STUDIES ON CONTRACTILITY AND EXCITABILITY OF RAT UTERINE SMOOTH MUSCLE

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ABSTRACT

The purpose of this research was to study electrical and mechanical events in smooth muscle under normal conditions and where excitability and contractility were modified. Changes in endocrine condition of the living animal can effect chemical or physical changes in smooth muscle cells of the uterus which are reflected in the behavior of *in vitro* preparations. This study was done on smooth muscle of rat uterus preparations *in vitro*. Some basic properties of rat uterus were examined under various hormonal influences to provide a basis for selection of a suitable preparation for the determination of changes that occurred after procedures modifying excitability and contractility.

Experiments on the effects of oestrogen, progesterone and pregnancy are reported. Uterus from oestrogen dominated and twenty-one day pregnant rats showed regular spontaneous contractions regulated by action potentials from a discrete pacemaker area located near the ovarian end of preparations. Uterus from animals either treated with oestrogen and progesterone or seventeen days pregnant showed irregularity of contractions and action potentials concluded to be due to multiple pacemaker areas firing asynchronously.

The uterus *in vitro* did not significantly differ in resting membrane potential from the *in vivo* preparation, indicating that the uterus *in vitro* could maintain the membrane potential. Variations in ionic content of tissues occurred without change in resting membrane potential, and vice versa. Determination of extracellular space and calculation of electrolyte content of uterus indicated that control of resting membrane potential in uterine smooth muscle is complex.

Uterine preparations could be specifically desensitized to one agon-

ist or to two different types of agonist in series. The muscle still had a normal resting membrane potential and gave a normal contraction in response to washout of the bath. When a preparation was desensitized to three different agonists, resting membrane potential was normal. The tissue contracted in response to washout, but without change of potential. The desensitization was still of the specific type, because unspecific desensitization results in a muscle which is unresponsive to washout and has a depolarized membrane. This appears to be the first clear demonstration of contractions in a normally polarized tissue without accompanying action potentials or membrane depolarization, occurring in a system where the effective stimulus normally includes action potentials and depolarization.

Caffeine disrupted close cellular junctions and invaginations and reduced the number of cytoplasmic vesicles. Increased calcium or lowered sodium concentration in the bathing fluid, or ouabain reversed the inhibition produced by caffeine. These experiments indicated that caffeine probably depleted or displaced calcium from membrane or other cellular sites.

Cocaine increased the duration of spontaneous contractions, increased conduction velocity, lowered the threshold for electrical stimulation, and induced multiple pacemaker areas. Reserpine increased the frequency and maximum developed tension of spontaneous contractions, increased conduction velocity, lowered the threshold to electrical stimulation, and increased the number of active pacemaker cells in a discrete pacemaker.

It is concluded that cocaine and reserpine produced their effects by altering calcium utilization by the cells of rat uterus.

To my beloved wife and children,

Norah, Ross, Linda and Morag,

my parents and my dear friend, Roddie.

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The bridegroom may forget the bride
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That smiles sae sweetly on her knee;
But Ian, I'll remember thee
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INTRODUCTION AND STATEMENT OF THE PROBLEM

Nasse, 1879 and Mayer, 1881 who reviewed the physiology of smooth muscle, commented on the paucity of existing information. This state of affairs still persisted in 1926, when Lovatt Evans stated that properties of smooth muscle in any one organ may differ markedly between species and that most smooth muscle receives a dual innervation of approximately opposing functions. In spite of the fact that almost one hundred years have elapsed, and literally thousands of papers on various aspects of smooth muscle have been published, similar comments are found in almost every review available on smooth muscle. (Fischer, 1944; Burnstock, Holman and Prosser, 1963). Recent reviewers have circumvented some problems by writing about advances made on a particular type of smooth muscle (Somlyo and Somlyo, 1968; Holman, 1969) or on a particular aspect of smooth muscle (Bohr, 1964; Ruegg, 1971).

There is general agreement why results and conclusions of experiments on smooth muscle are somewhat ambiguous. Smooth muscle usually occurs as part of a complex tissue, frequently associated with secretory epithelium, nerve cells, plexuses or endings. Cell size is usually 2-5 μ in diameter and this gives further problems in electrophysiological studies. The activity of smooth muscle is sensitive to small changes in environment and can give variable responses to stimuli. Some smooth muscles display automaticity and their tonus (expressed as basal length or tension) varies with time. Finally, the properties (physiological and pharmacological) of smooth muscle depend on the organ, age, sex and species and many more variables, e.g. hormonal state. A comparative approach is therefore mandatory

when analysing drug effects, since information gained from experiments on one type of smooth muscle cannot always be extrapolated to all types.

