is given by the Nernst Equation:

$$E_{K^+} = \frac{RT}{F} \ln\frac{(K^+)}{(K^+)}\frac{o}{i} \qquad E_{Na^+} = \frac{RT}{F} \ln \frac{(Na^+)o}{(Na^+)_i} \qquad E_M = \frac{RT}{F} \ln\frac{(X)}{(X)}\frac{o}{i}$$

Where E_M represents the resting membrane potential, E_K and E_{Na} the potassium and sodium equilibrium potentials respect-

ively, R is the gas constant, T is the absolute temperature, F is Faradays constant and $(\ )_o$ and $(\ )_i$ represent the extracellular and intracellular ion concentrations respectively. This equation has been revised to take into account the permeability, P, of each ion in the membrane and is represented by the Goldman Constant Field Equation:

$$E_M = \frac{RT}{F} \ln \frac{P_{K^+}(K^+)_o + P_{Na^+}(Na^+)_o + P_{Cl}(Cl^-)_i}{(K^+)_i + (Na^+)_i + (Cl^-)_o}$$

(Katz, 1966). In order to take into account the number of sodium ions pumped out for each potassium ion pumped in, a coupling ratio "r" was added to give the revised version:

$$E_M = \frac{RT}{F} \ln \frac{P_{Na^+}(Na^+)_o + rP_{K^+}(K^+)_o}{(Na^+)_i + (K^+)_i}$$

(Simmons, 1976). The assumption that chloride is passively distributed and rapidly permeant is adapted as a matter of convenience simplifying the mathematical treatment (Mullins and Noda, 1963).

It follows from the equation for E_M, that a selective increase in membrane permeability to one ion will shift the membrane potential towards the equilibrium potential of that ion, which will carry the membrane current in a direction downhill to it's electrochemical gradient. This implies that a selective change in ion permeability may depolarize or hyperpolarize the membrane if the equilibrium potential of the given ion is more positive or negative than the membrane

potential. A non-selective increase in membrane permeability will result in a downhill flux of all permeant ions (Somlyo and Somlyo, 1968a).

In most excitable tissues, the resting membrane potential, (E_M), is dependant on and determined by the passive distribution of potassium (Katz, 1966; Somlyo and Somlyo, 1968a; Simmons, 1976). The resting membrane potential of smooth muscle is generally smaller than in skeletal muscle and this may be due to the more prominant roles of the other ion constituents involved. It is this relatively low unstable potential which causes the spontaneous spike activity in single unit smooth muscle (Somlyo and Somlyo, 1968a). As might be expected from the classification of smooth muscle, the membrane potential deviates much more from the potassium equilibrium potential (E_K) than it does in other excitable tissues depending on the type of smooth muscle and the species being studied (Holman, 1958; Kuriyama, 1963; Simmons, 1976).

Any deviation of the recorded RMP from the calculated equilibrium potential of the major contributing ion, signifies a disequilibrium which must be maintained. Since the maintainance of a diffusion potential (e.g. of K^+) entails passive diffusion down a transmembrane concentration gradient of the ion, an active pump is necessary to counteract this constant outward leakage of K^+. In addition there is a small

but significant permeability to external Na^+ and, therefore, Na^+ is constantly moving into the cell under the influence of the high driving force of it's electrochemical gradient. This, along with the passive loss of K^+ due to the membrane's relatively high permeability to K^+ would eventually abolish the Na^+ and K^+ concentration gradients and therefore, the membrane potential. It is, therefore, essential that some mechanism be involved to maintain the high K^+ concentration inside and the low Na^+ concentration outside (Katz, 1966; Simmons, 1976). The extrusion of Na^+ and uptake of K^+ against their electrochemical gradients appears to be mediated by a system which has the properties of a Na^+-K^+ dependant ATP-ase, (Somlyo and Somlyo, 1968a). Of the many mechanisms that have been proposed, it is generally agreed that an electrogenic sodium potassium pump is involved in at least some part of this activity.

Although the ratio of ions transported across the membrane is variable, depending on the type of muscle being studied, it is generally agreed that the pump is capable of transferring more Na^+ than K^+ thus producing a net transfer of outward charge which contributes to the RMP (Simmons, 1976). It should be mentioned, however, that the contribution of the electrogenic pump to the transmembrane potential is usually very small, and some shunting does take place across the membrane.

Most of the recent evidence would lend support for the existence of such a Na^+-K^+ ATP-ase dependent pump. There appears to be an unusually high dependence of this system upon external K^+. Removal of extracellular potassium from the bathing medium results in a 90% decrease in Na^+ efflux from the tissue (Somlyo and Somlyo, 1968a). Ouabain and metabolic inhibitors produce a rapid depolarization which is abolished after removing these agents from the bathing medium (Daniel, et al, 1962; Casteels and Kuriyama, 1966). Ouabain or cold decreases the E_M of the guinea pig portal vein 20 - 26mV and the normal electrogenic Na^+-pump is estimated to contribute 10-20mV to the E_M, (Kuriyama, et al, 1971; Prosser, 1974). The KCl induced relaxation of Na^+-rich isolated cat carotid artery (Bose and Innes, 1974) and rabbit pulmonary artery (Somlyo and Somlyo, 1970) is consistent with stimulation of an electrogenic Na^+-pump.

So there is evidence that the RMP in smooth muscle is at least partially due to an electrogenic Na^+-K^+ pump. It has been calculated that taenia pumps Na^+ and K^+ at a ratio of 3:2 (Hutter, 1961; Brading, 1973) and contributes 15 - 20mV to the RMP (Casteels, 1971; Simmons, 1976).

Pump activity appears to depend on many of the factors which it regulates. An increase in intracellular Na^+ or extracellular K^+ stimulates the pump while a decrease in the membrane resistance inhibits pump activity and an in-

crease in membrane resistance has the opposite effect, (Prosser, 1974). Low temperatures and metabolic inhibitors can, as would be expected, abolish pump activity, resulting in an accumulation of intracellular Na^+, a loss of intracellular K^+ and membrane depolarization. High K^+ concentrations invariably depolarize the membrane by decreasing the E_K. An indirect depolarization which is neurally mediated is also seen with K^+ concentrations in the order of 5 to 20mM, (Somlyo and Somlyo, 1968a). Alternating current stimulation of vascular strips results in a greater indirect component of contraction mediated by the release of catacholamines and K^+ releases catecholamines from adrenergic sites. The increase in tone elicited by 9 to 15mV external K^+, in the isolated pulmonary artery (Bevan and Osher, 1963) may be due to direct, indirect or combined effects. Peripheral vasoconstriction elicited in the hamster cheek pouch by 9 to 12mM K^+ is abolished by denervation or adrenergic blockade (Sudak and Fulton, 1962).

The contraction in rat portal vein elicited by removal of external K^+ is associated with depolarization and discharge of AP (action potential) (Axelsson, et al, 1967). The depolarization and contraction induced by the withdrawal of K^+ are probably direct effects, and not due to release of endogenous catecholamines.

The importance of K^+ and potassium conductance, (G_K)

for excitability has been demonstrated in a variety of studies. Multiunit muscles generally have higher RMPs which would be consistent with a relatively high G_K/G_{Na} ratio. Tomita has suggested that a low G_K might be the reason for the low RMP and spontaneous activity observed in taenia (Kuriyama, 1963; Tomita, 1966; Bulbring, et al, 1970).

It has been demonstrated that high K^+ increases G_K (decreases membrane resistance), decreases E_M and converts bursts of spikes and accompanying contractions to single spiking and greatly reduced contractions while Ba^{++} reduces G_K and increases the number of spikes per burst and contraction amplitude (Daniel, et al, 1962). Therefore, a reduction in the G_K/G_{Na} ratio should produce a depolarization, increase membrane resistance and possibly initiate spontaneous contractions (Simmons, 1976).

Very low extracellular K^+ can result in a contraction, however, it may also result in a delayed relaxation (Somlyo and Somlyo, 1968a). The depolarization and contraction induced by withdrawal of K^+ are probably due to direct effects and not due to the release of endogenous neurotransmitters (Somlyo and Somlyo, 1968a). There appears to be a K^+ concentration which is optimal for muscle relaxation, above or below that concentration results in muscle depolarization and contraction. This depolarization in K^+-free solution can be explained by a decrease in G_K with a shift towards the E_{Na}

or by the removal of a K^+-coupled electrogenic Na^+ pump's contribution to the E_M. Low K^+ can result in a depolarization of about 10mV. Adding back K^+ causes a hyperpolarization, and this hyperpolarization may drive the E_M as negative as -90mV (i.e. more negative than E_K). Ouabain acts like zero K^+ in that it also depolarizes the membrane about 10mV, however, if ouabain is present and K^+ is added back no hyperpolarization is seen. The hyperpolarization produced by adding back K^+ is not due to a decrease in G_K because the membrane resistance is increased only slightly, (Prosser, 1974). In zero K^+, smooth muscle cells lose K^+ and gain Na^+(the loss of K^+ results in the depolarization). When K^+ is added back, Na^+ is pumped out, the membrane becomes hyperpolarized and K^+ is taken up, unless ouabain is present or the temperature is low (Prosser, 1974). The G_K and therefore the RMP appears to be directly related to the amount of Ca^{++} bound to the membrane (Westfall, et al, 1975). Removing Ca^{++} from the bathing medium enhances the K^+ withdrawal contraction but the same effect abolishes the high K^+ contraction. It would seem, therefore, that the contraction seen in K^+ free solutions is not simply the result of depolarization, but to some interference with a K^+ dependent Ca^{++} pump or Ca^{++} permeability, (Barr, et al, 1962; Midrio and Bettini, 1967; Somlyo and Somlyo, 1968a). If membrane bound Ca^{++} does regulate the membrane activity then specific

changes should be observed if the bound Ca^{++} is displaced by lanthanum (La^{+++}). La^{+++} displaces extracellular Ca^{++}, blocks Ca^{++} influx and inhibits contraction by preventing Ca^{++} entry (Prosser, 1974). Such circumstances lead to a rapid inhibition of a K^+ induced contraction as in aortic smooth muscle where potassium stimulates calcium influx and La^{+++} blocks this influx. Lanthanum also blocks contractions produced by adding calcium to a calcium free medium (Van Breemen, 1973).

In most excitable tissues depolarization is caused by an increase in Na^+ permeability while hyperpolarization is assumed to be due to an increase in K^+ permeability or by an electrogenic Na^+ pump. It is Ca^{++}, however, that appears to play an essential but often regulator function. There is evidence of both antagonism and potentiation between these two anions at the cell membrane. The effects of Ca^{++} are more pronounced but Na^+ also has effects on smooth muscle spike; there is some antagonism between Na^+ and Ca^{++}, (Anderson, 1971). When, with taenia, sucrose is substituted for NaCl, spike amplitude increases, rate of rise increases, slight hyperpolarization occurs, spontaneous activity eventually stops, but spikes can be triggered; if Ca^{++} is decreased at the same time as the decrease in Na^+ nearly normal spikes remain. In cat intestine muscle spikes show no change over a 2-3 fold range of extracellular Ca^{++} if Na^+ is also changed

to keep the ratio $(Na^+)^2/(Ca^{++})$ constant.

In taenia the effect of reduced extracellular Ca^{++} is greater in high Na^+; after loss of spikes in low extracellular Ca^{++} a reduction in extracellular Na^+ leads to recovery, therefore there is some antagonism between Na^+ and Ca^{++}. Na^+ is needed for recovery from K^+ contracture but the contraction phase requires extracellular Ca^{++}.

In estrogen dominated uterine smooth muscle of the rat, the rate of rise of spikes increases with high extracellular Na^+, and is more in high Ca^{++} than in low Ca^{++} mediums. Neither Ca^{++} nor Na^+ alone can support spikes; La^{+++}, Mn^{++} or Co^{++} reduces or abolishes the inward current and TTX has no effect. It is suggested that uterine muscle may have a single conductance channel requiring both Na^+ and Ca^{++}. Voltage clamping indicates that the outward current is carried by K^+. Since neither Ca^{++} nor Na^+ alone can support spike activity, both would appear to be essential. In a Ca^{++} free medium there is a slight depolarization and a decrease in membrane resistance, but no spikes occur and contraction fails (Prosser, 1974), while Na^+ withdrawal results in a depolarization and a slight contracture which is said to be associated with an increased Ca^{++} influx (Somlyo and Somlyo, 1968a). The rate of rise of the spike is increased with high external Na^+ and is greater in high Ca^{++} outside than in low Ca^{++} outside

(Prosser, 1974). In those muscles which are spontaneously active it has been suggested that Ca^{++} is needed for triggered spikes and Na^{+} for the pacemaker potential (Prosser, 1974).

Smooth muscles vary in the importance of Na^{+} channels, and it is not clear, whether synergism between Na^{+} and Ca^{++} occurs, i.e. does Na^{+} influence Ca^{++} release. There is evidence to suggest that this is so (Prosser, 1974). However, whether or not there are parallel channels, or a common channel for both, or whether both interact with a common carrier molecule is unknown. Spikes of most, of not all smooth muscles, are based on a transient, rapid inward Ca^{++} current followed by a delayed outward K^{+} current which repolarizes the membrane. There may also be a slower inward Na^{+} current producing a plateau effect and a rapid outward K^{+} current which limits spike height (Frankenhaeuser and Hodgkin, 1957; Bulbring and Kuriyama, 1963; Brading, et al, 1969). It also appears that the membrane bound Ca^{++} stabilizes membrane conductance by altering G_{Na} and G_{K} and altering the regenerative process for spike production, and, as mentioned earlier, Na^{+} may compete with Ca^{++} in carrying inward current (Prosser, 1974; Simmons, 1976). The observation that spike parameters, especially the rate of rise of the action potential (AP), are increased in high extracellular calcium and then abolished by giving D-600, would be strong evidence for a dominant role of calcium in the action potential (Kohlhardt, et al, 1972;

Prosser, 1974). D-600 inhibits all spikes and is assumed to work by inhibiting only the entry of "trigger calcium" associated with spiking (Prosser, 1974). There is also evidence available to show that depolarization of smooth muscle by various means and/or agents, is accompanied by an increase in Ca^{++} influx (Prosser, 1974).

A RMP governed by a membrane permeable to only K^+ and Cl^- and by a passive Gibbs-Donnan distribution would follow the equilibrium potential for potassium as specified by the Nernst equation. There is clearly, therefore, a disequilibrium in smooth muscle since the Nernst equation predicts a slope for E_M verses the log of the external K^+ concentration of 61mV per 10 fold change in the external potassium concentration, while that observed is actually only 38mV per 10 fold change in the external potassium concentration. This may be due at least in part, to Na^+ with it's high electrochemical gradient (Simmons, 1976). However, recent work has shown that chloride ion plays a major role in determining the low resting membrane potential (Casteels and Kuriyama, 1966; Casteels, 1970). The chloride equilibrium potential in taenia coli is 25 to 35mV less negative than the resting membrane potential. The primary reason for this is suggested to be the presence of an inwardly directed chloride pump. Such a pump would increase intracellular Cl^- thereby decreasing the E_{Cl^-} and thus bring the RMP towards zero (Casteels

and Kuriyama, 1966; Casteels, 1970). This is supported by the finding of high intracellular chloride concentrations (Kao and Nishiyama, 1964). Since in a chloride free medium the electrogenic sodium pump is estimated to contribute only about 6mV it is calculated that the Cl^- pumping in taenia contributes about 15mV to the RMP (Droogmans and Casteels, 1976). The observed increase in chloride efflux after giving ouabain or high potassium would suggest Cl^- uptake may be coupled to the Na^+- K^+ pump (Casteels, 1971; Prosser, 1974). Another factor that could contribute to the more positive E_M in smooth muscle is the relatively high chloride permeability of the membrane. This is suggested by the rapid chloride efflux and the effects of replacing Cl^- with a less permeant ion such as isethionate (Barr, 1959; Durbin and Monson, 1961). Such a replacement results in a transient depolarization (Holman, 1958), a decrease in the difference between the slopes of E_M and E_K versus the log of the extracellular potassium concentration (Kuriyama, 1963), a 50% decrease in membrane resistance (Ohashi, 1970), a decrease in K^+ efflux (Casteels and Meuwissen, 1968) and suppression of the secondary depolarizations of the intestinal slow waves or control potentials (El-Sharkawy and Daniel, 1975c).

According to the Nernst equation, a decrease in extra cellular sodium should make the sodium equilibrium potential less positive and, therefore, slightly hyperpolarize the membrane. When sucrose is used to substitute for NaCl the

effects of chloride deficiency and decreased ionic strength must be considered, therefore, the depolarization initially seen with sucrose substitution is likely to be due to a decrease in the chloride equilibrium potential. This, in turn would tend to depolarize the muscle membrane (or at least reduce the eventual hyperpolarization) and increase membrane resistance and possible account for the greater spike amplitude observed (Kuriyama, 1963).

During the initial period of anion substitution, the E_{Cl^-} becomes more positive due to a decrease in extracellular Cl^-. At the same time active chloride uptake is decreased due to a lack of substrate, therefore, intracellular Cl^- leaks out of the cell under the influence of it's new electrochemical gradient. Thus the chloride equilibrium potential becomes progressively more negative (hyperpolarizing the membrane) and becomes established at a new level determined by the nature of the replacement anion. The chloride equilibrium potential becomes more negative with a more permeant anion like nitrate than with a less permean anion like isethionate. With isethionate the final RMP is more positive than it is in a Cl^- solution (Holman, 1958; Kuriyama, 1963; Simmons, 1976). The overall effect, therefore, of replacing chloride with a less permeant anion is an eventual decrease in Cl^- flux, when intracellular chloride is depleted resulting in membrane repolarization and an increase in mem-

brane resistance (Simmons, 1976).

Intracellular chloride is much higher in smooth muscle than in skeletal muscle resulting in a more negative E_{Cl} and E_M in striated muscles. It was traditionally assumed that chlorides contribution to the resting activity of the nerve and striated muscle cell was barely minimal and has often been neglected. More recent evidence would imply that this may not be the case. Denervated skeletal muscle often becomes rhythmic and these spontaneous oscillations may be related indirectly or directly with the chloride activity at the denervated muscle membrane. The membranes of normal mammalian skeletal muscles are highly permeable to Cl^- and the low membrane resistance values recorded in such muscles are probably due to high chloride conductances in these muscles (Lorkovic and Tomanek, 1977). It has been suggested that the mammalian striated muscle fiber requires a high chloride conductance in order to maintain stability of it's excitable surface membrane and prevent abnormal repetitive firing or sensitivity to depolarization (Camerino and Bryant, 1976a). In the denervated, spontaneously active preparation the influence of the motor nerves (AP'S or "trophic factors") are absent and this is suggested as being responsible for preventing the muscle fiber from maintaining it's normally high chloride permeability (Bryant and Camerino, 1976b).

Slow waves (SW) or control potentials (CP) function

as control activity by changing the excitability of intestinal smooth muscle to chemical and electrical stimuli which produce spikes and contractile responses. The occurence of the SW determines the occurence in time and space of the spike activity (El-Sharkawy and Daniel, 1975c).

Slow wave activity consists of repetitive depolarizations of 12 to 25mV amplitude and two second duration which occur at a frequency characteristic of the species and of the level of the intestine from which they are recorded. The spike activity consists of one to several action potentials which occur superimposed on the depolarized phase or plateau of the SW. Each spike is preceded by a small depolarization or prepotential (Bortoff, 1961; El-Sharkawy and Daniel, 1975b). The slow wave activity is myogenic; it originates in the longitudinal muscle layer and spreads electrotonically to the circular muscle layer (Daniel,et al, 1960; Bortoff, 1961a; Bortoff, 1961b; Kobayashi, et al, 1966; Bortoff, 1964; Bortoff and Sachs, 1970).

It has been reported that in a few cells, a diastolic intercontrol potential depolarization exists. These are progressively slow depolarizations of up to 6 to 7mV which occur between the end of each slow wave and the onset of the next. It is suggested that these may serve as a trigger for the SW, with the fastest intercontrol potential depolarization rate being the dominant driving oscillator in the pre-

paration (El-Sharkawy and Daniel, 1975a).

The coupling of SWs between the smooth muscle cells can be best described by the bidirectional coupled relaxation oscillator model of Sarna, Daniel and Kingma (Sarna, et al, 1971; Daniel and Sarna, 1978). This model predicts that a) the SW is electrically excitable, b) the coupling of the SW occurs by current flow between cells and c) the frequency of the SW in an isolated segment of the muscle or in an intact preparation, is determined by the highest frequency oscillator. In terms of the triggering mechanism proposed here, this would be the cell, or group of cells exhibiting the fastest rate of intercontrol potential depolarizations (El-Sharkawy and Daniel, 1975a).

Various studies have shown that the smooth muscle cells of the intestine, uterus, taenia and many others can be electrogenic (Liu, et al, 1969; Casteels, et al, 1971; Conner and Prosser, 1974; Prosser, 1974; Bose, 1975; El-Sharkawy and Daniel, 1975b). The role of this electrogenic pump in smooth muscle automaticity and the extent of it's contribution to the resting membrane potential of the muscle membrane is debatable. Potassium has at least two actions at the smooth muscle membrane; one is it's effect on the membrane potential as described by the Goldman equation and the other is it's action in stimulating the Na^+- K^+ pump (Connor and Prosser, 1974). The resting potential of

intestinal smooth muscle is greater at an external potassium concentration of 1.75 mM than at the normal extracellular potassium concentration of 3.5 mM or at zero extracellular potassium (Somlyo and Somlyo, 1968a). The peak resting potential at 1.75 mM (K^+ outside) is not observed at 22°C or after the administration of ouabain; these observations, together with the depolarization seen in a potassium free medium, are taken as evidence of a contribution of a Na^+- K^+ pump to the resting membrane potential in intestinal smooth muscle (Conner and Prosser, 1974). The random discharge of action potentials in phasic muscles or graded depolarizations in tonic muscles may be responsible for the tone observed in some smooth muscles, particularly vascular smooth muscle (Axelsson, et al, 1967; Somlyo and Somlyo, 1968a and b). It is also suggested that the tone observed in these muscles could be due to an increase in the permeability of the membrane to calcium or by an inhibition of calcium pumping, either of which may occur relatively independantly of changes in the membrane potential (Somlyo and Somlyo, 1968a).

Evidence indicating a pump component of other smooth muscles is as follows: guinea pig taenia coli, when freshly dissected or stored in the cold, lose K^+ and gain Na^+; if then soaked in Krebs Henseleit solution at 37°C, normal ionic gradients are restored within a few hours and this restora-

tion is blocked by ouabain or zero potassium (Casteels, 1966; Casteels, 1970). Exposure of taenia coli to potassium free medium causes depolarization and readmission of potassium results in hyperpolarization below the potassium equilibrium potential, an effect prevented by ouabain (Tomita and Yamamoto, 1971; Casteels, et al, 1971a and b; Prosser, 1974; Bose, 1975). The hyperpolarization on readmission of potassium is greater with high intracellular sodium than with low intracellular sodium (Bolton, 1973). It has been calculated by Casteel s et al, that in normal Krebs-Henseleit solution the pump current accounts for 10-15 mV of the resting potential in taenia (Casteels, et al, 1971). Not only does the sodium/potassium pump contribute to the resting membrane potential but it is also suggested to be directly involved in the slow wave activity of the intestinal smooth muscle (Job, 1969; Liu, et al, 1969; Conner and Prosser, 1974). The depolarization phase of the slow wave is reported to be due to a passive sodium influx; this inward current of depolarization pressumable provides the basis for the known conduction of the slow waves (Prosser and Bortoff, 1968; Job, 1969). On the basis of effects of inhibitors, particularly ouabain, it was suggested that the slow wave may be dependant on an electrogenic sodium pump (Daniel, 1965). This is supported by the finding that there is an enhanced efflux of sodium during the repolarization phase and that

this efflux is not only against an electrochemical gradient for sodium but also is opposite in direction to the change in electrical driving force. It is reasonable, therefore, to assume that the increased efflux of sodium during the repolarization phase is due to an active process (Job, 1969). That the generation of slow waves involves an active process is also supported by the studies of the effects on slow waves of inhibitors of oxidative phosphorylation (cyanide, anoxia, DNP, PCP); by the effects of specific inhibitors of active ion transport (ouabain), and by the effects of sodium substitution in the medium (Bortoff, 1961a; Daniel, 1960; Daniel, 1965; Tamai and Prosser, 1966; Kobayashi, et al, 1967; Liu, et al, 1969; Job, 1969). Further evidence that SWs result from a rhythmic efflux of sodium is that the slow waves but not the spikes, are lost in a sodium free or a potassium free medium; the slow wave amplitude varies with the log of the external sodium concentration with a slope of 10mV and this is the same at two calcium concentrations and the amplitude of the slow wave is increased when sodium, but not K^+, is iontophoretically injected into the cell. The slow wave also exhibits a high temperature coefficient and under voltage clamp, current pulses are recorded having the time relations predicted if the slow waves were due to a rhythmic electrogenic pump (Liu, et al, 1969; Job, 1969; Conner and Prosser, 1974). From this, then, it is postulated that the

pump, which contributes to the resting potential, can also oscillate to give slow waves (Papasova, et al, 1968; Liu, et al, 1969; Job, 1969; Conner and Prosser, 1974; Prosser, 1974).

The prepotentials and spikes in intestinal smooth muscle and taenia coli are believed to be due to an increase in Ca^{++} conductance (Liu, et al, 1969; Kuriyama and Tomita, 1970; Conner and Prosser, 1974). This is indicated by: 1) the reduction of spike amplitude in low extracellular calcium and complete cessation of spike activity in zero calcium, 2) the action of competitive inhibitors, manganese and cobalt in very low concentrations, 3) the relative insensitivity of the spikes to external sodium and their insensitivity to tetrodotoxin, 4) the linear relation of spike height with log of the external calcium concentration, 5) enhanced influx of calcium at the time of spiking and the voltage clamp studies indicate that the inward current is carried by calcium (Job, 1969; Liu, et al, 1969; Kumamoto and Horn, 1970; Conner and Prosser, 1974). The effects of calcium on spike amplitude cannot be due to changes in the resting potential since the membrane potential is relatively insensitive to external calcium (Liu, et al,1969; Conner and Prosser, 1974). In taenia coli, spikes are rapidly abolished in calcium free medium even when depolarization is prevented (Brading, et al, 1969). In addition to increased

calcium conductance associated with spikes, variations in the extracellular calcium concentration may alter membrane resistance. Spikes are absent in calcium free medium and both the amplitude of the spike and the rate of rise and fall are maximum at an intermediate concentration. In addition to the change in calcium conductance seen at low concentrations, there may also be a reduced permeability of the membrane at higher concentrations of calcium which would counteract increased conductance during a spike (Liu, et al, 1969).

There is evidence for a sodium-calcium interaction in intestinal smooth muscle. The frequency and amplitude of the spikes increases when the external sodium concentration is reduced or when the slow waves are eliminated, as by ouabain (Liu, et al, 1969). Sodium conductance increases in rat uterus during a spike and the rate of depolarization with high sodium is more in high extracellular calcium than in low extracellular calcium (Conner and Prosser, 1974). In ureter both sodium and calcium contribute to the spike; the plateau is lost in sodium free medium but the spikes remain, and the rate of rise of the plateau depolarization with high extracellular calcium is greater in high external sodium than in low sodium, hence the spike component may be primarily due to calcium and the plateau to sodium (Kobayashi, 1969; Kuriyama and Tomita, 1970). In a calcium

free medium with 0.5mM Mg^{++} some spiking occurs in taenia coli. This appears to be dependent on external sodium, hence there can be some Na^{+} current which is normally overbalanced by the calcium current (Sarna, et al, 1971). Spontaneous activity in taenia coli stops in sodium free medium but spikes can still be triggered if calcium is present (Brading, et al, 1969). It has been suggested that Ca^{++} binds to external anionic sites on the membrane, that calcium is released to give an inward current of an action potential and that sodium can compete with calcium for the binding sites but not substitute for calcium as a current carrier (Conner and Prosser, 1974). However, it has also been reported that a removal of external calcium can result in a reduction of slow wave activity, a decrease in the rate of rise and fall of the slow wave and eventually complete loss of the slow wave and a depolarization of the membrane (Tamai and Prosser, 1966; Liu, et al, 1969; El-Sharkawy and Daniel, 1975c). In the presence of verapamil, however, slow wave activity persists while all spikes and mechanical activity are abolished (El-Sharkawy and Daniel, 1975c). This has led to the suggestion that calcium may not have a current carrying function in the generation of the intestinal slow wave but that the inward Na^{+} current is responsible for the slow wave generation and this sodium

current may depend on the presence of external calcium or, alternatively, the triggering mechanism for the slow wave may be calcium dependant (El-Sharkawy and Daniel, 1975c).

The theory of slow wave generation presented, i.e. an oscillating sodium electrogenic pump, was first proposed by Daniel in 1972. Since then Job has proposed two other hypotheses, the first, to which reference has been made, is essentially the same as that proposed by Daniel, with the exception that an increase in sodium permeability which is assumed to cause the depolarization phase of the slow wave, is taken into account; in conjunction with the oscillating sodium pump (Job, 1969). The third theory of intestinal rhythmicity and the second proposed by Job, is an oscillating Na^{+} permeability hypothesis (Job, 1971). According to this hypothesis a build up in ATP concentration at the membrane, "turns on" an increase in sodium permeability which leads to the depolarization phase of the slow wave and an increase in the inward leak of sodium ions. The increase in intracellular sodium coincident with the high level of ATP stimulates the sodium pump. The pump depletes the ATP to subthreshold values again, the increase in sodium permeability is turned off and the membrane repolarizes.

Slow waves in the cat stomach consist of an initial rapid component which is sodium dependant, is propagated and not correlated with contractions; and a second slow

component which is calcium dependent, is abolished by Mn^{++}, may be propagated but is always found when contractions occur. Spikes may appear on the second component and enhance contraction. Tetrodotoxin has no apparent effect on either slow wave component, while the frequency of spontaneous waves is reduced by low external calcium and enhanced by high external calcium. This effect of extracellular calcium requires the presence of sodium (Papasova, et al, 1968). El-Sharkawy and Daniel (El-Sharkawy and Daniel, 1975a; 1975b; and 1975c) have also observed slow waves in intestinal muscle which appear to be made up of two components which they term initial and secondary depolarizations. The first component of the slow wave in the stomach is analogous to the intestinal slow wave. It is reduced or abolished by ouabain and its function is believed to be that of synchronizing activity in large fields of muscle fibers (Papasova, et al, 1968). The second component is reduced in amplitude or eliminated when calcium is reduced; and it is believed that this second component and the spikes are due to enhanced calcium influx. Spiking is enhanced in both jejunum and stomach by ouabain (Govier and Holland, 1965; Papasova, et al, 1968). This action of ouabain may be due to an increased calcium permeability or to a sodium-calcium exchange mechanism whereby an increase in intracellular sodium due to inhibition of the pump could result in an increased calcium influx due to a

passive exchange with sodium (Papasova, et al, 1968; Bose, 1975; Blaustein, 1977). At very low external calcium concentrations, the first component of the stomach potential is also reduced, hence while the first component of the slow wave is primarily sodium dependent, calcium may affect the sodium permeability (Papasova, et al, 1968).

The frequency of the spontaneous waves is relatively insensitive to sodium when calcium is in normal concentrations. However, the frequency is reduced when external calcium is lowered and is increased when calcium is raised in concentration. The effect of calcium on frequency requires a sodium-containing medium, therefore, there appears to be a coupling of the two ions in the regulation of frequency (Papasova, et al, 1968). High external potassium reduces all slow wave and spike activity, probably due to the depolarization as in intestinal muscle (Tamai and Prosser, 1966).

A notch, which appears early in the plateau phase of the intestinal slow wave has been observed by a number of investigators (Tamai and Prosser, 1966; Job, 1969; El-Sharkawy and Daniel, 1975a). El-Sharkawy and Daniel (El-Sharkawy and Daniel, 1975a) dispute the suggestion that this notching is a mechanical artifact or due to the electrogenic spread of a spike from a neighbouring cell, since it could be recorded after the membrane had been hyperpolarized by adrenaline and their experiments showed that it does not seem to be related

to electrotonic interaction between cells out of phase with the impaled cell. It is suggested that the slow wave may result from the operation of two processes occuring in sequence. The first process leads to an initial depolarization and the second causes a secondary depolarization (El-Sharkawy and Daniel, 1975a). Often the two processes occur sufficiently close in time so that the slow wave appears as a depolarizing phase, a plateau and a repolarizing phase (unnotched slow wave). Less frequently the two processes may be temporarily separated enough so that a notch appears on the plateau phase reflecting the turning off of the initial process (repolarizing phase of the notch) before the turning on of the second process becomes sufficient to maintain the initial depolarization. It is suggested that notching cannot be accounted for by any of the postulated ionic mechanisms for the slow waves since both the oscillating electrogenic pump hypothesis (Daniel, 1965; Liu, et al, 1969) and the oscillating sodium permeability hypothesis are obviously single "on and off" events (El-Sharkawy and Daniel, 1975a; and 1975c).

Temperature studies further substantiate the finding that slow waves are made up of two components. The rate of the initial depolarization had a Q_{10} of 1.56 where as the duration and rate of repolarization of the secondary depolarization had an appreciably higher temperature coefficient

(El-Sharkawy and Daniel, 1975a). Other investigators have also found the slow wave of the intestinal smooth muscle to have a higher Q_{10} (Daniel, et al, 1960; Job, 1969). It is possible that the rate of intercontrol potential depolarization and/or the level of membrane excitability are sensitive to cooling. It is interesting that neither the maximum resting membrane potential nor the slow wave amplitude exhibit appreciable temperature dependence (El-Sharkawy and Daniel, 1975a). This finding is inconsistent with the oscillating electrogenic sodium pump hypothesis for the slow wave generation, (Daniel, 1965; Liu, et al, 1969) from which it follows that at maximum polarization between the slow waves the pump contributes some 18mV (the height of the slow wave) to the membrane potential.

If an oscillating electrogenic sodium pump is responsible for the generation of slow wave activity in intestinal muscle three conditions must first be satisfied. First, the sodium pump in these muscles must be electrogenic. Second, the magnitude of the contribution of this pump to the membrane potential has to be at least equal to the amplitude of the slow wave, and thirdly, it must be shown that the pump oscillates spontaneously (El-Sharkawy and Daniel, 1975b). Various studies have shown that smooth muscle cells of the intestine, uterus, taenia coli etc. can be electrogenic (Taylor, et al, 1969; Liu, et al, 1969; Casteels, et al,

1971; Prosser, 1974; Connor and Prosser, 1974; El-Sharkawy and Daniel, 1975b). However, a theoretical analysis of the contribution of the pump to the E_M has shown it to be unlikely that the pump can, under normal conditions, provide more than a few millivolts to the membrane potential (El-Sharkawy and Daniel, 1975b and 1975c).

Using a modified version of the Goldman equation that does not assume either passive chloride distribution or equality of net passive and active sodium fluxes, El-Sharkawy and Daniel have calculated, on the basis of reasonable assumptions (about ionic permeabilities, coupling ratios etc.) that the contribution of the sodium electrogenic pump activity to the E_M may not exceed more than a few millivolts. They have concluded that such a small contribution is inconsistent with the hypothesis that the electrical slow waves (the amplitude of which is 18mV on the average) results from the turning on and off of the electrogenic sodium pump (El-Sharkawy and Daniel, 1975b). Another discrepancy with this hypothesis reported by these same investigators was the finding that slow wave activity in the presence of ouabain or in a sodium free solution was not accompanied by a membrane depolarization to the peak depolarization (Tamai and Prosser, 1966; Liu, et al, 1969). Furthermore, the time course of the increase and decrease of the slow wave amplitude upon starting and terminating, respectively, of intra-

cellular injection of sodium (Liu, 1969) is not what one would expect if the slow waves were due to oscillations of an electrogenic sodium pump (Bortoff, 1972). Also, the slow waves of the intestinal smooth muscle average about 18mV in amplitude that arise from a maximum "resting" potential of -55mV, but neither the slow wave amplitude nor the maximum resting potential exhibit the high temperature sensitivity that would be expected if the pump contributed 18mV to the maximum resting potential (El-Sharkawy and Daniel, 1975a and 1975c).

Inhibition of the sodium pump by a variety of procedures reversibly abolishes the slow wave activity but never depolarizes the membrane at the time the slow wave activity first disappears to the potential level at the peak depolarization of the slow wave as predicted by the oscillating pump hypothesis (El-Sharkawy and Daniel, 1975c).

Readmission of potassium to a sodium rich tissue causes a rapid hyperpolarization and the reappearance of slow waves of near normal frequency but much smaller amplitude. Since the hyperpolarization is sensitive to sodium pump inhibition, it reflects the stimulated activity of the electrogenic sodium pump (El-Sharkawy and Daniel, 1975b). Under these conditions of maximum stimulation of the sodium pump, one may expect either that the pump, because of it's maximal stimulation cannot be "turned off" and is, therefore,

on all the time (no oscillations) or that it could still somehow oscillate despite it's maximum stimulation. In the first case no slow waves should appear until enough sodium has been extruded and the pump activity slowed down, while in the latter case slow waves of much larger amplitude are expected to appear upon potassium admission and their amplitudes should decrease progressively concomitant with the return of the membrane potential to it's normal value. Neither of these predictions were observed by these investigators, instead, small amplitude slow waves appeared at the time of maximum hyperpolarization and their amplitude increased as the pump activity slowed down and the membrane hyperpolarization diminished (El-Sharkawy and Daniel, 1975c).

Replacement of chloride by less permeant ions, (isethionate, propionate) initially depolarizes the membrane and increases the amplitude of the secondary but not the initial depolarization. This is followed by a membrane hyperpolarization and a drastic shortening of the duration of the slow wave and a decrease in their frequency. This shortening is reported to be due to the elimination or "drastic reduction" of the secondary depolarization with only little effect on the initial depolarization (El-Sharkawy and Daniel, 1975c).

A substitution of a more permeant ion (nitrate) for Cl^- slightly depolarizes the membrane and increases the frequency of the slow waves without altering their configuration.

There is also intense spiking in the presence of nitrate (El-Sharkawy and Daniel, 1975c).

It is suggested that chloride may normally play a role in the genesis of the secondary depolarization of the slow wave. This secondary depolarization may result from a transient increase in chloride permeability which shortly follows or occurs concomitant with, but slower than, the transient increase in sodium permeability responsible for the initial depolarization. It may be that the increase in chloride permeability is a consequence of the initial depolarization caused by the preceding increase in sodium permeability, since it has been reported that in sodium free and sodium poor solutions no potential oscillations attributable to changes in chloride permeability could be recorded (El-Sharkawy and Daniel, 1975c). It is suggested that the initial transient effects of chloride replacement by less permeant ions (isethionate) i.e., the increase in amplitude of the secondary depolarization in conjunction with the slight membrane depolarization, might be due to an immediate shift in the chloride equilibrium potential toward a more positive value. As redistribution of chloride occurs, the secondary depolarization of the slow wave gradually disappears coincident with the membrane hyperpolarization between the slow waves (El-Sharkawy and Daniel, 1975c). With regard to the effects of nitrate, a more permeant anion, it is much

more difficult to predict the effects on either the maximum resting potential or the slow wave activity. Such effects would depend on whether nitrate utilizes the same channels as those used by Cl^- at rest and during slow wave depolarizations, or whether nitrate is actively pumped by an inwardly directed pump and on the relative extent to which the membrane potential is determined by the chloride equillibrium potential and the nitrate equillibrium potential. According to El-Sharkawy and Daniel, during the initial period of anion substitution, the chloride equilibrium rium potential should become more positive (decreased extracellular chloride) and a large negative nitrate equilibrium potential develops. Following this initial period, the depletion of intracellular chloride and the increase in intracellular nitrate would cause the chloride equillibrium potential to creep to more negative values and the nitrate equilibrium potential to go to more positive values, respectively. The changes in the resting potential would be a reflection of the magnitude of these two equilibrium potentials and the membrane permeability to both ions. Similarly, after anion distribution has reached a steady state, the contribution of nitrate to the secondary depolarization of the slow wave would depend on whether nitrate is passively or actively distributed across the membrane (El-Sharkawy and Daniel, 1975c).

According to El-Sharkawy and Daniel, the effects of a less permeant anion presents another inconsistency with the oscillating sodium electrogenic pump hypothesis. Chloride replacement, in guinea pig taenia coli, by less permeant anions increases the resting membrane resistance (Ohashi, 1970). It is suggested that a similar effect could take place in intestinal muscle, and that such an effect would have to cause an increase in the size of the slow waves if they were due to oscillations in the sodium electrogenic pump current (Rang and Ritchie, 1968; Taylor, et al, 1969). Such an increase in the slow wave was not however, observed (El-Sharkawy and Daniel, 1975c). Chloride currents involved in repolarization and in spikes have been observed in both skeletal and cardiac muscles (Dudel, et al, 1967; Peper and Trautwein, 1968; Hiraoka and Hiraoka, 1973; Fozzard and Hiraoka, 1973; Fukuda, 1974). The properties of these chloride currents in both of these tissues show similarities to the chloride currents underlying the secondary depolarizations of the intestinal slow wave (El-Sharkawy and Daniel, 1975c).

It is suggested that the intercontrol potentials (those slow depolarizations of up to 6mV which are observed between slow waves) may serve as the trigger for the initial event in the slow wave generation; i.e., the increase in sodium permeability (El-Sharkawy and Daniel, 1975a). The findings that the frequency of the slow waves could be decreased by

replacing chloride by less permeant and increased by more permeant, anions (El-Sharkawy and Daniel, 1975c), implicates chloride in the generation of intercontrol-potential depolarizations. These authors have suggested that it is possible that these depolarizations may result from an increase in chloride permeability distinct from the one that underlies the secondary depolarization of the slow wave. A common feature of the intercontrol-potential period and the secondary depolarization of the slow wave, besides their sensitivity to chloride substitution, is their sensitivity to temperature which may reflect the possibility that the membrane permeability to chloride may be regulated by cellular metabolism (El-Sharkawy and Daniel, 1975c).

Repolarization and Relaxation

Both the spike amplitude and the repolarization of the action potential in smooth muscle appears to be dependent on the membrane permeability to potassium, however, it has also been suggested that calcium binding may also bring about repolarization (Prosser, 1974). There seems to be little question, though, that the outward current is carried by potassium (Hutter, 1961; Prosser, 1974). Stimulation of an electrogenic sodium pump, which would drive more sodium out than potassium in, would also effectively hyperpolarize the muscle membrane. The hyperpolarization, which is observed to

go beyond the potassium equilibrium potential, after adding back potassium to a sodium loaded preparation is undoubtedly due to pump activation since the response can be abolished by metabolic inhibitors or a decrease in temperature (Somlyo and Somlyo, 1968a). It has been proposed that the fast outward current, observed in some smooth muscle preparations is a result of an initial fast calcium influx which transiently increases the local calcium concentration at the inner membrane surface and thereby momentarily increases potassium permeability resulting in the repolarization (Vassort, 1975; Gelles, 1976).

Hyperpolarization and relaxation should not be considered as synonymous events. Although hyperpolarization can result in muscle relaxation this does not appear to be the general mechanism involved in the relaxation process. Since the presence of Ca^{++} is required for the development of tension in smooth muscle it would seem reasonable to assume that removal of calcium be necessary for the muscle to relax. Active extrusion of calcium into the ECF would be an ideal method of muscle relaxation, however, there is little reason to believe that this is a general phenomenon. As a matter of fact, calcium efflux studies have shown that there is little, if any, calcium extrusion during smooth muscle relaxation (Goodford, 1965; Samlyo and Somlyo, 1968a; Katase and Tomita, 1972). It should be mentioned, however, that

there is reason to believe the existence of a sodium-calcium exchange mechanism in some smooth muscles which may be involved in the relaxation process (Blaustein, et al, 1977; Ma and Bose, 1977). Ma and Bose have presented evidence of a transmembrane Na^{+}-Ca^{++} exchange mechanism in taenia and clear cut evidence of transmembrane calcium movement during sodium mediated relaxation of sodium-free contracture (Ma and Bose, 1977). It would appear that an active process is involved in removing Ca^{++} from the myoplasm, at least in some muscles like aorta, but such a process centres around the sarcoplasmic reticulum (Van Breemen, et al, 1972). Just what contribution the sarcoplasmic reticulum plays in the relaxation of smooth muscle is difficult to calculate because of the small amount of sarcoplasmic reticulum and the short diffusion distance in smooth muscle. The small vesicles found on the inner surface of the muscle membrane are known to accumulate calcium in concentrations equal to that of the ECF. These vesicles may be involved in the muscle relaxation process by means of removing Ca^{++} from the myoplasm (Somlyo and Somlyo, 1968a; Prosser, 1974). It is possible that some drugs may stimulate intracellular active calcium uptake or, as has been suggested, increase intracellular concentrations of some form of soluble relaxing factor (Somlyo and Somlyo, 1968a). It should be mentioned, however, that definite evidence of an endogenous inhibitory agent that relaxes smooth muscle has yet to be obtained.