Although the effect of a given drug on smooth muscle with subsequent contraction or relaxation is usually interpreted in terms of an interaction between the drug and a specific receptive substance or receptor of the cells of the effector organ system, smooth muscle activity is also controlled by activation or suppression of electrical activity over the cells of a smooth muscle organ. That smooth muscles can respond to chemical stimuli by contraction or relaxation (Evans, Schild and Thesleff, 1958; Daniel, Sehdev and Robinson, 1962) though completely depolarized led to the concept of "pharmacomechanical coupling" (Somlyo and Somlyo, 1968). However many workers ignore the fact that ability of a drug to further stimulate a tissue already depolarized does not exclude the requirement of depolarization as a crucial part in the normal excitation process.

The final response of many pharmacological experiments is represented on some type of recorder as a trace of the mechanical events of a tissue, and some workers tend to ignore that between the application of a given stimulus and the resultant mechanical effect, there has occurred a complex series of events usually classified as excitation-contraction coupling. Though widely studied in smooth muscle, excitation-contraction coupling is poorly understood (Somlyo and Somlyo, 1968). There is, however, general agreement that with many drugs the process involves (1) receptor occupancy (2) membrane phenomena, usually depolarization of the cell membrane and ion fluxes (3) movement of calcium ions to the contractile proteins, actin and myosin. The resultant contraction or

increase in tension is due to the sliding of filaments of the contractile proteins over one another (Hanson and Huxley, 1955).

The sequence of events with drugs which have not as yet been identified with specific receptors, e.g. papaverine, or when the stimulus is physical rather than chemical, e.g. stretch or light, has not been extensively studied and offers intriguing alternatives for activation of the contractile mechanisms.

A more comprehensive discussion of phenomena involved in excitation and contraction processes follows.

CELL MEMBRANE

Since the formulation of the cell theory of life by Schwann and Schleiden about 1840, it has been customary to regard the function of any organs, or indeed of the whole body, as the sum of the function of its constituent cells. Nageli and Cramer (1855) observed that cells swell in hypotonic solutions and shrink in hypertonic ones and compared the cell with an osmometer. The analogy required a membrane, an idea reluctantly accepted because no one had even seen a cell membrane, which is not surprising as it was only with the advent of the electron microscope that cell membranes were really visualized.

Studies by many workers on permeabilities, electrical resistance, surface tension and refringence of cell membranes gave rise to the Davson - Danielli model of a cell membrane, which consists of a bilayer of lipids coated with protein. Preparation of artificial membranes and determination of the effects of drugs on various properties of such membranes is now a full time occupation of quite a number of scientists and has pro-

vided useful information especially in predicting effects of drugs on membranes of living cells.

MEMBRANE COMPOSITION

Before analysis of the chemical composition of the cell membrane can be done, one has first to separate it from the rest of the cell. Disruption of cells is usually performed by sound, osmotic disturbances, or mechanical homogenization. The disrupted membranes rearrange into small vesicle-like structures which can be separated from other cellular components by density gradient centrifugation and other biochemical techniques. The problems of whether membranes extracted in this manner are representative of the intact membrane, whether some membrane fragments go into the discarded cellular fractions, and whether membrane fractions might also contain intracellular components are not yet resolved. A succinct discussion of the purification - recognition problem is given by Wallach (1965).

The three major components of membranes are proteins, lipids and water, and between cells from different tissues there is a wide variation in the proportion in which proteins and lipids enter into their composition (Maddy, 1966). The extraction and purification of membrane components which retain the physiological properties with which they were endowed when present in the intact cell has not yet been highly successful and points out the fact that the organization of the intact cell structure may well confer many of the properties peculiar to a particular component of a cell. In smooth muscle there are few reports on the chemical composition of the cell membrane or on the properties of isolated

membranes, though Allen and Daniel (1970), have reported on the isolation of rat myometrial membrane preparations and the activity of related sodium-activated ATPase.

MEMBRANE ULTRASTRUCTURE

Electronmicroscopy established that individual smooth muscle cells have clearly defined membranes about 15 mμ thick in rat uterus and about 25 mμ thick in rat ureter. Caesar, Edwards and Ruska (1957) showed that the cell membrane of smooth muscle can be differentiated into three regions: an opaque basement membrane, an interspace and a dense plasma membrane. Plasma and basement membranes vary greatly in thickness and the densest or thickest areas often lie at corresponding areas in adjacent cells. In some muscles the membranes of neighbouring cells lie approximately parallel; in others the membranes are extremely convoluted and irregular and form complicated relationships with each other (Prosser, Burnstock and Kahn, 1960).

The most distinctive feature of visceral smooth muscle is the profusion of vesicles, found as spherical invaginations of the plasma membrane and lying free in the cytoplasm. Sizes reported vary between 10 to 400 mμ. Mitochondria are often abundant behind regions populated with vesicles. The possibility that these vesicles may play a role in the exchange of ions or other physiological and pharmacological agents is indeed of great interest.

The question of the relationships of individual muscle cells to one another is of great functional importance. Is there a true syncytium? If not, as electrical transmission is impossible over large intracellular

distances because of short circuiting by extracellular fluid, how is cell to cell communication established, so that the whole muscle can act as a functional syncytium? Areas of close apposition of cell membranes have been suggested as the regions where conduction of excitation might occur (Caesar, Edwards and Ruska, 1957; Moore and Ruska, 1957; Yamamoto, 1960; Dewey and Barr, 1962). Areas where adjacent cell membranes appear fused are called nexuses, and areas of close apposition have been called tight junctions, peg and socket junctions, etc. Controversy still exists as to the functional importance of such areas and if indeed they actually exist in living cells or are artifacts produced by histological procedures.

MEMBRANE POTENTIALS

Among the most intriguing features of all living cells is that the interior contains an entirely different ionic composition from that in the fluid surrounding the cell and there is an electric potential difference across the cell membrane. Some mechanism(s) must be present to maintain intracellular ion concentrations reasonably constant since there is continual exchange of ions across the cell membrane. There are three mechanisms generally invoked to explain the existence of the membrane potential. These mechanisms may act alone or in concert.

DONNAN EQUILIBRIUM

Donnan equilibrium can account for a sizable membrane potential without participation of any active transport process. In a Donnan equilibrium the membrane potential is generated by the diffusion of permeating ions down their concentration gradients, the asymmetric distribu-

tion of ions is caused by the presence of charged nondiffusible ions, e.g. proteins, inside the cell.

ELECTRICALLY NEUTRAL ION PUMPS

This hypothesis says that there is a metabolic transport system for sodium and potassium which can move both these ions uphill, i.e. from regions of lower to higher electrochemical potential. This sodium and potassium transport is mediated by a single system so that the outward movement of sodium is coupled to an inward movement of potassium. Implicit in the hypothesis of a coupled sodium-potassium pump is the idea that it is electrically neutral. At first it may seem that an electrically neutral pump could not be responsible for a large potential difference across the cell membrane. However most cell membranes have selective permeability properties and so the large differences in concentrations created by a sodium-potassium pump could generate large membrane potentials. If the membrane is permeable to several ions, sodium, potassium and chloride then the membrane potential (E_m) will be a function of the permeabilities and concentration differences for all these ions. This is stated mathematically by the Goldman equation.

ELECTROGENIC ION PUMPS

Until recently ion pumps were generally thought to be electrically neutral. However there is no reason why an ion transport system cannot cause a net movement of charge and thus make a direct and immediate contribution to the membrane potential. This type of ion pump is described as electrogenic. There is now a growing body of evidence that ionic transport processes can be electrogenic (Keynes, 1969; Taylor, Paton and Daniel,

1970; Bose, 1971), although the unequivocal demonstration of an electrogenic pump is not an easy task because various combinations of membrane permeabilities can prevent the pump from greatly influencing the membrane potential. The demonstration of electrogenic pumping in tissues which, after sodium enrichment in cold potassium-free medium, were made to recover in the presence of potassium in the external medium is thought-provoking but not conclusive evidence such pumps exist under physiological conditions.

ASSOCIATION INDUCTION HYPOTHESIS

Ling (1953) proposed an alternate hypothesis to the classical "pump" hypothesis and stated that the distribution of substances across a cell membrane is not based on "uphill" movement but in the adsorption of solutes on specific sites or exclusion of solutes from a non-liquid cell water. Ling also stated that active transport of materials can and does occur in cells but does not explain all the movements of substances in and out of living cells.

In more recent years (Ling, 1967; Cope, 1969; Wiggins, 1970) the use of X-ray, infrared and nuclear magnetic resonance analysis to study the state of water in cells has alerted some biologists to the fact that cell water may not simply be there as a stage for biological reactions but may be present in an ordered or structured form and may be interacting with ions in such a way that the observed distribution of ions in living tissues is due to the difference in structure of intracellular and extracellular water. The organization of proteins and lipids in cells is such that most cell water is probably close enough to a hydrophilic surface to be influenced by it.