Excitation Contraction Coupling

Spikes in smooth muscle are not always accompanied by contraction. As a matter of fact, spike electrogenesis is not the only link in excitation-contraction coupling (E-C coupling). Graded depolarizations, rather than action potentials appear to be the normal electrical response in certain mammalian smooth muscles. There is little question, however, that the spontaneous action potentials in phasic muscles trigger the associated contraction. It is possible that the action potentials are calcium spikes which also provide the activator calcium for contraction (Somlyo and Somlyo, 1968a). The major alternative to this hypothesis is that the spikes could cause a translocation of calcium from the intracellular storage sites, similar to that which occurs in striated muscle; i.e. a trigger calcium mechanism. It is suggested that in the absence of a T-tubule system, the Ca^{++} spikes permit a more direct coupling than occurs in striated muscles. This direct connection between membrane spikes and contraction is the reason why smooth muscle fibers are of necessity small in diameter (Prosser, 1974; Bose, 1972; Uvelius and Johanson, 1974).

Depolarization of the smooth muscle membrane could transiently increase the membrane's permeability to Ca^{++} resulting in a sudden influx of Ca^{++} which could initiate the contractile response or be sufficient to stimulate the re-

lease of Ca^{++} from internal storage sites. It is proposed that membrane bound Ca^{++} regulates the permeability of the membrane to ionized calcium itself as well as other solutes. Depolarization removes some of the membrane bound Ca^{++} and the resultant permeability increase is transient in phasic and sustained in tonic smooth muscles (Somlyo and Somlyo, 1970).

The threshold for activation of contractile proteins is 10^{-7}M Ca^{++} and maximum activation occurs at 10^{-6}M (Van Breemen, et al, 1972). A very large calcium gradient across the membrane allows for rapid influx of Ca^{++} if the permeability is suddenly increased (Van Breemen, et al, 1972). This is a possible mechanism governing E-C coupling. Lanthanum, which displaces extracellular bound Ca^{++} and blocks Ca^{++} influx, could stabilize the membrane by binding to sites on the membrane which are involved in the transport of Ca^{++} and thus inhibit the E-C coupling process. Such methods result in complete inhibition of the potassium-induced contracture, while adding back Ca^{++} to a high K^{+} bathing solution leads to redevelopment of tension (Van Breemen, et al, 1972). This is suggested as being supportive evidence of a sodium-calcium exchange mechanism at the smooth muscle membrane.

Cardiac glycosides produce a depolarization and a contraction in many smooth muscles. The ouabain-induced contracture requires Ca^{++} in the bathing medium and is associated

with an increase in (Kasuya, 1969) Ca^{++} uptake (Briggs and Shibata, 1966; Reuter, et al, 1973; Blaustein, 1977). The depolarization produced by ouabain is presumably due to an inhibition of the Na^{+} pump (Casteels, 1966; Bose and Innes, 1973; Bose, 1975). There is reason to believe that the contraction seen during pump inhibition may be due to a Na^{+}-Ca^{++} exchange process. When pump activity is decreased by metabolic inhibitors, low temperature or removal of extracellular K^{+}, the cells accumulate Na^{+} (Casteels, 1966; Reuter, et al, 1973; Bose and Innes, 1973; Bose, 1975; Blaustein, 1977). Under such conditions Ca^{++} efflux is inhibited and Ca^{++} influx increases resulting in the contraction of the muscle (Maengwyn-Davies, 1968; Reuter, et al, 1973).

This Na^{+}-Ca^{++} exchange process can apparently be reversed such that Na^{+} moving down its electrochemical gradient can impart energy, produced in this process, for the active extrusion of Ca^{++} against its electrochemical gradient (Blaustein, et al, 1977). Ma and Bose have reported evidence of a Na^{+}-Ca^{++} exchange mechanism in the guinea pig taenia coli, and it has been suggested that such a mechanism, which causes Ca^{++} translocation from intracellular to extracellular sites, may be involved in the relaxation process in conjunction with intracellular sequestration of Ca^{++} by the sarcoplasmic reticulum and the mitochondria (Ma and

Bose, 1977). It is suggested that 3 sodium ions exchange for one calcium ion, and that such a coupled exchange could maintain a Ca^{++} concentration gradient across the membrane (Blaustein, 1977). Such a gradient could be maintained without the benefit of direct energy input from ATP. Although ATP does effect the kinetics of Na^{+}-Ca^{++} exchange in some tissues, it is not absolutely required and may not in fact power the exchange (Blaustein, 1977).

It has been shown that ouabain in relatively high concentrations can relax rather than contract smooth muscle (Bose, 1975). It would appear that very high levels of intracellular Na^{+} have an inhibitory effect on the contractile proteins that can override the contractile effect of the intracellular Ca^{++}, (R. Bose, personal communications). In guinea-pig taenia coli, ouabain produces a depolarization along with an increase in tension and frequency of spikes. This is followed by what has been termed a "secondary relaxation", believed to be due to non-electrical mechanisms (Bose, 1975). Ouabain is known to inhibit the Na^{+}-K^{+} pump, and it is this that is believed to cause the accumulation of intracellular Na^{+} (Van Breemen, et al, 1972; Blaustein, 1977; Bose, 1975).

The strongest evidence for an intracellular inhibitory role of Na^{+} comes from the experiments where the effects of Na^{+} replacement was compared with the results of substi-

tuting lithium for sodium. Sodium ions can be pumped out when the pump recovers and this may explain the gradual recovery from inhibition (Bose, 1975). Lithium, on the other hand, can enter the cell but cannot be pumped out by the Na^+-K^+ pump (Skou, 1965). Lithium can substitute for Na^+ in many instances,(Katase and Tomita, 1972) and it is possible that the inhibitory effect on taenia is no exception. Axelsson has shown that in the taenia coli, lithium substitution for Na^+ abolished mechanical responses without affecting electrical activity (Axelsson, 1961). It is possible that a similar effect of Na^+ is not normally seen because the Na^+-K^+ pump prevents intracellular accumulation of Na^+ (Bose, 1975).

It is interesting to note that sodium is also involved in promoting relaxation in the rat portal vein (Biamino and Johansson, 1970). Whether sodium induced relaxation is prominent in muscles where sarcolemma plays a greater role in relaxation than the sarcoplasmic reticulum remains to be established.

Pharmacomechanical Coupling

The discovery that depolarized smooth muscles can be contracted or relaxed by drugs (Evans, et al, 1958; Schild, 1967) has been known for some time. Action potentials in spike-generating smooth muscles can be abolished without

depolarization, while the contractile effect of the drug persists (Somlyo and Somlyo, 1968b). In the same study it was shown that the inequality of the maximal contractile response of polarized smooth muscle to different drugs persists in the depolarized state. It would appear that there is only a limited correlation between the electrical and mechanical responses evoked by drugs, (Cuthbert and Sutter, 1965; Somlyo and Somlyo, 1968b) such observations led Somlyo and Somlyo to describe the phenomenon of pharmacomechanical-coupling: the role of non-electrical processes in mediating drug induced contractions (Somlyo and Somlyo, 1968a and 1968b).

The effects could be due to drugs which can translocate Ca^{++} into the cytoplasm from a compartment not accessible to depolarization. Another possibility is that these drugs produce a longer and more persistent increase in membrane permeability to Ca^{++} than to K^{+}. If this were the case, Ca^{++} bound to the basement membrane and any contaminant Ca^{++} in a Ca^{++}-free solution (which could be as high as 10^{-5}M without a chelating agent present) could activate contraction (Somlyo and Somlyo, 1968a). The decrease in drug induced contractions in a Ca^{++}-free medium can be accelerated by a chelating agent (Edman and Schild, 1962; Hinke, 1965). This would imply that drug induced contractions do not preferentially use intracellular Ca^{++} (Somlyo and Somlyo, 1968a).

There is evidence to believe that drugs have a more pronounced effect on Ca^{++} permeability than does depolarization (Edman and Schild, 1962; Hinke, 1965). It is generally believed that there are two permeability barriers to Ca^{++} in series (Sparrow and Simmonds, 1965). The external barrier, containing high affinity sites for Ca^{++}, could concentrate the cation from the external medium and, if fully utilized, support maximal contractions in the presence of very small amounts (0.2mM) of total tissue Ca^{++}. It is suggested that the basement membrane probably has the required binding properties (Somlyo and Somlyo, 1968a).

The inner barrier in this model would be the membrane, whose permeability to Ca^{++} and other ions would be determined by the E_M, by labilizing and stabilizing drugs and by Ca^{++} itself. As long as the membrane stabilizing effect of Ca^{++} on the membrane permeability exists, influx from the external sites (basement membrane or free in the ECF) would be subject to "autoinhibition". In contrast, drugs which eliminate the stabilizing effect of Ca^{++}, either by removing it from the membrane or through some other mechanism, would permit maximal influx from the external sites into the myoplasm. This is the mechanism by which acetylcholine has been proposed to work (Hurwitz, 1965; Hurwitz, et al, 1967).

Although the exact cellular mechanism of action of acetylcholine has not yet been totally elucidated, it is

believed that it works by increasing membrane permeability to Ca^{++} and other ions. Studies on endplate permeabilities and intestinal smooth muscle show that there is a clear increase in permeability associated with acetylcholine (Born and Bulbring, 1956; Burn and Hobbs, 1959; Katz, 1966; Burgen and Spero, 1968).

It is suggested that the process involved in mediating the drug-induced contraction in depolarized muscle is an increase in membrane permeability to Ca^{++}, that certain membrane active agents act by displacing membrane bound Ca and that tonic contractions are associated with a maintained increase in Ca^{++} influx (Shanes, 1961; Briggs, 1962; Feinstein, 1964; Somlyo and Somlyo, 1968b).

It has been proposed that the membrane bound Ca^{++} regulates the permeability of the membrane to ionized calcium itself as well as to other solutes (Rothstein, 1968; Somlyo and Somlyo, 1968a). Depolarization removes some membrane bound Ca^{++}, the resultant permeability increase is transient in phasic and sustained in tonic smooth muscles (Somlyo and Somlyo, 1968a). Some drugs may release membrane bound Ca^{++} and exert an even greater stabilizing action than Ca^{++} itself (Feinstein, 1964). Other agents like acetylcholine, noradrenaline, and other active amines or peptides, may eliminate the stabilizing effect of Ca^{++} with or without removing it from the bound sites (Hurwitz, 1965; Hurwitz, et al, 1967;

Somlyo and Somlyo, 1968a). Activator Ca^{++} under these conditions may arise in varying quantities from the membrane itself, from binding sites within the ground substance or from the extracellular fluid (Somlyo and Somlyo, 1968a). The unequal efficiency of pharmacomechanical-coupling obtained with different drugs has been attributed to an unequal ability of the drugs to overcome the stabilizing action of Ca^{++} and thereby produce a sustained increase in membrane permeability (Somlyo and Somlyo, 1968b).

According to Somlyo and Somlyo the major assumptions inherent in the model of E-C coupling, based on permeability changes in the membrane, are: 1) excitatory drugs primarily act by a common mechanism of increasing the ion permeability of the smooth muscle membrane; 2) the quantitatively most important, though perhaps not the only source of activator calcium mediating drug induced contraction, is a site external to the membrane and, 3) the primary determinant of contraction (or relaxation) is the rise (or fall) of intracellular free Ca^{++} ion concentrations. There is at least a semiquantitative correlation between the maximum contraction and the maximum permeability change produced by a given drug. An increase in calcium permeability is produced by several and perhaps all excitatory drugs and this effect appears to be the primary mechanism of near-maximum and maximum contractions. The unequal maximum contractile effects of dif-

ferent drugs are believed to be an expression of the unequal maximal increase in calcium permeability produced by them (Somlyo and Somlyo, 1970). The assumption that excitatory drugs act through increasing the calcium permeability implies that the major source of activator Ca^{++} is extracellular, i.e. the basement membrane, ground substance or the extracellular fluid. There is also the possibility that drugs may increase the membrane permeability by displacing stabilizing calcium (Somlyo and Somlyo, 1968b).

In phasic striated muscles the existence of a stable Ca^{++} pool is well known and is located in the sarcoplasmic reticulum. It is functionally demonstrated by the large caffine contractures that can be produced in severely calcium depleted striated muscles in the presence of high concentrations of EDTA (Blanchi, 1961; Somlyo, 1965). Although there is much less sarcoplasmic reticulum in smooth muscle, in certain types of smooth muscle large numbers of vesicles have been observed, which as previously mentioned, may serve a similar function (Somlyo and Somlyo, 1968a, 1976). Unlike skeletal muscle, smooth muscle does not respond to drugs after severe calcium depletion, however, smooth muscle does contract with caffeine if the muscle is bathed in normal Ca^{++} medium because caffine, in addition to translocating calcium from the sarcoplasmic reticulum also increases the membrane permeability to calcium in skeletal muscle (Blinks, et al,

1972). It's effect on smooth muscle could be produced by the latter response.

Bozler suggested that in Ca^{++} free medium the contractile effects of acetylcholine are due to an action of the drug on the sarcoplasmic reticulum of the smooth muscle. Although there is no evidence to suggest that E-C coupling in smooth muscle utilizes intracellular calcium sites, it would seem that such sites represent a plausible mechanism for smooth muscle contraction.

It is generally believed that drugs increase calcium permeability and this change in calcium permeability could trigger the release of Ca^{++} from the sarcoplasmic reticulum. The sarcoplasmic reticulum in smooth muscle, however, is sparse which suggests that there is not enough stored intracellular calcium for the activation of a sizable contraction. Although relaxation in some smooth muscles may be due to active extrusion of calcium there is no reason to believe that this is a general phenomena. As a matter of fact, calcium efflux studies have shown that there is little if any calcium extrusion during smooth muscle relaxation with most agents, however, it should be mentioned that more recent studies by Kroeger and Chow have demonstrated calcium extrusion during relaxation under such circumstances in the rat uterus (Kroeger, et al, 1975; Chow and Bose, 1978). It is possible that some drugs may stimulate intracellular active

calcium uptake or increase intracellular concentrations of some form of soluble relaxing factor.

Somlyo suggests three possible mechanisms for relaxation: 1) Relaxation could result from a decrease in calcium influx into depolarized smooth muscle, e.g. local anesthetics. Some agents acting on passive membrane properties might not decrease the resting ion permeability of smooth muscle but inhibit drug stimulated ion fluxes, e.g. ethanol, which inhibits the cholinergically stimulated transmembrane potassium fluxes in intestinal smooth muscle, 2) stimulation of active pumping of cytoplasmic calcium into extracellular compartments or an intracellular storage site, 3) relaxing agents that do not decrease stimulated calcium influx into smooth muscle and have a relaxing effect that cannot be accounted for by extracellular calcium pumping.

Anoxia usually produces relaxation as well as an indirect effect on sympathetic discharge. The relaxation seen with anoxia may be due to a release of local metabolites (Somlyo and Somlyo, 1970; Folkow and Neil, 1971). Large decreases in P_{02} generally produce a modest decrease in resting tone of vascular smooth muscle, while acute anoxia may lead to a modest decrease in the contractile response to drugs, but it is generally agreed that glycolysis can support contraction for several hours (Furchott, 1955; Carrier, et al, 1964; Shibata and Briggs, 1967).

Denervation and Rhythmicity

Denervation of muscle, skeletal or smooth, results in profound changes in the effector cells. The results of numerous studies over the years led Cannon and Rosenblueth to formulate the Law of Denervation. The law deals primarily with the supersensitivity of the effector tissue(s) that accompanies chronic denervation, (Cannon and Rosenblueth, 1949) the causal mechanism of which has yet to be found. Of the many interesting phenomenon that take place in the supersensitive tissue, one that is of particular interest is the spontaneous or induced rhythmic behavior of the denervated muscle.

Reference to such rhythmic behavior in airway smooth muscle was made as early as 1903 when Dixon reported the induction of oscillatory mechanical activity in the vagotomized dog trachea and bronchi by pilocarpine and muscarine (Dixon and Brodie, 1903). Many of the early investigators observed that the lack of motor innervation initiated or enhanced the peristaltic activity in tracheal smooth muscle (Dixon and Brodie, 1903; Reinberg, 1925; Macklin, 1929; Ellis, 1936; Wich, 1952). Of interest too, is the early finding that mechanical stimulation of the tracheal smooth muscle could often induce phasic contractions. It was proposed that foreign matter or pathological changes in the muscle

could be responsible for the rhythmic activity (Jackson, 1917; Ellis, 1936; Widdicombe, 1963; Gold, 1972). It may not be surprising, therefore, to find that in in vivo experiments carried out by Loofbourrow in 1957, tracheal oscillations were noticed. These experiments involved recording intraluminal tracheal pressure changes upon stimulation with various agonists. Loofbourrow observed the tracheal phasic contractions and reported that cholinergic agonists and asphyxia enhanced the rhythmicity, while hyperventilation and atropine reduced or abolished it (Loofbourrow, et al, 1957).

Chemically Induced Rhythmicity

Tetraethylammonium (TEA)

It is generally believed that under normal physiological conditions tracheal smooth muscle does not generate AP's or exhibit mechanical oscillations (Kirkpatrick, 1975; Kroeger and Stephens, 1975; Kamburoff, 1976; Suzuki, et al, 1976; Bose and Bose, 1977). Such properties have resulted in tracheal muscles being classified as multiunit muscle, the characteristic properties of which have been previously mentioned.

The reason stated for trachea as being a quiescent nonspiking muscle is said to be due to its membrane rectifying properties (Kirkpatrick, 1975; Stephens, 1975; Kroeger and Stephens, 1975; Suzuki, et al, 1976). Rectification could

be described as the inability of the membrane to offer equal resistance to the flow of current in opposite directions, across the membrane, in response to a given stimulus. That is to say that the amplitude of hyperpolarization produced by an anodal (outward) current is greater than the amplitude of depolarization produced by the same amount of current passed inward, (i.e. cathodal) or the resistance to outward current is much less (or the conductance is much greater) than to inward current flow (Kirkpatrick, 1975).

Because of this property it is much more difficult to depolarize tracheal muscle to threshold and therefore, to cause spike generation. This situation can be remedied with TEA, which reduces G_K and reduces or abolishes membrane rectification of tracheal and other multiunit smooth muscles (Mekata, 1971; Kirkpatrick, 1975; Kroeger and Stephens, 1975; Suzuki, et al, 1976). The result is a decrease in the E_M (i.e. a depolarization) of about 10mV (Stanfield, 1970; Kirkpatrick, 1975). Since this depolarization persists in the presence of atropine, it is unlikely that it is due to a cholinergic action of TEA (Kroeger and Stephens, 1975, Suzuki, et al, 1976). TEA has two basic actions: the first is a blockade of the steady state potassium channel (Tasaki and Hagiwara, 1957), (a time and voltage dependent block) and the second is a reduction in the resting G_K, which produces the decrease in the E_M and an increase in the membrane

resistance (Stanfield, 1970). The primary action of TEA on excitable membranes is, however, its ability to reduce G_K and prevent G_K from rising during the AP (Tasaki and Hagiwara, 1957; Mekata, 1971; Kroeger and Stephens, 1975; Stephens, et al, 1975; Kirkpatrick, 1975; Suzuki, et al, 1976). TEA also makes the E_M unstable, so that there is a tendancy for spontaneous spikes to be discharged, or for the spontaneous release of neuromuscular transmitter to increase (Tasaki and Hagiwara, 1957; Bergmann, et al, 1968; Kirkpatrick, 1975). The membrane permeability to K^+ is normally too great in quiescent muscles to permit a regenerative increase in permeability to Na^+. TEA, by inhibiting permeability to K^+ might, therefore, relieve this inhibitory influence and allow spontaneous phasic electrical spiking. The rhythmic behavior seen with TEA may possibly be due to a decrease in permeability to K^+ and a secondary "weak activation" of permeability to Na^+ with depolarization (Kroeger and Stephens, 1975). There is also evidence that TEA displaces membrane bound Ca^{++} and increases G_{Ca} by modifying G_{Na} and directly increasing excitability (Beaulieu and Frank, 1967; Suzuki, et al, 1976). It is suggested that the depolarized activated Ca^{++} and or Na^+ current(s) produced in tracheal smooth muscle are masked by a high resting G_K. When this blocked by TEA, Ca^{++}-dependant action potentials are seen (Simmons, 1976). TEA tends to increase the ampli-

tude and maximum rate of rise of the action potential in electrically excitable smooth muscle (Ito and Kuriyama, 1970). Kroeger finds that in the multiunit dog trachea, TEA results in a decremental action potential with low amplitude, low maximum rate of rise and a low conduction velocity (Kroeger and Stephens, 1975). However, one must take into consideration that this preparation is normally quiescent.

The electrical wave form in trachealis muscle is described as consisting of a rapid spike potential often followed by a long plateau of depolarization and rhythmic mechanical contractions are associated with the electrical spontaneous activity (Casteels and Kuriyama, 1965; Kirkpatrick, 1975, Kroeger and Stephens, 1975; Bose and Bose, 1977). The control potentials or slow waves of intestinal smooth muscle appear to have similar morphological characteristics, (Papasova, et al, 1968; Job, 1969; El-Sharkawy and Daniel, 1975a, 1975b, 1975c). It may be that the plateau seen in the action potential of the tracheal smooth muscle could be due to changes in chloride conductance as suggested by El-Sharkawy and Daniel (1975b). It is pointed out however, that a change in chloride conductance is unlikely with TEA, at least in rat uterine smooth muscle, as a decrease in chloride condustance would account for the greater membrane resistance but this would tend to hyperpolarize the membrane since the

chloride equillibrium potential is less negative than the resting membrane potential (Kroeger and Stephens, 1975; Kirkpatrick, 1975).

The inward current of the smooth muscle action potential is primarily due to Ca^{++} influx which may be potentiated or modified by a Na^{+} current, while the outward current is carried by potassium (Kao and McCullough, 1975), and is markedly reduced by TEA (Mekata, 1971; Inomata and Kao, 1976). This outward current has been differentiated into two components, a fast early outward current that is blocked by TEA, and a delayed outward current that causes delayed rectification (Kao, 1971; Vassort, 1975). The fast outward current appears to account for the low amplitude of the action potential and it's fast repolarization. This is supported by the finding that the amplitude and duration of the action potential are increased by TEA (Mekata, 1971; Vassort, 1975; Inomata and Kao, 1976). The plateau phase in the smooth muscle action potential in the presence of TEA is, therefore, probably due to a decrease in potassium conductance which would slow repolarization (Hille, 1967; Koppenhofer, 1967; Kirkpatrick, 1975). It should be mentioned too, that this plateau has also been observed in ureter smooth muscle preparations and has been atributed to a slow inward Na^{+} current (Kobayashi, 1965; Kuriyama, et al, 1967; Kuriyama and Tomita, 1970).

Under normal physiological conditions tracheal smooth muscle responds with a tonic contraction and a graded depolarization that is proportional to the strength of the stimulus. Under such conditions a myogenic contraction cannot be produced in response to quick stretch (Kroeger and Stephens, 1975; Stephens, et al, 1975). Experimental conditions such as hypoxia and acidosis or the presence of barium, acetylcholine or high external potassium were also unable to elicit a myogenic response (Stephens, et al, 1975). It has been reported, however, that trachealis muscle undergoes a conversion from a multiunit to a single unit muscle in the presence of TEA which allows a myogenic response to take place (Stephens, et al, 1975; Kroeger and Stephens, 1975). It is suggested that the myogenic response observed in the presence of TEA is produced by stretch-induced depolarization which initiates action potentials from normally latent pacemakers whose inward current is carried by calcium ions (Stephens, et al, 1975; Kroeger and Stephens, 1975).

The myogenic response to stretch, observed in the presence of TEA containing medium, varies in amplitude with the external calcium concentration, and it appears that the ability of the muscle to produce this response is absolutely dependant on this external calcium and is completely blocked by D-600 (Stephens, et al, 1975; Kroeger and Stephens, 1975).

Histamine (10^{-6}g/ml) has been reported to produce

rhythmic contractions in bovine tracheal smooth muscle similar to that of TEA. The electrical slow waves observed were in phase with the oscillations in tension. Acetylcholine, on the other hand, resulted in smooth maintained contractions of the muscle and no spikes were observed (Kirkpatrick, 1975). In a Ca^{++}-free bathing solution containing EGTA, the histamine response is reduced as is that of acetylcholine, however, the latter does not appear to be affected as much. Similar results have been reported in the presence of lanthanum (La^{+++}), (Kirkpatrick, 1975; Suzuki, et al, 1976). Unlike the response observed with histamine and acetylcholine, the tissue is unresponsive to TEA in a Ca^{++}-free EGTA bathing solution or if the muscle is exposed to La^{+++}. The effects of TEA are also blocked by agents which decrease membrane permeability, such as Mg^{++}. However, the normal TEA response does not return when calcium is added back (Mayer, et al, 1972; Stephens, et al, 1975; Kroeger and Stephens, 1975; Kirkpatrick, 1975; Suzuki, et al, 1976). It is suggested that calcium comes from two sites, the ECF and that sequestered in intracellular sites. Action potentials or slow waves might increase calcium permeability allowing Ca^{++} from the ECF to enter and activate contractile proteins. In a Ca^{++}-free solution or in the presence of La^{+++}, there is not enough extracellular calcium available for TEA to produce a contraction. The sequestered stores of calcium within

the cell would not be available to agents which act solely by producing permeability changes in the membrane, but might possibly be available to substances which could react with specific receptors which determine the release of sequestered Ca^{++}. In this way both histamine and acetylcholine would be able to elicit a response in the absence of extracellular Ca^{++} or in the presence of La^{+++}. Histamine could produce a considerable part of its effect by increasing permeability to Ca^{++}, such that Ca^{++} could enter with each slow wave, (Kirkpatrick, 1975). It may be that smooth muscle contraction involves just a redistribution of intracellular Ca^{++} stores rather than an influx and the effect of Ca^{++} deprivation might be to deplete these intracellular stores. A more prolonged washing in a Ca^{++}-free EGTA solution causes a further decrease in the contractile response to acetylcholine and histamine suggesting still further depletion of the stores, (Kirkpatrick, 1975; Suzuki, et al, 1976). Kirkpatrick has proposed that histamine and acetycholine have access to mechanisms of E-C coupling which are not available to TEA and which are somewhat resistant to Ca^{++} deprivation and La^{+++} (Kirkpatrick, 1975).

Local Anesthetics: Cocaine; Reserpine; 6-Hydroxydopamine

It is interesting to note that rat and guinea pig vas deferens, which are normally quiescent can also be made

to contract rhythmically when exposed to cocaine, procaine, lignocaine, piperoxan, thymoxamine or mepyramine (Cliff, 1968). Although other investigators have observed such rhythmic behavior of normally quiescent muscles in the presence of local anesthetics this behavior is not completely associated with these agents (Cliff, 1968; Birmingham, 1970; Bose and Innes, 1974). Innervation of the muscle is not required for such activity and it has been reported that the oscillations become even more pronounced in the denervated preparation (Cliff, 1968).

Cocaine, which impairs neural uptake of catecholamines, is reported to change multiunit cat splenic capsular smooth muscle to a single unit type. After such treatment quick stretch results in a myogenic response and rhythmic contractions are observed on administration of noradrenaline. Tetrodotoxin was found not to abolish rhythmic oscillations due to the combined effects of cocaine and noradrenaline, suggesting that the oscillations were not neurogenic but myogenic in origin (Bose and Innes, 1974).

Reserpine, which depletes stores of catecholamines and effectively causes denervation is also reported to cause rhythmic activity in both the rat and guinea pig vas deferens and the nictitating membrane of the cat (Rothballer and Sharpless, 1961; Green and Fleming, 1967; Lee, 1975; Fleming, et al, 1975). Such forms of chemical denervation are re-

ported to be accompanied by a decrease in the tissue calcium stores as well as a decrease in the sodium and potassium content of the muscle. These changes have been suggested as a possible mechanism responsible for the slight depolarization seen in the denervated muscles. It would not seem unreasonable to assume that with the possible lower membrane calcium levels, the membrane permeability to sodium and potassium may increase. This would allow these two ions, especially sodium, to come to a new equillibrium level. A partial depolarization of the smooth muscle membrane and a lower threshold would then exist for the response of the muscle to various agonists (Hudgins and Harris, 1970; Fleming, et al, 1975; Carrier, 1975). Spontaneous activity in reserpine treated preparations are also uneffected by TTX, phentolamine and atropine (Lee, 1975). Chemical denervation by 6-hydroxydopamine, which destroys adrenergic nerve endings within an hour after administration is also reported to result in rhythmic activity of splenic smooth muscle (Tranzer and Thoenen, 1968; Thoenen and Tranzer, 1973; Bose and Innes, 1974). Epineural injection of colchicine which in an appropriate dose produces many of the effects of denervation without block of motor activity or muscle fiber contraction is also reported to result in fibrillatory activity of skeletal muscles (Hofmann and Thesleff, 1972; Camerino and Bryant, 1976). Similar results were observed as those seen in chemical

denervation of smooth muscles, i.e. a 10mV depolarization rhythmic activity and TTX resistant action potentials. Treatment with this agent also results in a significant increase in membrane resistance and a decrease in chloride conductance. It was suggested that the low chloride conductance was responsible for the increase in membrane resistance, the abnormal excitability and the rhythmic activity of the muscle and that the mammalian skeletal muscle fiber requires a high chloride conductance to maintain stability of it's excitable surface membrane to prevent abnormal repetitive firing or sensitivity to depolarization (Camerino and Bryant, 1976).

Metabolic Depletion

Bose observed mechanical oscillations in canine trachealis smooth muscle while studying the biochemical changes associated with metabolic depletion. It is reported that under normal conditions canine tracheal smooth muscle responds with a graded contraction to acetylcholine, histamine and elevated potassium, with the removal of glucose from the bathing medium, however, acetylcholine and histamine stimulation results in phasic contractions that become regular with time, while elevated potassium continues to give a maintained tonic contraction. Not only does the tracheal muscle contract rhythmically but under these same condi-

tions it exhibits a myogenic response to quick stretch (Bose and Bose, 1977).

In skeletal muscle the resynthesis of ATP from ADP and creatine phosphate, catalyzed by phosphporyl creatine transferase (Lohmann Reaction) prevents the net decrease of ATP during contraction, furthermore the small decrease in creatine phosphate is obscured by the normal high levels found in skeletal muscle. Smooth muscle, however, contains very little creatine phosphate and ATP breakdown can, therefore, be shown without interference from creatine phosphate (Somlyo and Somlyo, 1970).

Work done during an isometric contraction is greater in smooth muscle than in skeletal muscle because of the much larger series elastic component of smooth muscle, 15 to 20% compared with 3 to 4% in skeletal muscle (Somlyo and Somlyo 1970). In skeletal muscle the creatine phosphate, 10 to 30 μmoles/g is the immediate source of rephosphorylation of ADP. In smooth muscle the concentration of creatine phosphate is much lower, about 0.2 to 0.8 μmoles/g and it's contribution to the resynthesis of ATP is much smaller than in skeletal muscle. In smooth muscle much of the ATP is synthesized from glycolysis and oxidative-phosphorylation during contraction which is undoubtedly facilitated by smooth muscles slower speed of contraction.

Addition of beta-hydroxybutrate, a substance which would lead to the production of ATP only through the mitochondria, abolished rhythmicity. When glucose was added back to the substrate depleted muscle, a rapid increase in tension and inhibition of the phasic mechanical contraction was seen (Bose and Bose, 1977).

Because ATP in canine trachealis muscle must come from either glycolysis or oxidative-phosphorylation and since tonic contractions could not be maintained by anerobic glycolysis alone it was suggested that mitochondrial ATP was required to produce the tonic contractile response of acetylcholine and histamine. Both ATP and creatine phosphate were found to be significantly decreased in the substrate depleted preparation. In the presence of carbachol the reduction in ATP concentration was even greater. It was suggested that since potassium produced a maintained contraction during metabolic inhibition, it was possible that ATP concentrations were not fluctuating in the area of the contractile proteins and that it may be possible that cytoplasmic calcium levels caused rhythmicity when carbachol was given to a tracheal strip in glucose free medium. Bursts of spikes were associated with each mechanical oscillation and there were oscillations in the E_M associated with slight oscillation in the tonic contraction long before the rhythmicity appeared. The membrane depolarized 7-15mV before the oscil-

lations in E_M were seen. Studies with the sucrose gap technique showed similar results and the E_M decreased when glucose was added back to the medium. It is reported that D-600 in relatively low concentrations preferentially abolished the phasic contractions without affecting much the tonic component of the carbachol response. Higher concentrations of D-600 completely relaxed the muscle suggesting that alterations in calcium movement had occurred under conditions of ATP depletion.

Removal of extracellular calcium had similar effects as D-600, showing that rhythmic muscles had a greater sensitivity to calcium removal. Increasing calcium concentration increased the amplitude of the contractions. It has been suggested that the decrease in ATP may be at least partly responsible for the production of rhythmicity. It is suggested too that tonic contraction is sustained only when ATP production, by mitochondria, is available since glycolysis alone could not maintain a tonic contraction.

A decrease in ATP levels could alter the membrane characteristics by altering the membrane bound calcium thereby affecting G_{Na} and directly increasing excitability. Transmembrane flux of calcium was found to be important when the muscle was depolarized with potassium or when metabolically depleted. Similarly canine trachealis muscle which is metabolically depleted appears to depend more on external cal-

cium. It is suggested that some internal store of calcium which is necessary for maintaining a tonic contraction with carbachol is depleted and unavailable for contraction.

It has been suggested that pacemakers may be masked under normal conditions and unmasked with glucose deprivation (Bose and Bose, 1977). In a similar manner it is possible that stable membranes may be labilized to produce action potentials.

It should be mentioned too, that Souhrada, et al,(1976) also observed what they term "spontaneous" rhythmic behavior in tracheal smooth muscle. Such spontaneous activity is seen, however, only after lengthy equilibration periods which will ultimately impair metabolic function.

Denervation

Since Reisseisen's anatomical description of airway smooth muscle in 1822 and Varnier's observations in 1779 of it's powers of contraction, the control of airway smooth muscle has been frequently investigated. Although our knowledge and understanding of smooth muscle has grown tremendously since those early years, the state of smooth muscle physiology still lags far behind that of skeletal and cardiac muscles. This is understandable when one considers the vast functional diversity of this muscle throughout the

mammalian system.

An attempt to cover the physiological role of smooth muscle, at the tissue and cellular levels, is beyond the scope of this thesis, however, it is important that some fundamental concepts be established in order to form a foundation for further discussion.

Some of the early investigators, that have been previously mentioned, have made reference to denervation and its effect on airway smooth muscle. Some, like Ellis, found that the peristaltic activity was intensified by bilateral vagotomy, (Ellis, 1936) while others have made reference to pathological processes involving airway smooth muscle innervation and the resultant rhythmicity of the smooth muscle which accompanies it, (Reinberg, 1925; Macklin, 1929; Loofbourrow, et al, 1957). More recently, however, numerous investigators have observed that inhibition of motor control of various muscles (smooth and striated muscle) can result in either spontaneously active rhythmic behavior or phasic responses that can be induced by a variety of stimuli.

Rhythmic activity has been reported in numerous denervated skeletal muscle preparations, mouse diaphragm, rat gastrocnemius, soleus and extensor digitorum longus, goat gastrocnemius and many others (Bowman and Raper, 1967; Smith and Thesleff, 1976; Camerino and Bryant, 1976a; Bryant and Camerino, 1976b; Lorkovic and Tomanek, 1977).

Many investigators have reported similar observations but the actual mechanism(s) linking denervation with rhythmicity has yet to be found. A decrease in the E_M of approximately 10mV appears to be a characteristic phenomenon of the chronically denervated skeletal muscle. This depolarization of the muscle fiber has been reported anywhere from 3 to 18 days after denervation, (Smith and Thesleff, 1976; Camerino and Bryant, 1976a; Bryant and Camerino, 1976b; Lorkovie and Tomanek, 1977). Accompanying this depolarization are signs of rhythmic behavior, tetrodotoxin resistant action potential and a significant increase in the membrane resistance, (Bowman and Raper, 1967; Smith and Thesleff, 1976; Camerino and Bryant, 1976a; Bryant and Camerino, 1976b; Lorkovie and Tomanek, 1977). The membrane of normal mammalian skeletal muscle is highly permeable to chloride ions and the low membrane resistance values recorded in such muscles are probably due to their high chloride conductance (Lorkovic and Tomanek, 1977). After chronic denervation, however, the chloride conductance falls dramatically to near zero while the potassium conductance is reported to be unchanged or increased, and it is this which is suggested as being responsible for the increase in membrane resistance, and significant increase in membrane capacitance (Camerino and Bryant, 1976b; Lorkovic and Tomanek, 1977). This decrease in chloride conductance is also believed to be responsible for the

abnormal excitability and rhythmic activity of the muscle. These denervated muscles also show a certain degree of muscle tone which appears to be dependant on the frequency of the spontaneous contractions (Bowman and Raper, 1967). The mammalian skeletal muscle fiber apparently requires a high chloride conductance in order to maintain stability of its excitable membrane to prevent abnormal repetitive firing or sensitivity to depolarization (Camerino and Bryant, 1976a; Bryant and Camerino, 1976b). It has been suggested that in the experimentally myotonic or denervated muscle, both of which have been found to have low chloride conductance, influences from the motor nerves (i.e. action potentials or "trophic factors") are absent or somehow prevented from maintaining the normally large chloride permeability of the mammalian muscle fiber. As a result, there is an increase in the excitability and a tendency to fire repetitively in these muscles (Bryant and Camerino, 1976b).

Smith and Bowman et al, have found that catecholamines enhance the spontaneous activity in the denervated mouse diaphragm as well as the rabbit and cat tibialis and soleus muscles. The enhanced part of the activity, due to the amines, is unaltered by alpha blocking agents but is abolished by beta receptor blocking drugs in doses smaller than any that might depress the background rhythmicity (Bowman and Raper, 1967; Smith and Thesleff, 1976). Ouabain and a

potassium free bathing solution were observed to reversibly block the rhythmicity. It is suggested that catecholamines and ouabain could affect this activity either directly through an action on the membrane excitability or indirectly via the sodium-potassium pump, and that the spontaneous action potentials observed in these preparations result from a regenerative change in sodium conductance (Smith and Thesleff, 1976).

It is not known whether the changes in ion conductance that follows denervation are caused by the removal of a trophic effect of the nerve or by the lack of electrical activity, mechanical activity or both. Since artificial stimulation of denervated muscle reverses most of the changes in the electrical properties caused by denervation, it is likely that ionic conductance changes would also be reversed by muscle activity. It has been suggested that activity is involved in the maintenance of normal ionic conductance (Lorkovic and Tomanek, 1977).

Spontaneous phasic electrical and mechanical oscillations are commonly observed in many smooth muscles, particularly those classified as single unit, e.g. intestinal smooth muscle, uterus, mesenteric vein, pulmonary artery and many others (Somlyo and Somlyo, 1968a; Somlyo, et al, 1969; Prosser, 1974; El-Sharkawy and Daniel, 1975b). Such activity is not, however, a common observance in those muscles classi-

fied as multiunit, e.g. rat and guinea pig vas deferens, canine and bovine trachea and rabbit carotid artery, (Mekata, 1971; Kirkpatrick, 1975; Stephens, et al, 1975; Lee, 1975). In some multiunit smooth muscles, as in striated muscle, loss of motor innervation often results in what has been termed a conversion to a phasic type, (Bose and Innes, 1974; Stephens, et al, 1975; Kroeger and Stephens, 1975; Bose and Bose, 1977). The response of these smooth muscles to denervation in many ways parallels those observed in the chronically denervated skeletal muscles. Chronic postganglionic denervation or decentralization of either rat or guinea pig vas deferens or the cat nictitating membrane results in a slight depolarization of about 10mV which is often accompanied by spontaneous rhythmic contractions or rhythmicity that can be induced by various agents which are not prevented by TTX, phentolamine or atropine, (Langer, 1965; Kasuya, 1969; Birmingham, 1970; Lee, 1975; Fleming, et al, 1975; Carrier, 1975). As in skeletal muscle too, the mechanism linking denervation with this oscillatory behavior is still obscure. A release of transmitter agents from the degenerating nerve endings has been proposed, (Langer, 1965) however, this seems an unlikely possibility since both alpha and beta receptor blocking agents have been shown to be ineffective in abolishing the rhythmic response in adrenergically innervated muscles (Lee, 1975). The rhythmic activity

is generally believed to be due to some intrinsic property of the muscle itself, i.e. myogenic rather than neurogenic (Lee, 1975; Fleming, et al, 1975a).

A number of investigators have discussed the possibility that the rhythmic behavior of the muscle may be directly related to the decrease in the membrane potential seen after denervation, (Hudgins and Harris, 1970; Fleming, et al, 1975a; 1975b; Carrier, 1975; Westfall, et al, 1975). Chronic denervation of smooth muscle or cardiac muscle has been shown to cause an alteration in the binding of calcium to the cell membrane (Fleming, et al, 1975a, 1975b; Hudgins and Harris, 1970). Since calcium is involved in membrane stability, and since potassium conductance and, therefore, the resting membrane potential appear to be directly related to the amount of calcium bound, this could result in a reduction in membrane stability which could, itself or through stimulation by certain agents, alter the membrane permeability, which could result in the observed depolarization and rhythmic activity (Hudgins and Harris, 1970; Fleming, et al, 1975b). Such a depolarization could also account for the supersensitivity observed in these denervated muscles since the depolarization would bring the membrane potential closer to threshold so that less agonist or stimulus would be required to produce a response, (Lee, 1975; Fleming, et al, 1975a and 1975b; Carrier, 1975).

Some biochemical changes have been observed in the denervated smooth muscle preparation and Westfall has reported a substantial decrease in tissue ATP levels one day after denervation, however, the levels rose significantly on succeeding days. He has suggested that the decrease in ATP concentrations may be one of the initial events that occurs after denervation of smooth muscle (Westfall, et al, 1975).

There are reports that the sparsely innervated smooth muscles (single unit) have many nexal regions whereas densely innervated tissue (multiunit) have relatively few nexuses (Somlyo, et al, 1974). It has been suggested that the decrease in the density of innervation, such as that which occurs with denervation, results in an increase in the incidence of nexal regions. Following denervation, exposure to an agonist might result in a more co-ordinated contraction (Westfall, et al, 1972; Bose and Innes, 1974). An improvement in cell-to-cell communication may be more significant in the case of the response to bulky molecules which diffuse relatively slowly through the tissue and which does not act through a specific drug-receptor mechanism (Westfall, et al, 1972). This may be the reason why Bose observed phasic contractions in metabolically depleted canine trachealis in response to acetylcholine and histamine, while elevated potassium continues to give a maintained tonic contraction (Bose and Bose, 1972).

Chapter II

FORMULATION OF THE PROBLEM

The classification of smooth muscle into tonically active multiunit and phasically active single unit muscles has given rise to a series of physiological and anatomical parameters characteristic of each type (Bozler, 1948). In recent years evidence has emerged to suggest that some smooth muscles often exhibit shades of multiunit and single unit properties, or a characteristic multiunit muscle may behave like that of a phasically active single unit muscle when the appropriate treatment and/or stimulus is applied. Such tissues could be classified as intermediate muscles. The canine trachealis smooth muscle is such a tissue having multiunit features under normal conditions but showing single unit characteristics after undergoing various forms of treatment. Numerous examples of this conversion have been recorded in both skeletal and smooth muscle preparations (Bose and Innes, 1974; Fleming, et al, 1975; Camerino and Bryant, 1976).

Several studies (Bose and Innes, 1974; Kroeger and Stephens, 1975; Bose and Bose, 1977) have shown that it is possible to make multiunit tonically active smooth muscles to exhibit rhythmic activity with the help of drugs such as tetraethylammonium or metabolic depletion resulting from substrate deprivation or hypoxia. As mentioned previously, my interest in examining the effects of denervation stemmed from earlier observations (Westfall, et al, 1972; Bose and

Innes, 1974; Lee, 1975; Fleming, et al, 1975a, 1975b) that autonomic denervation of multiunit cat spleen capsular smooth muscle or guinea pig vas deferens also manifest rhythmic behavior. The tracheal smooth muscle was used as an object for study to test if the effects of denervation applied to multiunit smooth muscles located in different regions and subserving different functions. Furthermore, both spleen capsule (Fillenz, 1970) and vas deferens (Birmingham, 1970) possess a single autonomic innervation (sympathetic) which is motor in nature. Thus it is not known as to what will be the effect of denervating an inhibitory autonomic nerve. Canine tracheal smooth muscle, because of it's dual innervation (motor and inhibitory) lends itself ideally for such exploration.