RESTING MEMBRANE POTENTIAL

The membrane potentials of spontaneously active smooth muscles are rarely completely steady and the term "resting potential" is usually applied to the maximum value (inside negative) reached by the membrane potential during pauses between individual spikes or bursts of action potentials. The resting membrane potentials of spontaneously active smooth muscles are generally lower than those of non-rhythmic smooth muscles, but even the latter appear to have resting membrane potentials lower than those of skeletal muscle.

The successful use of intracellular electrodes for measurement of membrane potentials in smooth muscle was first reported by Bülbring and Hooton (1954) and this has now been done with a variety of smooth muscles. The penetration of individual smooth muscle cells with microelectrodes has not been observed microscopically and it is therefore necessary to rely on electrical recordings to assess whether or not successful impalements are obtained. A complete discussion of the problems involved, degree of injury to the cell, tip potentials, etc. is given in a review by Shanes (1958).

The resting membrane potential of smooth muscles is generally thought to be dependent on the concentration gradients of sodium, potassium and chloride across the cell membrane and the permeability coefficients of these ions. The ionic theory predicts an inverse relationship between resting potential and external potassium concentration and studies in many smooth muscles have shown that the experimental results do not match theoretical predictions, or only do so over a narrow range in changes of external potassium concentrations. These deviations may be

accounted for by the fact that internal potassium concentration may change as external potassium concentration is changed or that the permeability for sodium and chloride ions may be important in determining the resting potential. The findings of Daniel and colleagues (1962, 1963, 1964) that the fluxes of sodium in smooth muscle are many times faster than those of potassium raises considerable doubt that the ionic basis of the resting membrane potential in smooth muscle is similar to that of skeletal muscle.

The role of chloride ions in regulating the membrane potential has not yet been fully elucidated and the general assumption that chloride is passively distributed is challenged by the finding of a high intracellular chloride concentration (Casteels, 1970). The reports that depolarization occurs when chloride is replaced by sulphate (at normal potassium concentration) also suggest that chloride ions may be involved in determining resting membrane potential (Burnstock and Straub, 1958; Marshall and Csapo, 1961; Kuriyama, 1963; Holman, 1968.

ACTION POTENTIALS

When a smooth muscle is active the membrane potential falls and action potentials occur, sometimes with a reversal of membrane potential. Various types of action potentials are found in smooth muscle, from slow oscillatory changes in membrane potential to spike type potentials. In many visceral smooth muscles, the spikes are initiated from pacemaker cells which display prepotentials as seen in pacemaker cells in cardiac tissue. Action potentials appear to be myogenic, as they are not affected by various adrenergic and cholinergic antagonists, ganglionic blocking

agents, or tetrodotoxin, (Bülbring, 1955; Kuriyama et al., 1966).

According to the ionic hypothesis action potentials are due to a specific increase in sodium conductance which changes the membrane potential to a value near the sodium equilibrium potential, followed by a delaved increase in potassium conductance which returns the membrane potential to its resting value.

The relationship between external sodium concentration and the action potential differs from that predicted by the ionic hypothesis in most smooth muscles. Amphibian stomach muscle is able to give contractile responses in solutions containing little or no electrolyte (Singh and Acharya, 1957) and Bogler (1960) and Kolodny and Van der Kloot (1961) obtained spontaneous contractions from frog stomach in isotonic sucrose containing 2 mM calcium chloride. That action potentials persist in very low sodium solutions may be taken as evidence that the ionic basis of spikes in smooth muscle differs from that in nerve or skeletal muscle. Spikes in crustacean skeletal muscle are partly due to calcium ions contributing to the inward current in the rising phase of the action potential (Fatt and Ginsborg, 1958; Hagiwara and Naka, 1964) and crustacean muscle, like smooth muscle, is resistant to tetrodotoxin, which is believed to block sodium conductance specifically (Nonomura et al., 1966).

Effects of changes in external calcium concentration are confusing in that depression and excitation have been observed by various workers after prolonged exposure to both high and low calcium concentrations. One explanation for these results may be that calcium, besides acting as a current carrier during excitation, can also regulate membrane

stability by binding to sites in the membrane (Hurwitz, Joiner and von Hagen, 1967).

The results obtained with changes in ionic environment suggest that smooth muscle is unique in that action potentials may be due to fluxes of different ions and that the ion carrying the greater part of the inward current can be varied by experimental conditions.

EXCITATION-CONTRACTION COUPLING

The effect of a given drug on a given effector system is usually interpreted in terms of an interaction between the drug and a specific receptor substance or receptor of the cells of the effector system. There is general agreement on much of the sequence of events in excitation-contraction coupling in striated muscle, namely that depolarization of the cell membrane by action potentials or other means initiates the contractile process and that calcium ions are the link between depolarization and contraction (Frank, 1958, 1960 and 1961; Bianchi and Shanes, 1961). Excitation-contraction coupling in smooth muscle, though widely investigated, is still poorly understood (Somlyo and Somlyo, 1968) and research on interaction of drugs with tissue receptors is still in its infancy, albeit a prolonged childhood (Ehrenpreis, Fleisch and Mettag, 1969).

Daniel (1964) justifiably commented that many workers in the field of drug-receptor interactions ignore the fact that smooth muscle activity is also controlled by spread of electrical activity over the cells of a smooth muscle organ or suppression of such activity and it is conceivable that some drugs act exclusively by their effect on electrical activity of smooth muscle cells.

Bozler (1948) divided vertebrate smooth muscle into two categories, single-unit and multi-unit muscles, according to their degree of dependence on an extrinsic nerve supply and their ability to respond in an all or none manner to various stimuli.

SINGLE-UNIT MUSCLES

Muscles of this category generally exhibit spontaneous rhythmic mechanical and electrical activity. The rhythmicity does not appear to depend on innervation, although it can be modified by nerve activity. Electrical activity can be propagated over long distances; this is consistent with the histological findings that single-unit muscles have areas of close apposition between cells (p. 6) where electrical activity could readily be conducted from cell to cell. Marshall and Csapo (1961) studying uterine smooth muscle potentials with microelectrode and sucrose gap techniques pointed out that in the sucrose gap technique the muscle is assumed to act as a core conductor. This can only be possible if spread of electrical activity can occur between cells. Single-unit muscles also contract in a coordinated all or none manner to diverse stimuli, such as stretch, light, temperature change, electrical stimuli and various physiological or pharmacological agents. Examples of single-unit smooth muscles include most visceral smooth muscles, smaller diameter arterial vessels and some veins.

MULTI-UNIT MUSCLES

Multi-unit muscles do not exhibit spontaneous rhythmicity, normally contract only in response to excitation of their nerve supply, do not show a myogenic stretch reflex, and give graded contractile responses to stimu-

lating agents when tested in vitro. Because of the small cell size and the amount of connective tissue in such muscles, extensive electrophysiological investigations have not been carried out. Those reported agree that multi-unit muscles usually do not show spikes but show a graded depolarization in response to stimuli (Cuthbert and Sutter, 1965; Holman et al., 1968; Somlyo and Somlyo, 1968). Examples of multi-unit smooth muscles are nictitating membrane, pilomotor muscles, spleen, rat vas deferens and large arteries, e.g. aorta and pulmonary artery.

In view of Daniel's comments (1964) (p. 12) it is interesting to note that early workers in the field of drug-receptor interactions who had little or no knowledge of the electrophysiological properties of smooth muscle made multi-unit muscles their organs of choice for study, or they altered the basic properties of single-unit muscles by cooling or exposure to low calcium solutions so that the muscles responded in a graded fashion to stimuli, i.e. as multi-unit muscles.

The ability of a smooth muscle to behave under different conditions in a single-unit or a multi-unit manner indicates that Bozler's arbitrary division can be misleading, and smooth muscles can be expected to exhibit a spectrum of activities between the two extremes.

RELATIONSHIP BETWEEN ELECTRICAL AND MECHANICAL ACTIVITY

Many workers agree that tension changes in rhythmic visceral smooth muscles are directly related to the frequency and degree of synchronization of spikes of component muscle cells provided the preparation does not exceed a critical length. This relationship between spike frequency and tension is apparent only if the spontaneous activity consists

of large simple spikes which are conducted throughout the whole preparation (Bozler, 1945; Jung, 1956; Melton, 1956; Woodbury and McIntyre, 1956). If the activity in different cells is asynchronous then the resultant tension does not reflect the electrical activity as seen in any individual cell. Daniel (1959) reported that such asynchrony was the probable explanation for those reports where membrane potential changes were found to occur during or after the onset of contraction.

However, dissociation between electrical and mechanical activity has been reported. Bülbring and Lullmann (1957) found that spontaneous electrical activity persisted in guinea-pig taenia coli exposed to dinitrophenol although tension changes were abolished, and Axelsson and Bülbring (1961) showed similar results in taenia coli bathed in glucose-free Krebs solution. In 1961 Axelsson also showed that spike activity persisted in the absence of contractile changes when taenia coli preparations were bathed in solutions in which sodium had been replaced by lithium, hydrazine or choline. Tension changes can still be elicited by many agonists even when smooth muscle is completely depolarized by potassium sulphate (Evans, Schild and Thesleff, 1958; Falk and Landa, 1960; Robertson, 1960; Edman and Schild, 1962; Evans and Schild, 1962). Tension changes are abolished by removal of calcium from the bathing medium.