Chapter III

MATERIALS AND METHODS

Parasympathetic Denervation

Mongrel dogs of either sex, weighing between 7 and 15kg. were used. The animals were anesthetized by an intravenous injection of sodium thiopental (Pentothal sodium^{Rx}) (30mg/kg). The ventral surface of the neck was then shaved and a 5 to 8 cm. incision was made midway between the thyroid cartilage and the manubrium sternae. Blunt dissection was used to separate the muscles (sternohyoidius and the sternothyroideus) along the midline to reveal the trachea. Care was taken not to disturb the vasculature supplying these muscles or the trachea and only minimal exposure of the recurrent laryngeal nerves, which lie on either side of the trachea was done.

Ligatures were tied around the recurrent laryngeal nerves at a level midway between the larynx and the tracheal bifurcation. Both nerves were then sectioned at that point and a length of each nerve (3-4 cm.) anterior to the section was carefully removed. Another ligature was tied loosely around the trachea at the level at which the nerves were sectioned. This served as a point of identification to show exactly where the recurrent laryngeal nerves had been cut and to identify the innervated from the denervated sections of the trachea. The wound was then closed with Michell clips and cleaned with normal saline. To guard against in-

fection the animal was given ampicillin sodium (Penbritin-1000^{Rx}) 500 mg. I.M.

In order to establish an adequate control and to see what effect surgery alone might have, sham surgery was performed. The technique was identical to that for denervation except that the recurrent laryngeal nerves were not cut. Once it had been established that trachea from the sham operated animals behaved like that of normal unoperated animals and the tracheal muscle caudal to the section in the denervated dogs also behaved like that in normal unoperated animals, we felt it safe to assume that the posterior (innervated) half of each trachea could serve as the control for the anterior (denervated) half. Smooth muscle preparations were obtained 1, 2, 4, 10, 15 or 20 days after nerve section.

In 8 experiments, the main trunk of the vagus, on both sides, was sectioned at the same level as in the case of the recurrent laryngeal nerves. However, this procedure was likely to affect both parasympathetic as well as sympathetic fibers as the cervical vagus in the dog is combined with the sympathetic trunk.

The dogs were generally fasted for 24 hours prior to being anaesthetized by an intravenous injection of pentobarbital sodium (35mg./kg.). As much of the trachea as possible was quickly but carefully removed and immediately placed in a beaker of ice-cold physiological solution of the following

composition, (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.4; $MgSO_4$, 1.2; $NaHCO_3$, 25 and glucose, 11. The dog was then killed by an intracardiac injection of a saturated solution of pentobarbital sodium.

Isolation of Smooth Muscle Preparation

In order to clean the preparation, the trachea was placed in a dissecting tray filled with ice-cold Krebs-Henseleit solution and continuously bubbled with 95% O_2 and 5% CO_2. The tough membranous sheet of connective tissue which adheres to the muscle on the dorsal side of the trachea was carefully dissected free. The trachea was then transversely sliced into rings, each ring being given a letter, D or I (denervated or innervated) and a number designating it's position in relation to the point of section of the recurrent laryngeal nerves. (fig. 1)

Muscle strips 1.0-1.5 cm. long and 10-35 mg. wet weight were carefully removed from each ring. Each muscle was mounted in a 10 ml. jacketed organ bath and connected to a Grass FT-03C force displacement transducer for recording isometric contractions. Contractions were recorded on Grass polygraphs (Models 7 or 5), Brush 280 and Beckman RM recorders. The latter two had rectilinear pens. In some experiments the first derivative of the tension recording was obtained by electronic differentiation. This allowed emphasizing small

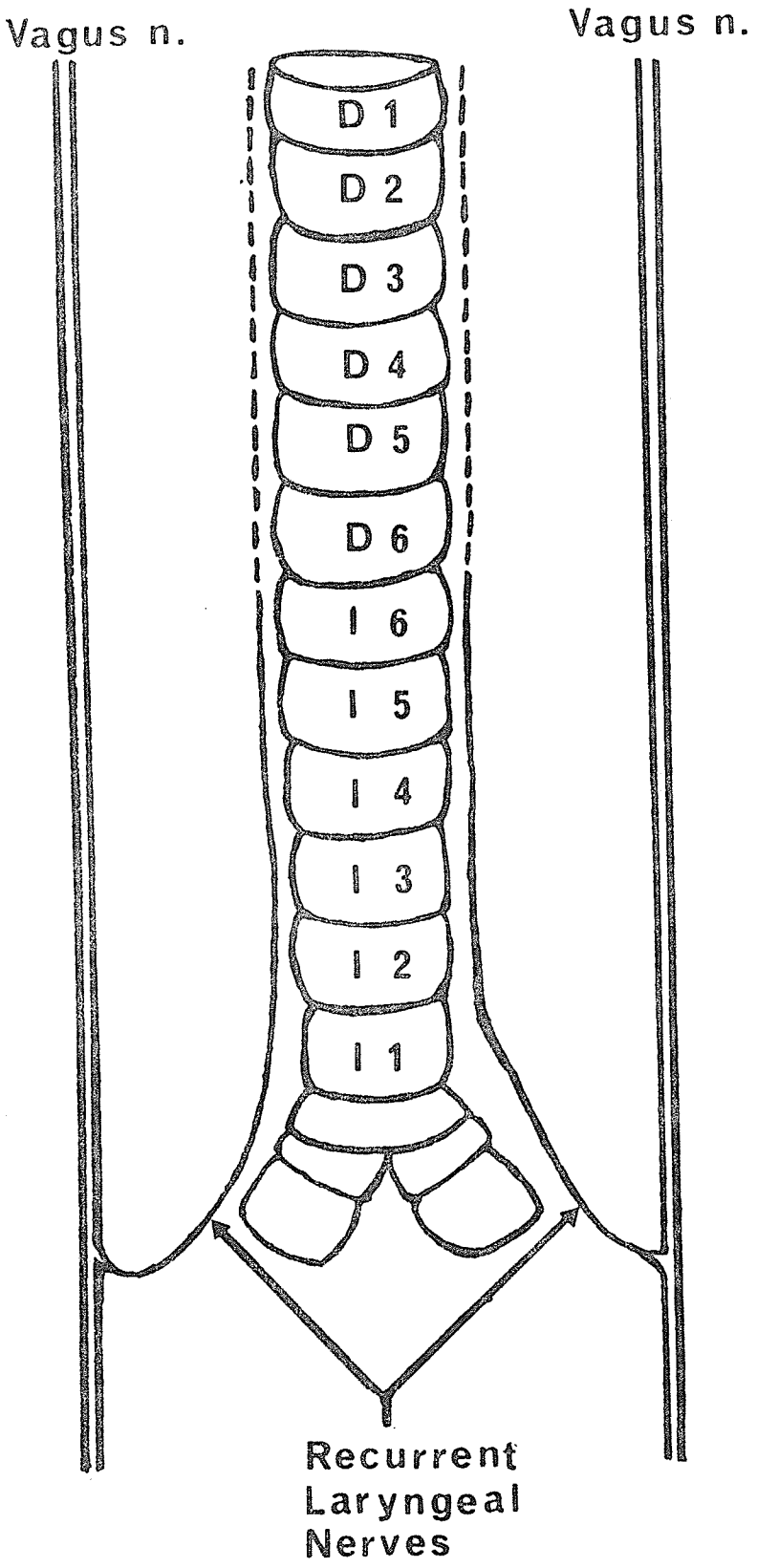

Vagus n.
Vagus n.
D 1
D 2
D 3
D 4
D 5
D 6
I 6
I 5
I 4
I 3
I 2
I 1
Recurrent
Laryngeal
Nerves

Fig. 1 Canine Trachea: showing anatomical demarcation of denervated and innervated sections.

but rapid oscillations superimposed slow but on large amplitude tonic tension changes.

The physiological solutions bathing the muscles were aerated with a 95-5% mixture of O_2-CO_2 and maintained at a pH of 7.3 at 37°C. Resting tension of the muscle was adjusted to obtain the optimal length (L_0) for development of active tension (P_0). Based on our previous experience, this amounted to a passive tension of 0.8-1 g.

After a 1 hour equilibration period, the muscles were exposed to 2×10^{-7} M carbachol, (Sigma Chemical Co.). Denervated muscle strips usually showed rhythmic behavior within 2 hours of being exposed to carbachol, while the innervated strips as well as muscles from sham and unoperated animals showed a maintained tonic contraction in the presence of carbachol.

Drugs and Chemicals

These were: Aminophylline, Sterilab; Ampicillin sodium, (Penbritin-1000^{R}x), Ayerst; Atropine methylnitrate, B.D.H.; Carbamylcholine chloride (Carbachol^{R}x), Sigma Chemical Co.; D-600 HCl, Knoll, A.-G.; Histamine dihydrochloride, Aldrich Chemical Co. Inc.; Isoproternol HCl (Isoprel^{R}x), Winthrop Laboratories; Ouabain octahydrate (Strophanthin-G^{R}x), Sigma Chemical Co.; Tetrodotoxin, Sankyo Co. Ltd.; 6-Hydroxydopamine hydrobromide, Aldrich Chemical Co.; Thiopental

sodium (Pentothal sodium^{R}x), Abbott Laboratories; (Ethylenebis (oxethylenenitrillio)) tetra-acetic acid (E.G.T.A.), Eastman; Pentobarbitone sodium, B.D.H. Pharmaceuticals; Tetraethylammonium chloride (TEA), Eastman; Isethionic acid (Sodium salt), Eastman and Sodium Nitrate, Fisher Scientific Co.

Quick Stretch and Release

Quick release studies and myogenic activity in response to quick stretch were done in 6 experiments by attaching the muscle strips to the transducer on one end and to a servo-controlled electromagnetic device, capable of rapidly stretching the muscle within 10 msec. on the other end. The amount of stretch was varied between 2 and 20% of the optimal length (L_0) and the maximum stretch was twice as large as the maximum extension of the series elastic component. Rectangular command signals were provided with a function generator (South West Technical Corporation, Rhapsody, Texas).

Temperature Studies

Temperature studies were performed on tracheal muscles from 6 animals. Three 15 cc. test tubes were embedded in an aluminum block mounted on a Pelltier effect thermoelectric device (Stir-Kool SK-14; Thermoelectrics Unlimited Inc.).

The aluminum block allowed an even distribution of heat. Two of the 15 cc. test tubes served as organ baths in which tissues were suspended from Grass Force-displacement Transducers FT-03C. The third test tube contained the same physiological solution as the organ baths but served as a medium for two thermistors. One of them led to the temperature controller (Thermistemp, Yellow Springs Instruments Co. Inc.) and the other to an electronic thermometer (Tele-thermometer, Yellow Springs Instruments Co. Inc.). With such a set up the temperature of the bathing solutions in the organ baths could be set or changed to any desired value.

Electrophysiology

Floating glass microelectrodes were used for recording intracellular electrical activity (n=5). The microelectrodes were drawn from glass capillary tubes (OMEGADOT; Fredrick Haer). The tip was filled with 2.7 M KCl by capillary action and the rest of the electrode was backfilled with a syringe. Electrodes of 25 to 50 M resistance and having tip potentials less than 5mV, were used and were connected to a high input impedance amplifier (Neuroprobe; Transidyne Corporation). Both electrical and mechanical activities and their first derivative were recorded on a Hewlett Packard 141B oscilloscope as well as on a Brush Mark 280 recorder.

Bathing Mediums

Composition of Physiological Solutions (in mM)

	NaCl	KCl	KH_2PO_4	$NaHCO_3$	$MgSO_4$	$CaCl_2$	Glucose
Krebs-Henseleit	118	4.7	1.4	25	1.2	2.5	11
Glucose Free	118	4.7	1.4	25	1.2	2.5	--
Ca^{++} Free	118	4.7	1.4	25	1.2	--	11
Cl^- Deficient Na^+ Isethionate	118	4.7	1.4	25	1.2	2.5	11
Cl^- Deficient $NaNO_3$	118	4.7	1.4	25	1.2	2.5	11
K^+ Free	118	--	1.4 (NaH_2PO_4)	25	1.2	2.5	11
K_2SO_4 Rich	--	K_2SO_4 122.7	1.4	25	1.2	2.5	11

Sympathetic Denervation

Because of the difficulty in isolating the adrenergic innervation of the trachea a chemical postganglionic denervation was done.

Five dogs were treated with two intravenous injections of 6-hydroxydopamine (30 mg/kg) on successive days. 6-hydroxydopamine acts by destroying sympathetic nerve endings; it does not appear to damage peripheral adrenergic nerve cell bodies or proximal axons (Thoenen and Tranzer, 1973).

Biochemical Determinations

Adenosine triphosphate (ATP), was assayed spectrophotometrically by the methods described by Fawaz and Fawaz (Schwartz, 1971).

Hexokinase Method for ATP

The following enzymes are needed for this method: glucose-6-phosphate dehydrogenase (G-6-PDH) and hexokinase (HK). The assay is best described in terms of the reactions involved.

$$\text{glucose} + \text{ATP} \xrightleftharpoons[\text{Mg}]{\text{HK}} \text{glucose-6-phosphate} + \text{ADP} \qquad (1)$$

$$\text{glucose-6-phosphate} + \text{TPN}^{+} \xrightleftharpoons{\text{G-6-PDH}} \text{6-phosphogluconate} + \text{NADH+H} \qquad (2)$$

In the presence of HK, Mg ions and an excess of glucose, ATP transfers a phosphate to glucose producing glucose-6-phosphate (reaction 1). The glucose-6-phosphate then generates NADH from NAD as it becomes dehydrogenated (reaction 2). At equilibrium, reactions one and two are in favour of the reaction products, and so ATP is quantitatively used up; 1 μmole of ATP forms 1 μmole of TPNH. A quantitative amount of ATP can be determined by means of the following formula:

$$V \times \frac{E}{k} \times \frac{1000\mu l}{x\mu l} \times \frac{I}{mg} = \text{ATP } (\mu \text{ moles})$$

Where V= Total volume of the extract
k = extinction coefficient (6.22)
x = volume of sample used in μl
mg = wet weight of muscle being sampled in mg
ΔE = change in absorbance

Assessment of Parasympathetic Denervation

At designated days after denervation dogs were anesthetized with pentobarbital sodium (30mg/Kg) and the intact vagi on both sides of the trachea were exposed. Bipolar platinum cuff electrodes were placed around each vagus and the central end of the nerve was clamped with a pair of haemostatic forcepts.

An endotracheal tube, equipped with two balloons (fig. 2) was then inserted into the trachea. The tube was positioned so that one balloon could be inflated in the posterior innervated section of the trachea while the other balloon was in the anterior denervated segment. The balloons were filled with water and connected to Statham P23 Db pressure transducers while changes were recorded on a Grass polygraph.

Stimulations were delivered with a Pulsar 6 stimulator (Frederick Haer and Co.), and consisted of square wave trains having frequencies of 1, 2, 3.3, 5, 10, 20, and 33Hz with a pulse duration of 0.5 msec. and a supramaximal stimulus amplitude. Both balloons were distended to equal pressures of about 50mmHg and a change in intraluminal pressure on nerve stimulation indicated contraction of the tracheal smooth muscle.

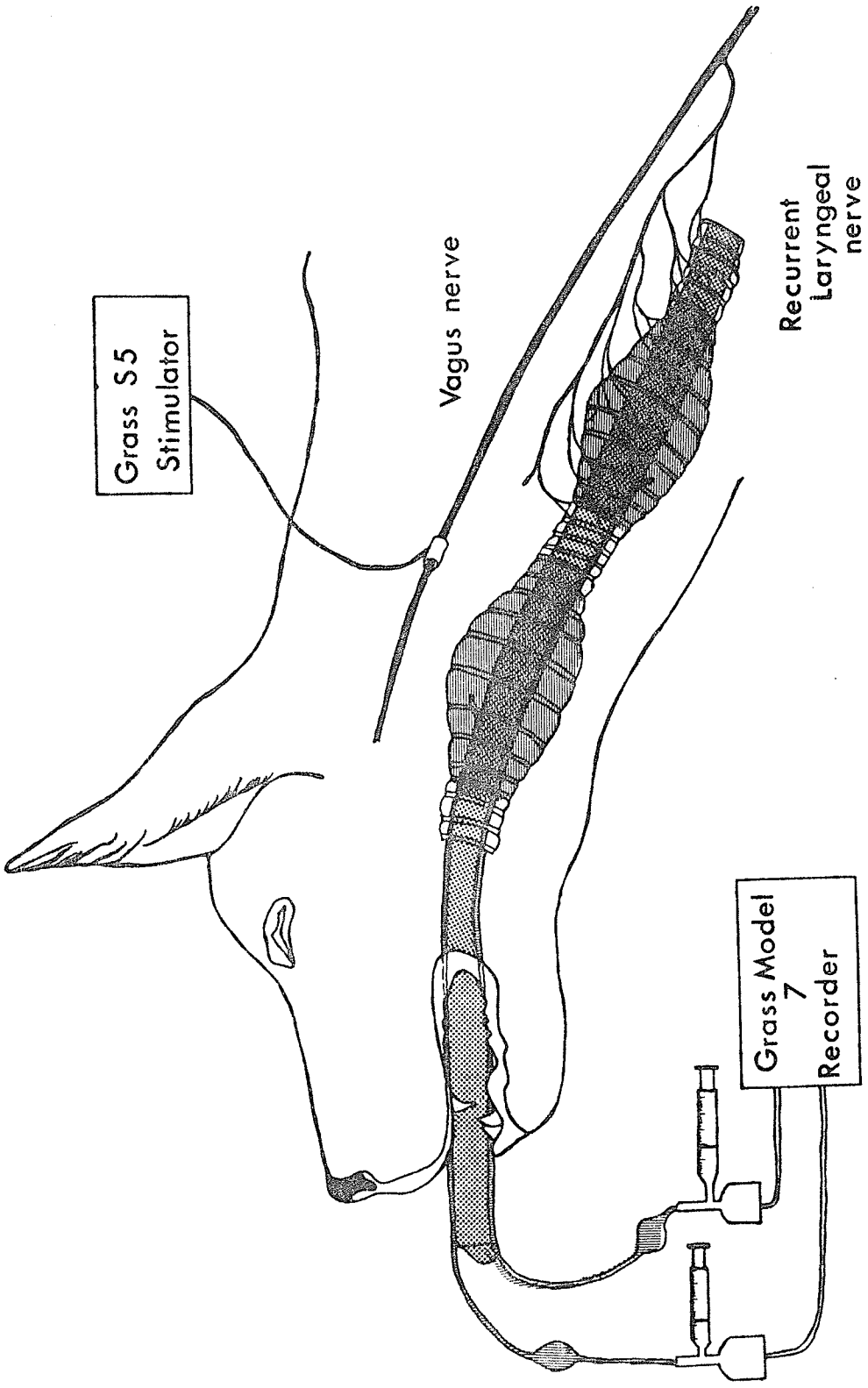

Grass S5
Stimulator
Vagus nerve
Recurrent
Laryngeal
nerve
Grass Model
7
Recorder

Fig. 2 Method of evaluating parasympathetic denervation in the dog. Two separate ballons in an intratracheal tube were connected to pressure transducers. The peripheral end of the transected cervical vagus nerve was stimulated bilaterally with rectangular pulses. The anterior half of the trachea was denervated by bilaterally sectioning the recurrent laryngeal nerves.

Chapter IV

RESULTS

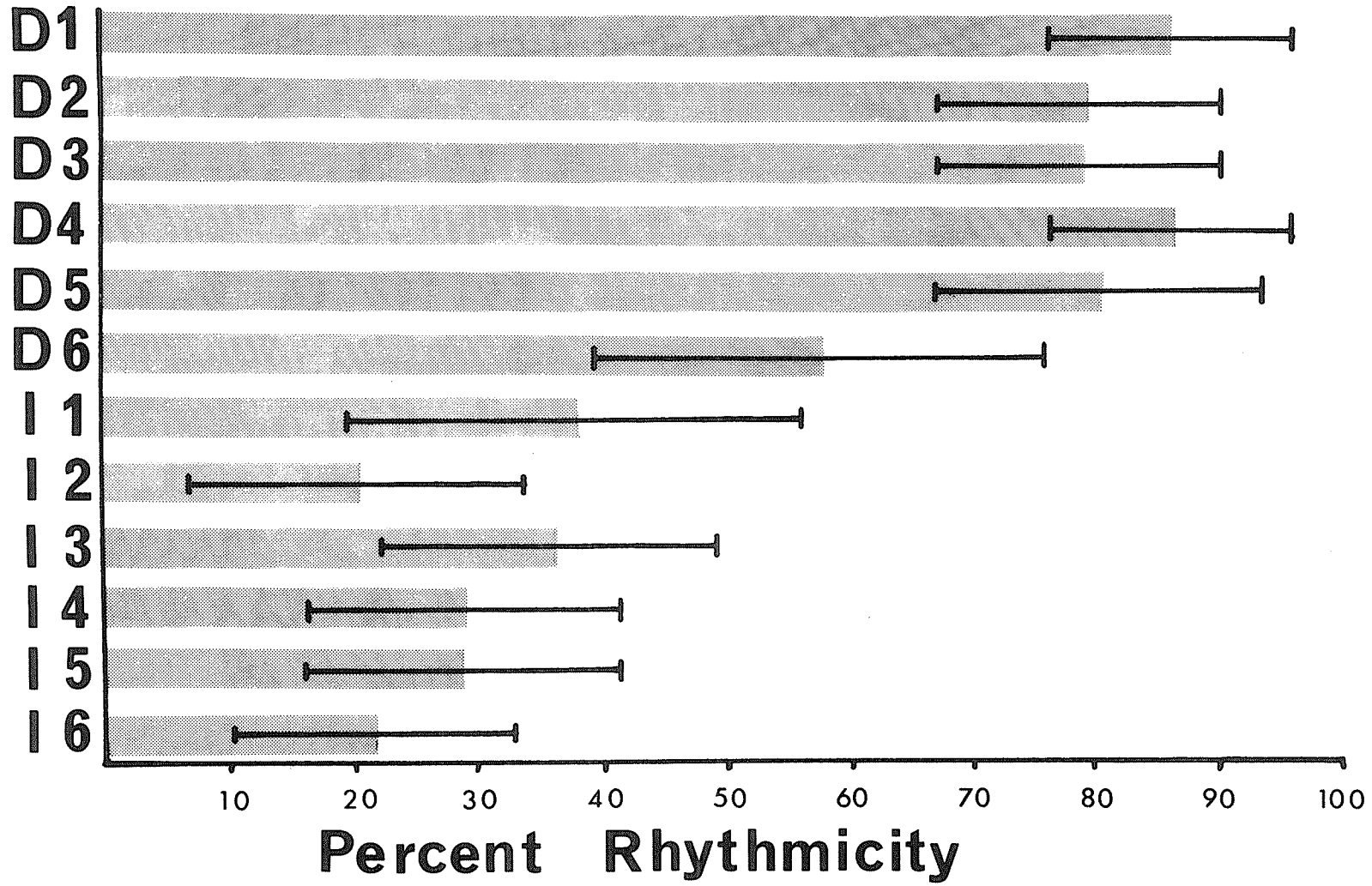
Relationship of Rhythmicity and Innervation

In those experiments where the trachea was half denervated and half innervated (n = 23) it was of interest to determine the efficiency of the surgical technique. Muscle strips from both anterior and posterior segments of the trachea were mounted in baths and stimulated with carbachol ($2x10^{-7}M$) after a suitable equilibration period. Analyzing each experiment and then graphing the mean incidence of rhythmicity (85%) (fig. 3) shows that the anterior segments of the trachea, i.e. the denervated muscle strips, showed a significantly greater ($P<.001$) incidence of rhythmic behavior after stimulation than did the innervated posterior segments, (15%).