The dissociation between potential changes and contractile responses led to the introduction of the term "pharmacomechanical coupling" by Somlyo and Somlyo (1968a), but none of the evidence bearing on this concept proves that depolarization of the membrane is not a prerequisite of activation of the contractile processes.

EXCITATION-CONTRACTION COUPLING

Excitation-contraction coupling is generally understood to mean those events that produce contractile responses or tension development after an excitable tissue has received an effective stimulus. The stimulus in smooth muscle can be chemical, e.g. neurohumoral transmitter, or other means (p. 13). Many workers describe the events after excitation of the tissue as being a chain of events, the length of this hypothetical chain being adjustable to suit the needs of the research worker. Only one fact is really known and accepted by all workers, that calcium ions are essential. This has been rediscovered by many workers since Ringer and Krebs showed that calcium ions were essential components of the bathing media they developed.

More direct evidence that calcium is intimately concerned with the contractile process was produced by Heilbrun (1940) who showed that of all the physiological ions that have been injected in small quantities directly into muscle cells, only calcium produced a contraction. Indirect evidence of the importance of calcium ions for contraction and contracture is substantial and has been extensively reviewed (Bohr, 1964; Daniel, 1964, 1965; Schatzmann, 1965). In striated muscle the major source of calcium that directly activates the contractile proteins actin and myosin is intracellularly located in the sarcoplasmic reticulium (Frank, 1958; Peachy and Porter, 1959; Bianchi, 1963).

The sinks and sources of calcium in smooth muscle are still an open and interesting question. Daniel, Sehdev and Robinson (1962) stated that release and combination of calcium at sites in the cell surface were the final common pathways for excitatory influences acting to

contract smooth muscle. Bohr (1964) stated that mechanical activity of smooth muscle was probably regulated by the concentration of ionized calcium in the neighbourhood of the myofilaments. The concept of various functional pools of calcium available for contractile processes arose from findings that contractile responses to various stimulating agents declined at different rates when calcium was withdrawn from the bathing medium (Durbin and Jenkinson, 1961; Edman and Schild, 1962; Hudgins and Weiss, 1968).

Daniel (1964, 1965) postulated an elegant scheme of interaction between various activators and inhibitors of smooth muscle and the various functional pools of calcium. Daniel (1963) studied diffusion time in rat uterus and proposed that a pool was weakly bound in the membrane and that this could regulate the access of extracellular calcium to the cell cytoplasm. Daniel in his later studies also called more tightly bound calcium "sequestered" and postulated that these two pools of calcium interacted together in a series, parallel or series/parallel fashion. A third pool of calcium which serves to stabilize the cell membrane has also been postulated (Daniel, 1965; Hurwitz et al., 1967).

The possible functional significance of vesicles found in smooth muscle membranes and cytoplasm has recently aroused interest in many workers. If the vesicles are produced by pinocytosis the calcium content could be as high as that of extracellular fluid, and could serve as a source of calcium for contraction; presumably vesicles could also act as a sink for calcium in the relaxation process (Somlyo and Somlyo, 1968; Somlyo and Devine, 1970; Wells and Wolowyk, 1971). Membrane and vesicular calcium may be the morphological correlates with the functional pools suggested by many workers.

STATEMENT OF THE PROBLEM

From the foregoing discussion it is obvious that knowledge of excitation-contraction processes in smooth muscle is still limited. The purpose of this research was to study electrical and mechanical events in smooth muscle under normal conditions and where excitability and contractility were modified. We chose to modify the responsiveness of smooth muscle by making tissues supersensitive and subsensitive because, by comparison, these procedures could help to elucidate mechanisms of excitation contraction in normal smooth muscle.

Also, studies on supersensitivity and subsensitivity have been carried out for more than a century and, though these problems have stimulated a vast amount of research, we still do not know the mechanisms which produce changes in sensitivity of tissues to stimulating agents. Electrical phenomena in supersensitive and subsensitive tissues have received very little attention. The objective of this study is therefore to determine relationships between electrical and mechanical events in normal, supersensitive and subsensitive smooth muscle.