Those muscle strips nearest the ligature showed some variability which may be related to some uncertainty regarding exact functional demarcation of the denervated zone.

The Effects of Agonists on Innervated and Dennervated Tracheal Smooth Muscle

Various drugs and chemicals are capable of inducing a contracture in tracheal smooth muscle. The response of this smooth muscle to carbachol, histamine and potassium chloride are well documented (Bose, 1975; Suzuki, et al, 1976; Bose and Bose, 1977). It was of interest, therefore, to see what

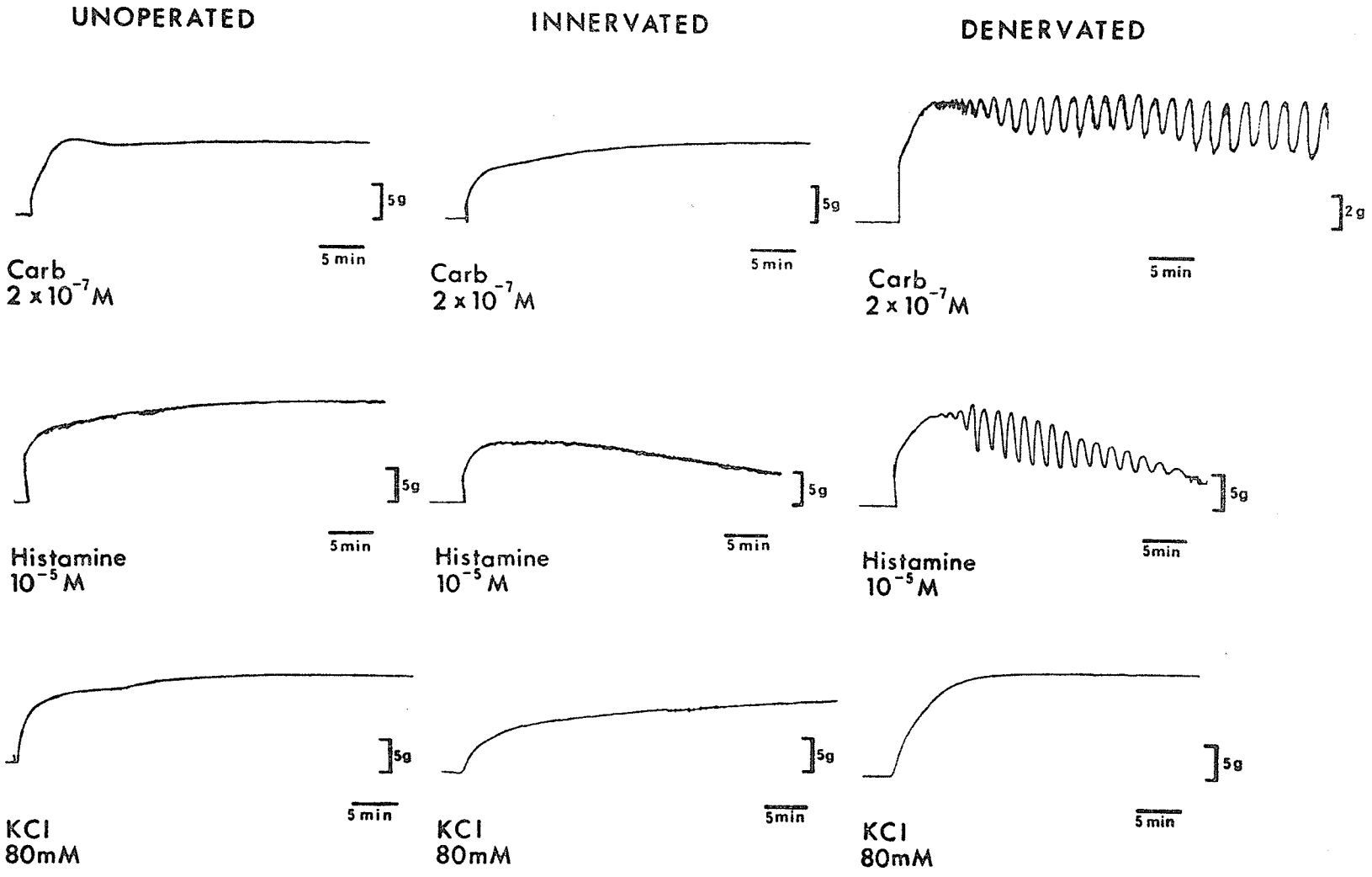

D1
D2
D3
D4
D5
D6
I 1
I 2
I 3
I 4
I 5
I 6
10
20
30
40
50
60
70
80
90
100
Percent Rhythmicity

(99a)

Fig. 3 Canine Trachealis: incidence of rhythmicity with carbachol (2×10^{-7} M) in innervated (I) and denervated (D) segments. Recurrent laryngeal nerves transected between D6 and I1.

type of response might be produced by the denervated preparation after stimulation by these agonists. Each treatment represents a minimum of 5 experiments.

In the untreated animal (normal trachea) the muscle strips, equilibrated for two hours prior to stimulation with carbachol ($2x10^{-7}M$), histamine ($10^{-5}M$) and potassium chloride (80mM), (fig. 4A), responded with a maintained tonic contraction typical of the documented multi-unit response. The posterior segments of the treated trachea, i.e. the innervated muscle, responded in a similar fashion to the same three agonists, (fig. 4B), indicating that the innervated muscle strips behaved like that of the normal preparation. The denervated, anterior segments, of the tracheal muscle responded much differently, however. Carbachol and histamine, in the same doses as those used in the control, produced oscillating or phasic type contractions within minutes of stimulation. The rhythmicity was initially often variable in amplitude and period resulting in a somewhat irregular pattern but gradually became more regular with time, (fig. 4C), responding in a manner typically described as that of a single unit smooth muscle. Potassium chloride, on the other hand, continued to show a maintained tonic contraction identical to those observed in the normal and innervated tracheal strips.


UNOPERATED
INNERVATED
DENERVATED
5g
5 min
Carb
2 x 10⁻⁷M
2g
Histamine
10⁻⁵M
KCl
80mM

(100a)

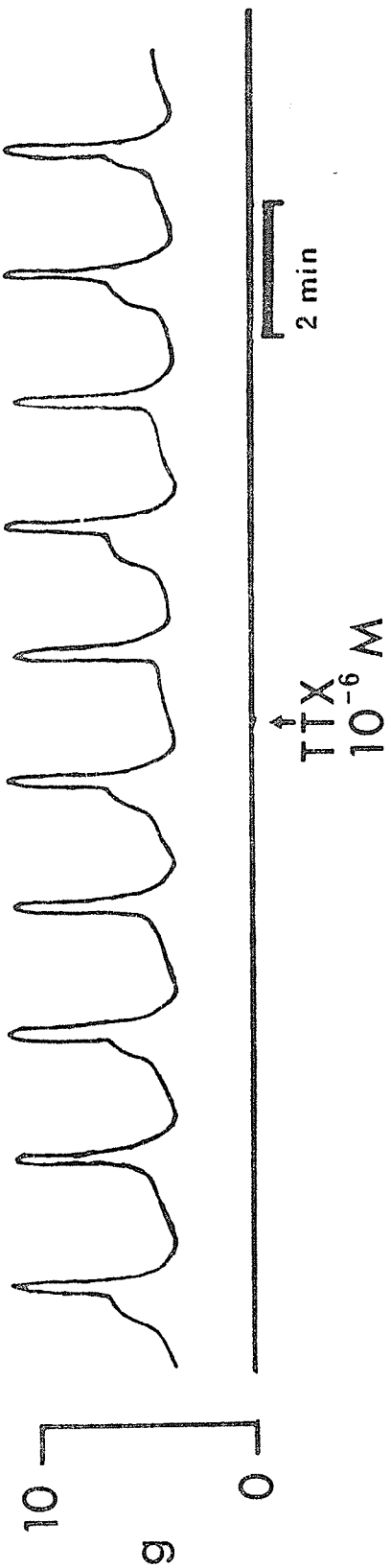
Fig. 4 Canine Trachealis: effects of various agonists, carbachol (carb.; 2×10^{-7} M), histamine (hist.; 10^{-5} M), and potassium chloride (KCl; 80 mM).

Sympathetic Denervation

Since the vagus nerve carries both sympathetic and parasympathetic innervation to the dog trachea, and results had shown that sectioning the recurrent laryngeal nerve caused rhythmic behavior, it was necessary to determine whether or not inhibition of the sympathetic collaterals off the vagus affected the mechanical responses of the tracheal muscle. Five dogs were treated with two intravenous injections of 6-hydroxydopamine (30mg/Kg) on successive days. The muscle strips taken out on the third day did not show any rhythmic behavior in response to carbachol.

Effects of Tetrodotoxin on the Phasic Contractions

In order to determine that the oscillatory behavior of the muscle was due to the muscle itself and not due to indirect effects mediated through transmitters released from nerve terminals that might still exist, six experiments were done with the agent tetrodotoxin. Tetrodotoxin is known to inhibit conduction in both preganglionic and postganglionic nerves and is often used as a physiological tool to differentiate between direct muscle response and those produced by neural stimulation. Figure 5 shows the effects of tetrodotoxin (10^{-6}M) on the rhythmically active denervated smooth muscle preparation. This potent neurotoxin did not abolish



(101a)

Fig. 5 Denervated Canine Trachealis: rhythmicity induced by carbachol (2×10^{-7} M). Effects of tetrodotoxin (TTX; 10^{-6} M).

the rhythmic behavior of the muscle.

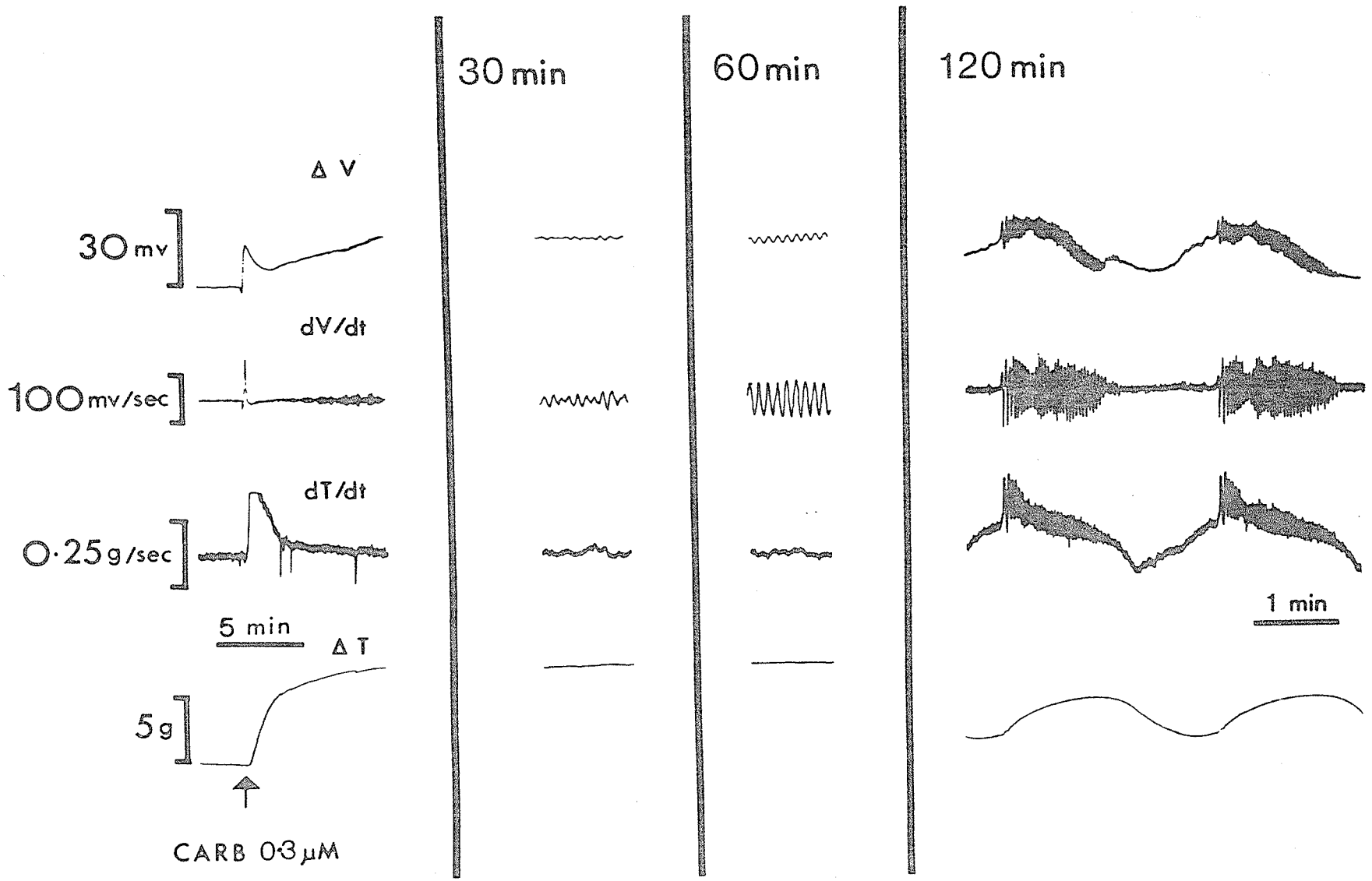
Components of the Phasic Contractions

In a number of experiments there was evidence that there were smaller oscillations superimposed on the primary rhythmic mechanical contractions. A closer look at the rhythmic contractions revealed that each slow oscillation, which had a period of 1-3 minutes, was made up of many smaller but faster oscillations which showed up much better when the first derivative of the isometric tension trace was obtained with the help of a differentiator (fig. 6). Even though such fast phasic contractions were not always evident on the tension trace (fig. 6), differentiation of the mechanical response invariably showed that the slow waves had a superimposed rapid phasic component, (n = 6).

Electrical studies using glass microelectrodes (n=5). showed that 1 -20 mv electrical oscillations were associated with each rapid phasic mechanical oscillation. The frequency of the spike like activity was highest during the peak of the slow rhythmic contractions and slowest during the relaxation phase.

Myogenic Response

A myogenic response of muscle to quick stretch is a characteristic property of a single unit rhythmic muscle.


30 min
60 min
120 min
Δ V
30 mv
dV/dt
100 mv/sec
dT/dt
0·25 g/sec
5 min
Δ T
5 g
CARB 0·3 μM
1 min

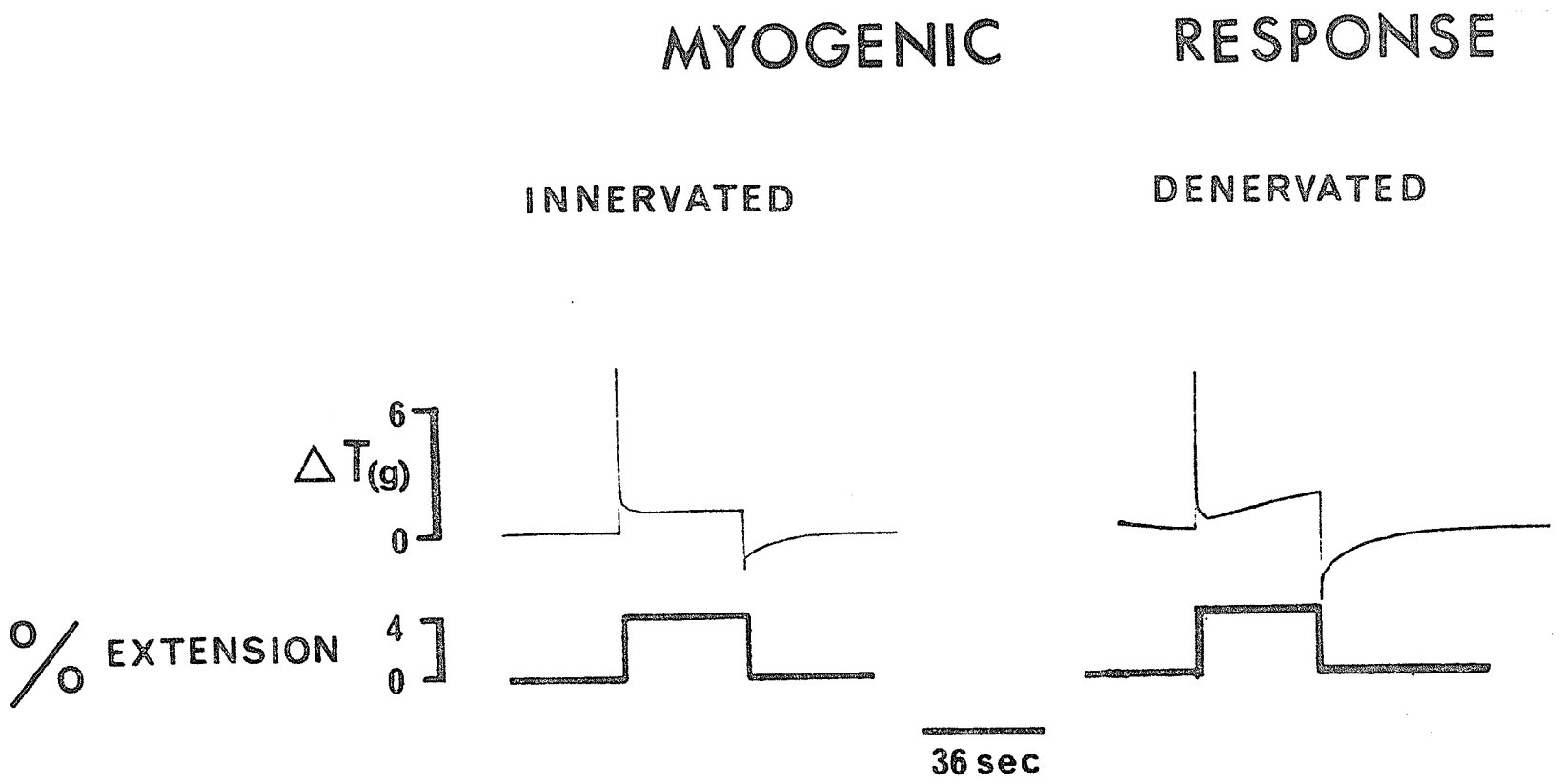
I02a

Fig. 6 Denervated Tracheal Smooth Muscle:

Electrical activity (transmembrane) and its first derivative are shown in the first and second panel respectively. Isometric tension changes and their first derivative are shown in the third and fourth panels respectively. Rhythmicity was induced by carbachol (2×10^{-7}M).

As there was reason to believe that the normally quiescent tracheal muscle had undergone a conversion of sorts from a multiunit type to a single unit type smooth muscle quick stretch experiments (n = 6) were performed in order to test this. Rapid stretch of the denervated muscle (4 percent of L_o) resulted in a rapid rise in tension followed by a decay towards prestretch levels, (fig. 7). Previous studies had indicated that an optimal response to quick stretch (myogenic response) was seen when the muscle was stretched to about 4 percent of L_o. This was followed within 5 seconds by a gradual but steady increase in tension development which continued to rise until the stretch stimulus was released or a peak contracture was reached of about 5 grams tension. Release of the stretch stimulus caused a rapid fall in tension well below the prestretch value. The muscle then slowly contracted until it reached it's prestretch level of tension.

Unlike the denervated preparation, normal tracheal muscle and those from the posterior segments of the operated animals did not show an increase in tension development with rapid stretch, (fig. 7). An equivalent stimulus produced an initial rapid rise followed by a fall in tension, like that observed in the denervated muscle. However, no secondary contracture was seen at any point in the duration of the stimulus. Sudden release of the muscle resulted in a rapid fall


MYOGENIC RESPONSE
INNERVATED
DENERVATED
ΔT(g)
6
0
% EXTENSION
4
0
36 sec

(103a)

Fig. 7 Quick stretch, (4% of L_0) of innervated and denervated canine tracheal muscles.

in tension to slightly below former resting values. The muscle then contracted to restore the tension to it's pre-stretch level. The rate of recovery after release, (an indication of the active state of the muscle), was much greater in the innervated preparation and the degree of relaxation, after release, was much less than that observed in the denervated muscle strips.

Ionic Requirements of Phasic Contractions

Earlier studies have reported that the induced rhythmic behavior observed in normally quiescent muscles may be due, at least in part, to an unstable resting membrane potential brought about by an altered membrane function in relation to it's ionic environment. Changes in the ionic constituents or ionic proportions in the bathing medium, brought about by changes in the membrane function and/or structure, may be partly responsible for the observed phasic responses in the denervated tracheal smooth muscle. In order to study the ionic requirements of the rhythmically active muscle a number of experiments were done in which the ionic environment was altered.

Calcium

Free calcium concentrations were calculated by the method described by Iamai and Takeda (1967). The normal external Ca^{++} concentration in Krebs Henseleit solution is

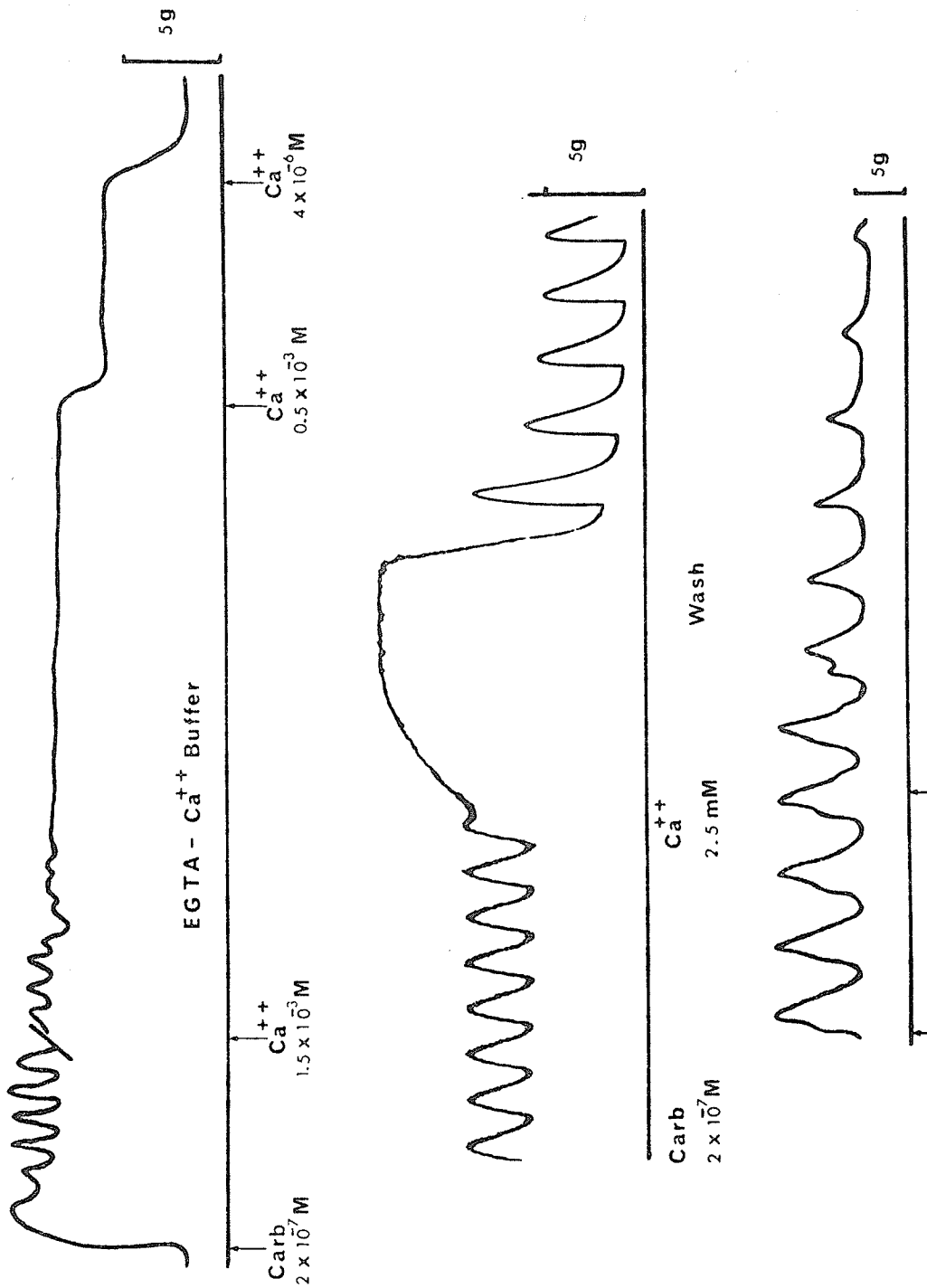

5g
EGTA - Ca++ Buffer
Carb
2 x 10-7 M
Ca++
1.5 x 10-3 M
Ca++
0.5 x 10-3 M
Ca++
4 x 10-6 M
5g
Carb
2 x 10-7 M
Ca++
2.5 mM
Wash
5g
Carb
2 x 10-7 M
D 600
10-7 M

Fig. 8 Denervated Canine Trachealis: Rhythmicity induced by carbachol (2×10^{-7} M). Effect of different indicated low calcium concentrations (top panel) or .5mM calcium to Krebs-Henseleit (middle panel) or D-600 (10^{-7}M) (bottom panel)

2.5 mM. Reduction of the external calcium concentration in 5 experiments, with the help of an EGTA-Ca^{++} buffer (fig. 8A) (calcium reduced to 1.5×10^{-3}M) rapidly and preferentially inhibited the rhythmic contractions while the tonic component of the carbachol response was appreciably reduced only when the calcium concentration was reduced more dramatically to 0.5×10^{-3}M and 4×10^{-6}M respectively.

While removal of calcium from the bathing medium resulted in a loss of rhythmicity and ultimately a decrease in tension a similar and opposite effect was observed with the addition of 2.5 mM calcium to muscles bathed in normal Krebs Hensleit solution (fig. 8B)(n = 4). The additional calcium had the effect of abolishing the oscillations, similar to that observed when the external calcium concentration was reduced, yet there was a substantial increase in tension.

The effects of D-600, a chemical tool known to block the active influx of calcium associated with action potentials was examined in 5 experiments. D-600 abolished the rhythmic contractions in rhythmically active denervated muscle strips in very small concentrations (10^{-7}M) that did not much effect the tonic contractions, (fig. 8C).

Chloride Replacement

Because chloride has been directly implicated in, and reported to be a possible source of the rhythmic activity observed in some skeletal and smooth muscles, (El-Sharkawy

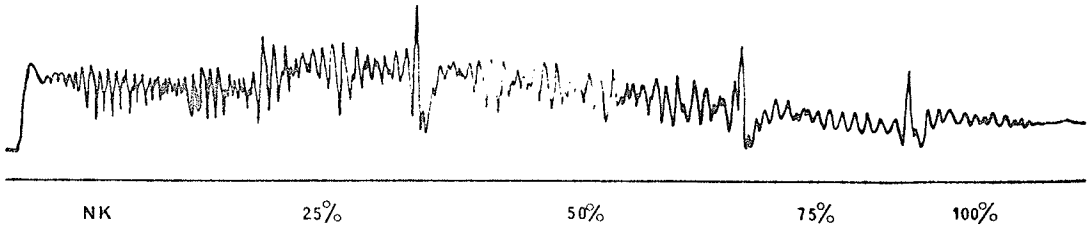
and Daniel, 1975; Camerino and Bryant, 1976), a study of this anion and it's possible role in the denervated tracheal muscle rhythmicity was examined in 8 experiments.

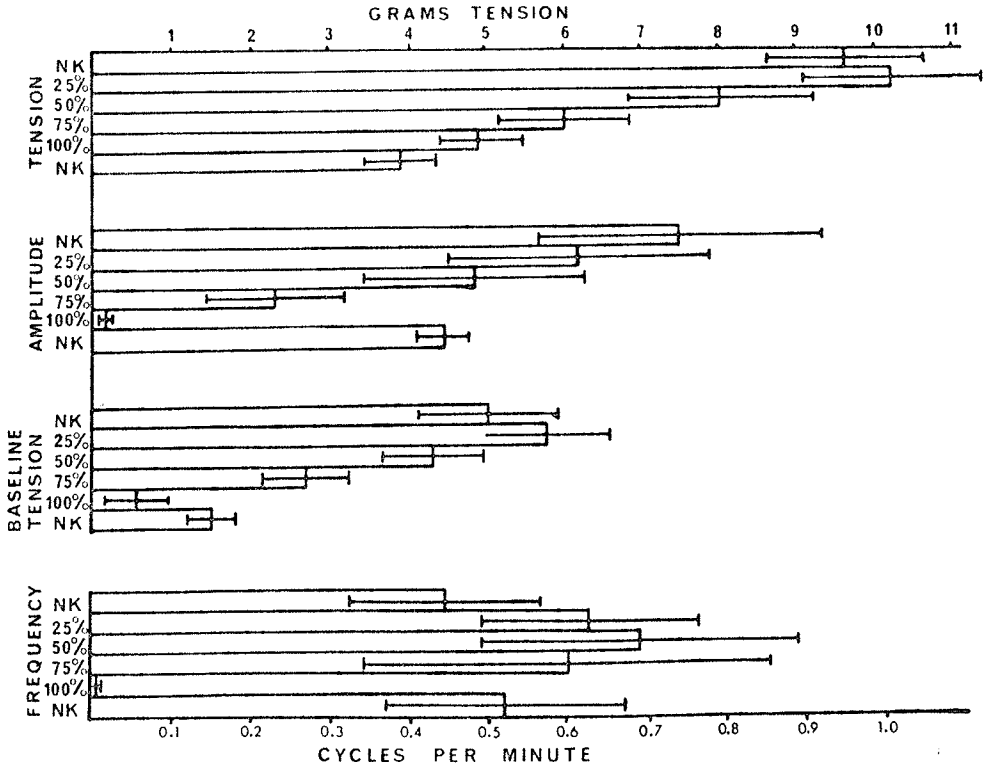
The effects of replacing the chloride concentration in the bathing medium with different ion substitutes are represented in figures 9 and 10. The response observed by replacing chloride in normal Krebs Henseleit solution with 25 percent sodium isethionate, a less permeant anion, was an initial increase in tension accompanied by a decrease in amplitude and a slight increase in baseline tension. The frequency of the oscillations increased only slightly (fig.9). With further replacement of chloride by this anion (50%-75%-100%) peak tension progressively decreased and all rhythmic activity was eventually lost. The rhythmicity usually returned and the tension recovered upon washing the muscle in normal Krebs Henseleit solution.

Replacement of chloride ion with a more permeant ion, sodium nitrate (25%) resulted in an initial contraction of the muscle similar to that observed with sodium isethionate (fig. 10). Further replacement of chloride with 50%, 75% and finally 100% sodium nitrate resulted in a gradual but steady decrease in muscle tension.

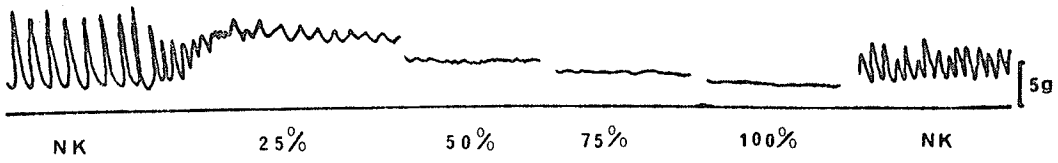
The amplitude of the phasic contractions appeared to be affected much more by the nitrate anion than by sodium isethionate, (fig. 10). The initial replacement of chloride

CHLORIDE REPLACEMENT WITH SODIUM ISETHIONATE





CHLORIDE REPLACEMENT WITH SODIUM NITRATE



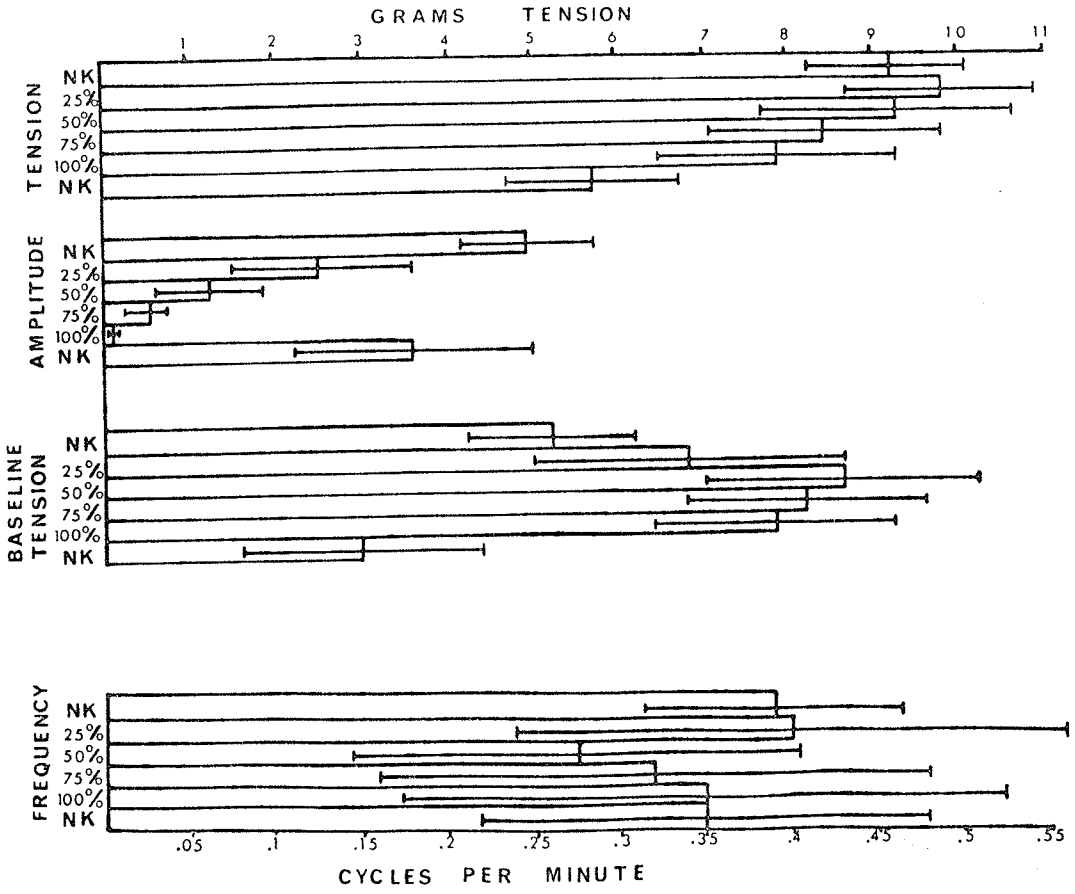


Fig. 9 Denervated Canine Trachealis: Effect of different degrees of chloride replacement with isethionate on various parameters of rhythmic contractions due to carbachol ($2x10^{-7}$ M).

Fig. 10 Denervated Canine Trachealis: Effects of different degrees of chloride replacement with nitrate on various parameters of rhythmic contractions due to carbachol ($2x10^{-7}$ M).

ion in the bathing medium by sodium nitrate caused a significant decrease in amplitude which progressively fell to near resting levels as the chloride concentration was sequentially reduced. Only when the chloride ion was fully restored, by washing the muscle several time in normal Krebs Henseleit solution did the amplitude approach near normal values.

Although the mean tension and amplitude decreased with the progressive decrease in chloride concentration, the frequency of the phasic contractions did not change significantly. It is interesting to note too, that the mean baseline tension showed a substantial increase which peaked when the chloride concentration was decreased to 50% that of normal (fig. 10). Further loss of chloride ion in the bathing medium resulted in a gradual decline in baseline tension. Washing out the sodium nitrate solution with normal Krebs Henseleit caused a significant decrease in baseline tension to values approximating those recorded before ion replacement.

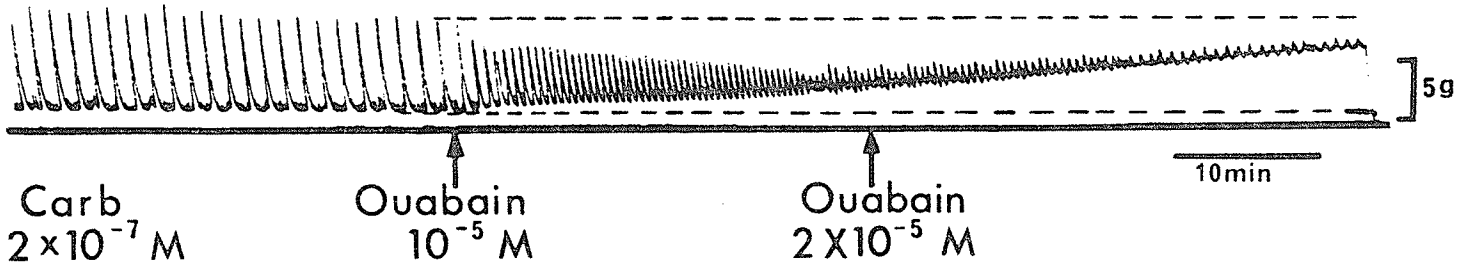
Role of the Electrogenic Sodium Pump

Because much discussion has centered around the role of pumping mechanisms, specifically the electrogenic sodium pump, in relation to the slow electrical oscillations observed in rhythmic muscles, it was of interest to study the effects that pump inhibition might have on the denervated

tracheal smooth muscle preparation.

Ouabain (10^{-5}M), an agent known to inhibit the sodium potassium pump, was administered to rhythmically active denervated muscle strips (fig. 11)(n = 5). If one assumes that the crests and troughs of the mechanical responses correspond to electrical depolarization and repolarization respectively, then an oscillating sodium electrogenic pump should be most active during repolarization and least active during depolarization. It was expected, therefore, that ouabain should cause a depolarization by nature of it's ability to inhibit the sodium pump. This in fact did occur, however, it was also expected that such an effect would have resulted in an increase in baseline tension associated with a decrease in rhythmic oscillations and that at the point of abolition of the rhythmicity the baseline tension should have reached the level of the peaks of the phasic contractions, (fig. 11A). Figure 11B represents the expected and actual results expressed in graphic form. If the amplitude of the phasic contractions before giving ouabain is designated "A", and the amplitude recorded after the administration of ouabain is designated "B", while the change in the baseline tension after ouabain is denoted as "C", then the percent decrease in amplitude of the phasic contractions can be expressed as:

$$\frac{A - B}{A} \times 100$$


5g
10min
Carb
2×10⁻⁷ M
Ouabain
10⁻⁵ M
Ouabain
2X10⁻⁵ M

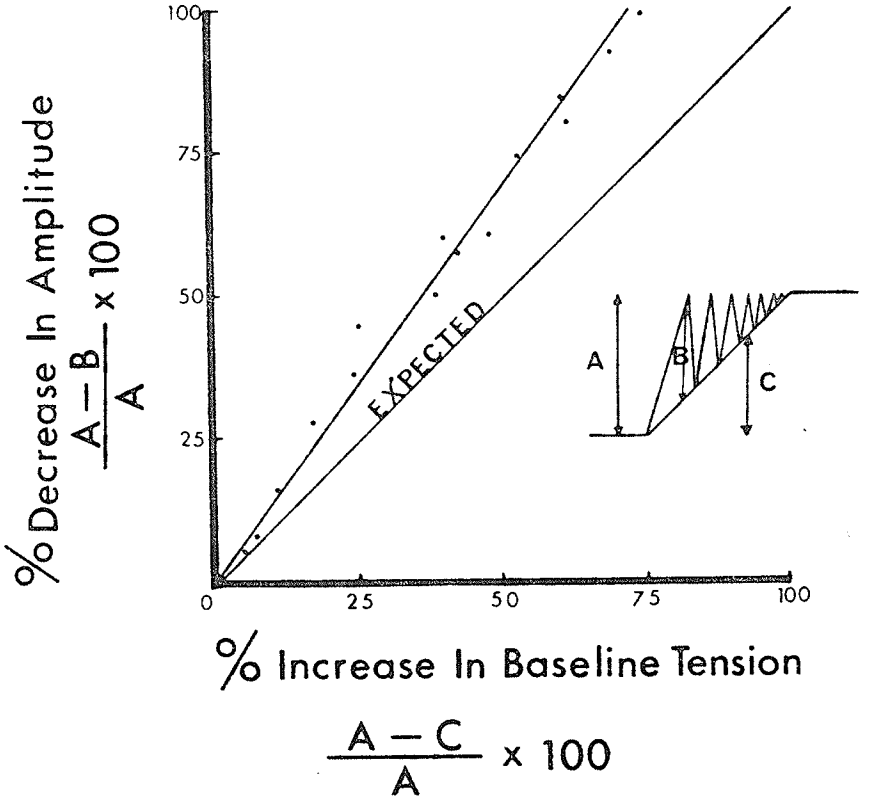

100
75
50
25
0
25
50
75
100
EXPECTED
A
B
C
% Decrease In Amplitude
(A − B)/A × 100
% Increase In Baseline Tension
(A − C)/A × 100

Fig. 11 Denervated Canine Trachealis: Effect of ouabain (10^{-5} M) on amplitude of rhythmicity and baseline tension, (upper trace). Expected versus observed response to pump inhibition by ouabain (10^{-5} M) (lower graph).

Inset shows schematical changes in baseline and amplitude of rhythmic contractions. Solid line with slope of 1 represents idealized situation where oscillations are totally dependent on fluctuations in electrogenic sodium pumping.

while the percent increase in baseline tension can be found by the formula:

$$\frac{A - C}{A} \times 100$$

The results, as shown in figure 11B were contrary to these expectations. The decrease in amplitude of the phasic contractions exceeded the increase in baseline tension.

Temperature

Maximum activity of the sodium potassium pump is dependant, in the mammal, on an optimal temperature, which generally centers around 37°C. Pump activity can be reduced and eventually abolished by a reduction in temperature below that optimum value. Figure 12 illustrates the results, observed in 6 animals, after such a decrease in temperature, on rhythmic activity in tracheal smooth muscle. A drop in temperature of only 3-4°C effectively abolished the rhythmic activity which was accompanied by a significant increase in baseline tension. Tension continues to increase as temperature decreases. In a number of experiments tension continued to rise until the temperature reached 15°C at which point a plateau stage in tension development was attained. Rewarming of the preparation resulted in an almost immediate loss of tension as the muscle relaxed to near baseline levels. As the temperature approached 37°C tension redeveloped to

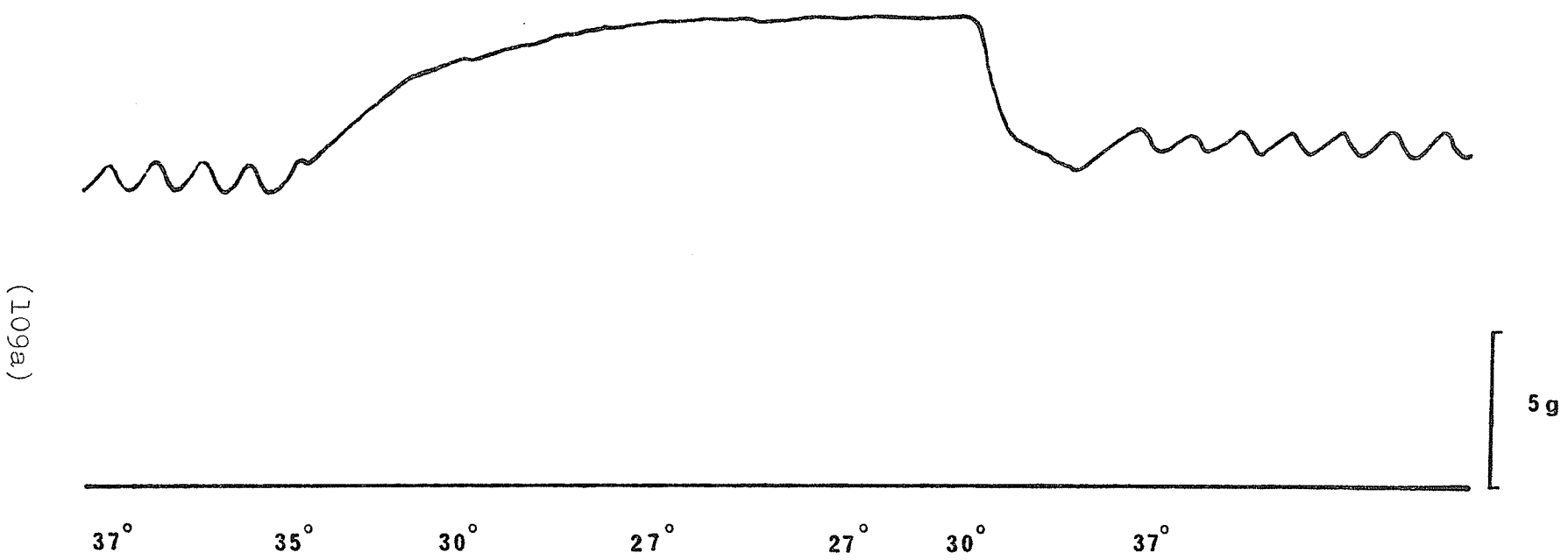

(109a)
5 g
37°
35°
30°
27°
27°
30°
37°

Fig. 12 Canine Trachealis: Rhythmicity induced by carbachol (2×10^{-7} M).
Effect of lowering the temperature.

near previous levels and mechanical oscillations were again observed.