SELECTION OF THE EXPERIMENTAL TISSUE

Most studies on modification of excitation-contraction coupling have been done on isolated tissues. These preparations have advantages over whole animal experiments, in that reflex effects are eliminated, more accurate predictions of actual drug concentrations at a site of action can be made, administration of drug is not limited by side effects, e.g. blood pressure, and enough strips can usually be obtained from a single organ to enable appropriate control experiments to be done at the same time.

Experimental conditions favoured by most workers in the field of supersensitivity and subsensitivity, e.g. low calcium, low temperature, are such that there is little or no spread of electrical excitation in tissues. Since we particularly wished to study this phenomenon, it was essential that the experimental tissue should satisfy certain basic requirements:

a) It should behave as a single-unit muscle so that conducted electrical responses could be studied.

b) It should have sufficient sensitivity to various stimuli to ensure responses adequate for quantitative analysis.

c) Supersensitivity and subsensitivity could be produced to stimuli tested in the in vitro preparation.

The organ chosen to satisfy these criteria was rat uterus, which behaves as a single-unit muscle when tested under "physiological" conditions. Previous work has shown that this tissue will respond to a variety of stimuli and that we can produce supersensitivity by various procedures (Davidson, 1970) and subsensitivity (Gaddum, 1937; Innes, personal communication). The uterus has other advantages in that the responsiveness of the tissue can be altered by hormones or pregnancy, various workers (Csapo, 1956; Marshall, 1959, 1962; Daniel, Sehdev and Robinson, 1962) have already established control values of some electrical parameters, and Daniel and colleagues have done extensive ionic and metabolic studies on rat uterus, so that we had some basic values with which we could make comparisons (Daniel and Daniel, 1957; Daniel and Robinson, 1960; Allen and Daniel, 1964; Batra and Daniel, 1970a, 1970b).

SECTION II

METHODS

GENERAL PROCEDURES

Female rats (Long Evans strain) were killed by a blow on the head. Uterine horns were removed as quickly as possible and placed in Krebs-Henseleit solution at 4°C. Strips or segments 20 mm long were cut and suspended horizontally in a plexiglass bath containing Krebs-Henseleit solution maintained at 37°C and bubbled with 95% oxygen and 5% carbon dioxide. The organ bath contained about 10 ml of bathing fluid and was drained and filled through openings at the bottom of the chamber. In some experiments an overflow procedure was used to change the bathing fluid. Strips were allowed to equilibrate for 1 h before tests were started. During this time the bathing fluid was changed every 5 min.

Tension developed by the muscle was recorded isometrically with a force displacement transducer (Grass FT 03), with a resting tension of 1 g. Electrical activity of single myometrial cells were measured with intracellular glass capillary microelectrodes filled with 3 M potassium chloride. Electrode resistance varied between 50-100 MΩ. Microelectrodes were mounted on a platinum wire of 0.004 inch diameter formed into a helix of 1 cm diameter and approximately 3 revolutions. Membrane potentials and tension were recorded on a Hewlett Packard 3960 tape recorder and monitored on a Hewlett Packard 141 B storage oscilloscope. Measurements of membrane potentials were made from projected photographic recordings.

EXPERIMENTAL PROCEDURES

I. VAGINAL SMEARS

A. STAGE OF OESTRUS

The stage of oestrus of the rats was determined by vaginal smears obtained by gently inserting into the vagina a cotton swab of the "Q Tip" type moistened with saline. From these slides were prepared and stained with methylene blue, and cells were examined cytologically.

B. FERTILIZATION TIME

Animals in oestrus were mated and the onset of pregnancy determined by vaginal smear. The findings of a bloody "plug" and spermatozoa in the vagina was regarded as evidence of fertilization. The day spermatoza were found was regarded as day zero of the twenty-two day gestation period.

II. TREATMENT OF ANIMALS WITH STILBOESTROL AND PROGESTERONE

Immature and mature virgin female rats were given intramuscular injections of stilboestrol and progesterone on the following regimen.

A. STILBOESTROL

Animals were given stilboestrol, 100 µg/kg, followed by a second injection of 100 µg/kg 24 h later.

B. PROGESTERONE

Animals were given progesterone, 2.5 mg/kg, daily for 3 days.

C. PROGESTERONE AND STILBOESTROL

Animals were given stilboestrol, 100 µg/kg, daily for four days. Animals were also given progesterone, 2.5 mg/kg, daily for three days, starting on the third day of stilboestrol treatment.

D. VEHICLE CONTROLS

With the same volumes and regimen the appropriate vehicle was injected to provide controls.