ATP Content in the Denervated Tracheal Muscle

Surgical denervation was performed on 6 animals. The neural innervation of the posterior section of the trachea was left intact so as to serve as an internal control for each experiment. The ATP content of both the anterior denervated and the posterior innervated segments of the muscle where obtained and a paired T-test was used to analyze the results (fig. 13). As can been seen from the graph, the innervated muscle segments showed a significantly greater ATP content (mean 1.1μM /gm wet tissue) than the denervated segments (0.5μM /gm wet tissue),(P=0.01).

Invivo Experiments

Whole animal experiments were performed on 5 dogs by the technique described in the methods and diagramatically represented in fig. 2.

The results of a typical experiment are shown in fig. 14. Stimulation of the vagus nerve at one hertz (Hz) produced a small contraction of 5 mmHg tension in the innervated muscle but no response was observed in the denervated muscle segment. An increase in the frequency of nerve stimulation from 1 Hz to 3.3 Hz, 10 Hz, 20 Hz and 33 Hz re-

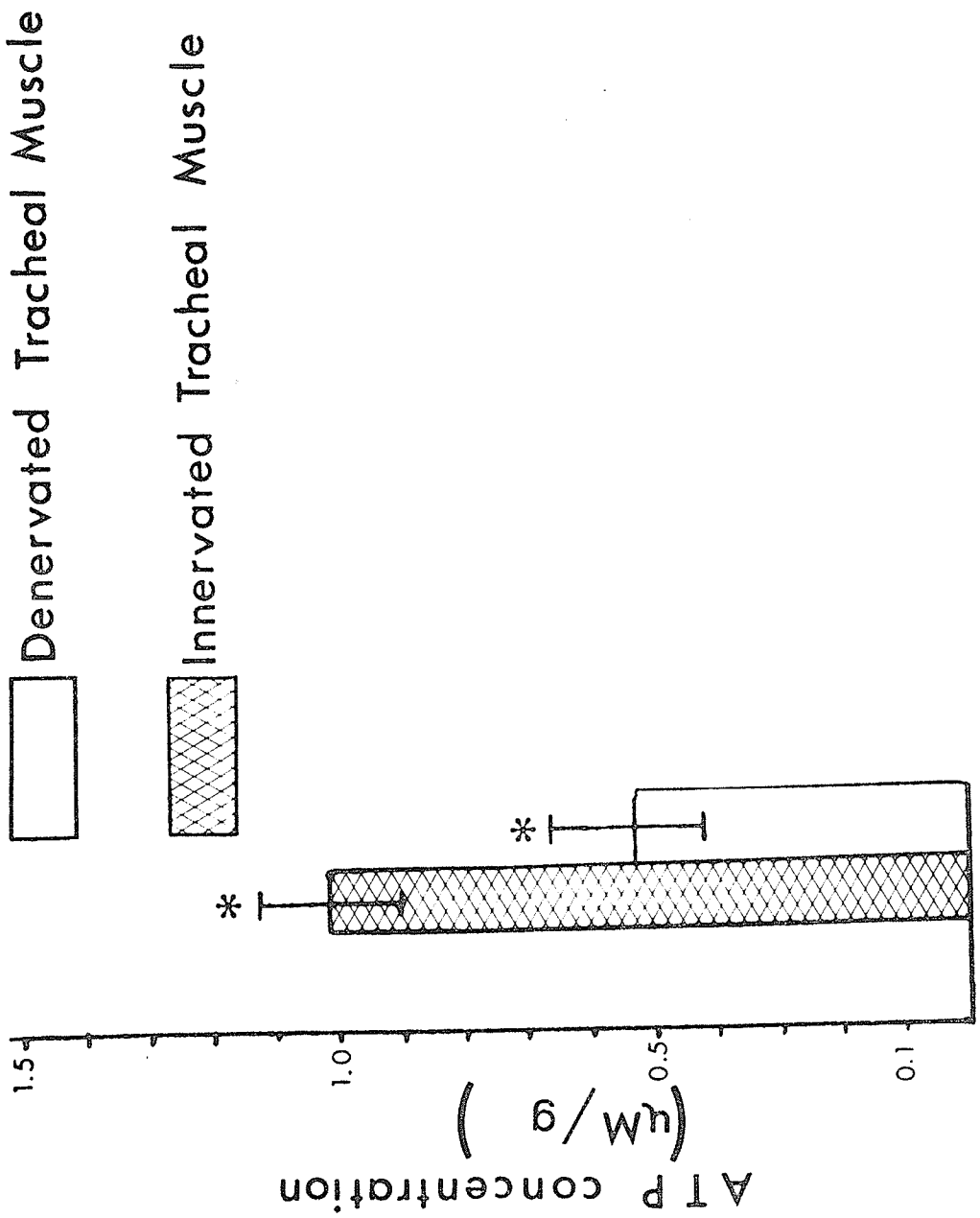
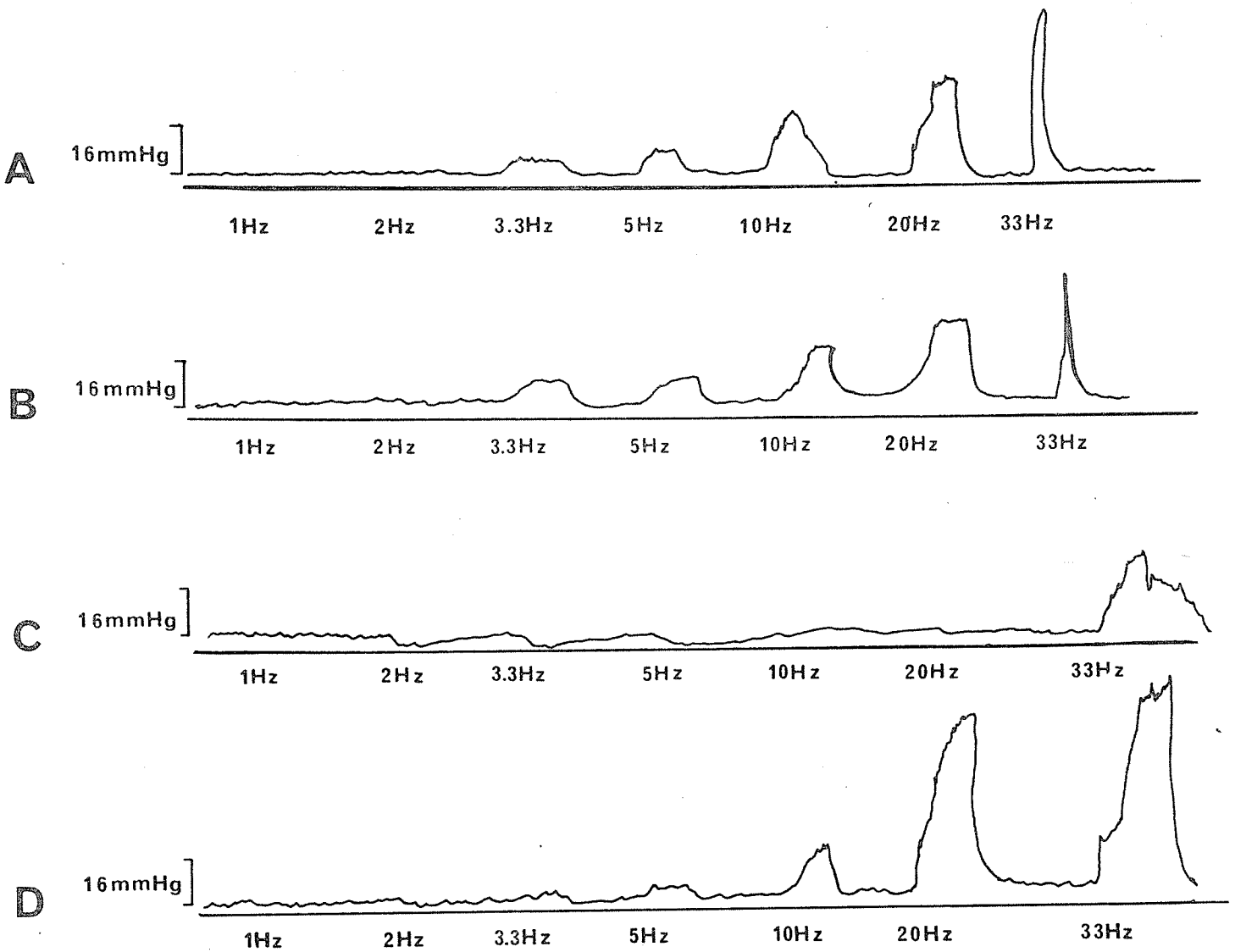

Denervated Tracheal Muscle
Innervated Tracheal Muscle
*
*
1.5
1.0
0.5
0.1
ATP concentration
(μM/g)

Fig. 13 ATP content of innervated and denervated canine trachealis muscle.

sulted in a progressive increase in the contraction of the innervated tracheal segments from 5 to 21 mmHg, while little or no response was observed in the denervated muscle at the lower frequencies. Occasionally a small contraction of about 2 - 5 mm was observed in the denervated preparation when stimulated at 33 Hz. This may have been due to a passive spread of the electrical stimulus from the innervated to the denervated segment of the muscle.

Control studies, in which no surgical denervation was performed, showed that the normal trachea behaved like that of the innervated tracheal segments. Increased neural stimulation resulted in an increased muscular contraction along the entire length of the trachea.


A
16mmHg
1Hz
2Hz
3.3Hz
5Hz
10Hz
20Hz
33Hz
B
16mmHg
1Hz
2Hz
3.3Hz
5Hz
10Hz
20Hz
33Hz
C
16mmHg
1Hz
2Hz
3.3Hz
5Hz
10Hz
20Hz
33Hz
D
16mmHg
1Hz
2Hz
3.3Hz
5Hz
10Hz
20Hz
33Hz

(111a)

Fig. 14 Evaluation of parasympathetic denervation in the dog.

A. represents posterior tracheal section in the normal dog

B. represents anterior tracheal section in the normal dog

C. represents anterior denervated tracheal section of the surgically denervated dog

D. represents theposterior innervated section of the surgically operated dog.

Chapter V

DISCUSSION AND CONCLUSIONS

Stimulation of the distal end of the trachea with carbachol, histamine or elevated potassium consistently caused tonic contractions. Since this end had intact innervation, it can be concluded that the surgical procedure, per se, did not alter the properties of this segment and allowed it to behave like the normal tracheal smooth muscle obtained from unoperated dogs. Likewise, it can be reasonably concluded from the pressure measurements and vagus nerve stimulation experiments that the proximal portion of the trachea, which showed rhythmic behavior to carbachol or histamine was indeed functionally denervated inspite of our not having done structural studies to prove the point beyond any doubt. The care taken in separating the cut section of the recurrent laryngeal nerve ensured that the denervation was well maintained over the maximum observation period of 20 days.

Our studies with 6-hydroxydopamine treated animals indicates that the denervation of inhibitory sympathetic nerves does not induce rhythmicity. In the previously cited studies done on spleen and vas deferens, it was sympathetic denervation which caused rhythmicity. The only common feature in these experiments and ours on the trachea was that in both cases rhythmicity was only produced by motor denervation.

Unlike receptor specific pharmacological agents, ele-

vated potassium caused toric contractions in both innervated and denervated muscles. The action of increased external potassium on tracheal smooth muscle differs from those agonists like acetylcholine in that tension development is related to a much greater extent on membrane depolarization (Farley and Miles, 1977). While it cannot be explained as to why agents depending on electromechanical coupling (e.g. potassium) are not successful in causing rhythmicity as compared to agents dependant on pharmacomechanical coupling, it does remain a consistent observation even in cases of rhythmicity induced by substrate deprivation (Bose and Bose, 1977).

Although it is generally accepted that tetrodotoxin specifically blocks the fast sodium channels in the membranes of excitable cells, there are reported exceptions in which this agent does not block sodium channels (e.g. puffer fish) (Gershon, 1967; Somlyo and Somlyo, 1970; Narahashi, 1972). This toxin is also known to block both preganglionic and postganglionic nerves, excited by transmural stimulation as well as through the action of various neurotransmitters including acetylcholine. The induced phasic activity observed in denervated canine tracheal smooth muscle appears to be completely unaffected by tetrodotoxin. This is not surprizing since it is known that tetrodotoxin does not inhibit spontaneous electrical activity in vertabrate smooth muscle or that produced by direct electrical stimulation (Kuriyama,

et al, 1966). Such a response, however, does indicate that the mechanical oscilations in the denervated tracheal muscle represents a phenomenon occuring in the muscle itself rather than an effect mediated through the release of transmitters from nerve terminals. It also provides evidence to suggest that the depolarizing phase of the electrical oscillations observed at the muscle membrane are due to some ion current other than sodium. If excitation and contraction are a coupled process, it would seem reasonable to suggest that the rising phase of the mechanical oscillations may be due to an increase in membrane permeability to calcium.

Differentiation of the mechanical trace revealed that the slow waves were made up of small but rapid oscillations which corresponded with the electrical changes in the membrane Whether the slow wave oscillations result from summation of the fast oscillations or the two represent independant phenomena is not yet clear. It is possible, however, that the slow phasic component may be due, at least in part, to fluctuations in chloride current. Such a mechanism has been proposed (El-Sharkawy and Daniel I975b) to describe the secondary depolarizations observed in the cat jejunum and for the fibrillatory behavior of the denervated skeletal muscle reported by Bryant and Camerino. Chloride is believed to have a substantial effect on the resting membrane potential of most smooth muscles

and it has been calculated that a chloride pump may contribute as much as 15 mV to the resting membrane potential of the taenia coli (Droogmans and Casteels, 1976). The membranes of normal mammalian skeletal muscle requires a high chloride conductance in order to maintain stability of it's excitable surface membrane and prevent abnormal repetitive firing or sensitivity to depolarization. In the denervated spontaneously active preparation the influence of the motor nerves (i.e. the action potentials or trophic factors) are no longer present and this is suggested as being responsible for preventing the muscle fibers from maintaining their normally high chloride permeability resulting in spontaneous phasic contractions (Bryant and Camerino, 1976).

Other characteristics of single unit muscles were also exhibited by the denervated muscle. One of them is the myogenic response to rapid stretching. Burnstock and Prosser (1960) have shown that myogenic response occured only in single-unit smooth muscle and is probably as a result of membrane depolarization in response to mechanical perturbation. Myogenic response was only elicited in the denervated tracheal muscle in the presence of carbachol after rhythmic contractions had been established. The response was absent in the resting and carbachol stimulated innervated muscles as well as in the resting denervated muscle. This implies that stretch induced membrane events can only occur after the excitability

of the cell is altered by carbachol. However, it seems that denervation sets up the conditions necessary to allow this to happen.

Multiunit smooth muscles show a characteristic absence of action potentials or oscillatory slow potentials when stimulated. It has been demonstrated that this is the case with canine tracheal smooth muscle (Kroeger and Stephens, 1975; Bose and Bose, 1977). TEA induced rhythmic activity is accompanied by a generation of action potentials (Kroeger and Stephens, 1975). Similarly it has been shown that rhythmicity due to substrate deprivation is accompanied by relatively rapid spike activity (ca. 1/sec) superimposed on slow oscillations of membrane potential (ca. 1/min.) (Bose and Bose, 1977). The pattern of electrical oscillations in the denervated tracheal muscle resembled that induced by substrate deprivation and there was a good correspondence between the rapid and slow components of depolarization and the accompanying mechanical oscillations. Given the limitations of the microelectrode method in which the electrical activity is recorded from a single cell whereas the mechanical response is the average response of all the smooth muscle cells in the preparation, the close coupling of the mechanical and electrical activities were striking.

Several other differences between tonic and phasic (rhythmic) contractions are obvious. Reduction of extracellular calcium caused preferential inhibition of phasic

contractions suggesting that different calcium pools may be involved in the two types of contractions. This impression was further strengthened by the observation that D-600, a calcium current inhibitor (Fleckenstein, et al, 1971), preferentially inhibited the phasic response. It will be interesting to see whether D-600 affects the rapid or slow components of the electrical oscillations. However, these will have to wait until further studies are done. The effect of elevated calcium in abolishing phasic activity is more difficult to explain. However, calcium subserves many different functions in the excitation-contraction coupling process. Besides acting as an activator, membrane bound calcium also acts as a stabilizer and controls ionic permeability (Frankenhaeuser and Hodgkin, 1957; Hurwitz, et al, 1967). This may be an explanation for the effect of high calcium concentrations on phasic oscillations. An increase in the tonic component of tension when the calcium concentration was increased could also result in an increase in the intracellular stores of calcium to the point where the active uptake mechanisms by the sarcoplasmic reticulum and/or the mitochondria became saturated and the contractile proteins would become maximally activated. The increase in tension and loss of phasic activity observed with the addition of 2.5mM calcium above the normal concentration may be due to a saturation and maximal activation of the contractile elements within the muscle fibers. It is just as likely, however, that the increased

extracellular calcium has a stabilizing effect on the excitable surface membranes of the smooth muscle cells.

The connection between metabolism and oscillatory phenomena in the cell has been postulated for a long time. Recent evidence to strengthen this comes from studies in which oscillatory behavior could be induced by the manipulation of the cell ATP content. Thus tracheal rhythmicity could be induced by substrate deprivation and this event was accompanied by a decrease in ATP content (Bose and Bose, 1977). Similarly the Poky mutant of neurospora crassa which has a defect in the oxidative phosphorylation process, exhibits electrical oscillations which are quite unlike the behavior of the wild strain which has a higher ATP content (Gradman and Slayman, 1975). It was interesting to note that parasympathetic denervation of tracheal smooth muscle also led to a decrease in ATP content compared to control innervated muscle strips obtained from the same animal. Thus the effect of denervation on ATP content in guinea pig vas deferens, first shown by Westfall, (1975) seems to be applicable to other muscle types as well.

The exact mechanism by which a low ATP concentration can influence muscle properties to cause the oscillatory phenomenon can only be conjectured. Fluctuations in the internal ATP concentration in the vicinity of the contractile proteins seem to be an unlikely possibility because in that

case elevated potassium should also have caused rhythmicity. This was not the case. There is wide spread belief that ATP within the cell may be compartmentalized (Gudbjarnson, et al, 1970). It is therefore conceivable that fluctuations in the ATP concentration in the vicinity of the plasma membrane can turn on or off an electrogenic sodium pump (El-Sharkawy and Daniel, 1975b). It is also possible that varying ATP concentrations may influence calcium pumping and may lead to fluctuations in the level of calcium in the vicinity of the inner face of the membrane. It is well known that intracellular calcium can influence potassium conductance and thus vary the membrane potential. Sperelakis and Schneider (1976) have proposed similar changes in metabolically impaired cardiac muscle.

Both cooling and ouabain , a specific inhibitor of the sodium-potassium pump (Skou, 1965) were found to abolish phasic activity. In both cases the abolition of rhythmic contractions was accompanied by an increase in baseline tension. At first sight this would seem to favour the possibility that oscillation of an electrogenic sodium pump is responsible for rhythmicity (Prosser and Bortoff, 1968). On closer examination of the ouabain effect, one could easily discern that phasic oscillations disappeared before the basal tension reached the level of the peak of each phasic contraction. If the entire rhythmic contraction was due to turning

off of the pump then inhibition of the pump should have caused the phasic contractions to subside only when the basal tension equalled the peak of the phasic contraction. Therefore, it is unlikely that the electrogenic sodium pump is the only mechanism governing rhythmicity even though ouabain had an inhibitory effect. Ouabain has been found to influence the intracellular concentration of other ions besides sodium and potassium. Casteels (1971) has found ouabain to decrease intracellular chloride concentrations. Normal intracellular chloride levels are in excess of the amount expected on the basis of the Gibbs-Donan equilibrium. This has resulted in the belief that an inwardly directed chloride pump exists in smooth muscle and that the resulting electrochemical chloride gradient can contribute to the membrane potential. Whether ouabain has an effect on the chloride pump independently of it's effect on the sodium pump or whether the two are linked remains to be known. Nevertheless, if oscillations in chloride permeability are contributing to the membrane potential oscillations, then ouabain is likely to abolish rhythmicity by reducing the electrochemical gradient. A possible role of chloride ions in the generation of rhythmic activity in cat jejunal smooth muscle has been suggested by El-Sharkawy and Daniel (1975c), and in view of the inhibitory effect of chloride depletion on rhythmic activity in tracheal smooth muscle, it is tempting to postulate that

chloride ions may be important in the oscillatory phenomenon in smooth muscle as has been suggested in the case of denervated skeletal muscle (Camerino and Bryant, 1976).

In summary, many of the changes in tracheal smooth muscle properties following motor but not inhibitory denervation are suggestive of a conversion from a multi to a single unit type. These changes are similar to the ones seen following rhythmicity induced by substrate deprivation. The precise mechanism by which low ATP concentrations can induce membrane instability remains to be elucidated.

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