E. Uterine tissues were removed 24 h after the last injection from all animals given hormones or vehicle.

III. UTERINE PREPARATIONS

A. VIRGIN ANIMALS

Uterine horns from virgin rats were dissected free of mesometrial attachments and segments of horn, 20 mm in length, were suspended in organ baths with undyed terylene thread. Two loops attached at the ovarian end of the tissue were connected to stainless steel hooks fixed in the walls of the bath and a single thread at the cervical end led under a stainless steel guide to the force displacement transducer. The preparations were suspended so that the linea uteri and mesometrial sites lay parallel to the sides of the organ bath; thus myometrial cells probed by microelectrodes were not cells of the linea uteri, which appears to be a specialized conducting system and not representative of uterine smooth muscle cells (Melton and Salvidar, 1967).

B. PREGNANT ANIMALS

Uterine horns from pregnant rats (17 or 21 days gestation) were dissected free of mesometrial attachments and strips 20 mm long and 3 mm wide cut from the following areas: (1) interplacental sites (2) a section of uterus midway between the mesometrial attachment and the linea uteri.

Strips were suspended in organ baths with undyed terylene thread, as described for uterine horns of virgin animals. Unless otherwise stated

the single thread was attached to the end of the strip that corresponds to the cervical portion of the uterus and the loops attached to the end corresponding to the ovarian portion.

C. IN VIVO UTERINE PREPARATIONS

Twenty-one day pregnant rats were anaethetized by chloralose-urethane (1%, 10%) or pentobarbitone. A midline incision in the abdomen was made and skin and peritoneal muscles were stitched to a plexiglass cradle and the peritoneal cavity kept moist with Krebs-Henseleit solution. The temperature of the animal was controlled at 38°C by a Thermistemp termperature controller. Reference and ground electrodes were placed in the abdominal cavity and membrane potentials recorded from various sites in the uterus, corresponding to strips used in the in vitro studies.

IV. TREATMENT OF ANIMALS WITH RESERPINE

Reserpine depletes rat uterus of catecholamines and induces supersensitivity (Davidson, 1970). Animals were given reserpine, 1.0 mg/kg, intraperitoneally 24 h before an experiment. A check that reserpine had acted upon uteri was made by a catecholamine content assay by the method of Euler and Lishajko (1961).

V. BATHING MEDIA

The following bathing fluids were made with glass-distilled deionized water.

A. KREBS-HENSELEIT SOLUTION

NaCl 118 mM; KCl 4.7 mM; $CaCl_2$ 2.5 mM; KH_2PO_4 1.1 mM; Mg_2SO_4 1.2 mM; $NaHCO_3$ 25 mM and glucose 11 mM. Calcium chloride and magnesium sulphate were added after the other components were dissolved and the solution equilibrated with 95% O_2, 5% CO_2 for 30 min.

B. MODIFIED KREBS-HENSELEIT SOLUTION

1. Calcium Free Solution

The composition was similar to that of Krebs-Henseleit solution except that calcium chloride was omitted. No substitution was made to compensate for the change in osmolarity of the bathing fluid due to the omission of calcium chloride. For replacement of calcium chloride in the bathing media appropriate volumes of stock calcium chloride solution were pipetted to the fluid in the organ bath or the bathing fluid was replaced by standard Krebs-Henseleit solution.

2. Low Potassium Solutions

The potassium content of Krebs-Henseleit solution was varied by substitution of equimolar concentrations of sodium chloride and sodium dihydrogen phosphate for potassium chloride and potassium dihydrogen phosphate respectively.

3. High Potassium Solutions

In these solutions varying amounts of sodium chloride were replaced by equimolar concentrations of potassium chloride.

4. Low Sodium Solutions

In these solutions varying amounts of sodium chloride were replaced by equiosmotic concentrations of sucrose. The most commonly used low sodium solution contained 36 mM sodium, i.e. approximately 25% of normal sodium content.

C. ISOTONIC SUCROSE

A solution of 10% sucrose solution was prepared. In some experiments calcium chloride, 2.5 mM, was also added to this solution.

VI. DETERMINATION OF EXTRACELLULAR SPACE

Strips or segments of uterus were incubated in Krebs-Henseleit solution with or without drugs or in a modified bathing media. Inulin was used as the extracellular marker since it is believed not to penetrate cells. Inulin used was always obtained from the same source to minimise variation between batches of inulin (Bihler, personal communication). Inulin-methoxy-^{3}H was added to the incubating medium 30 min. before tissues were removed for analysis. When the incubation was over, preparations were blotted gently, weighed and digested with 0.3 ml Nuclear Chicago Stabilizer at 50°C for approximately 2 h. When digestion was completed, the digest was cooled, 0.2 ml of 9 N acetic acid was added and the solution thoroughly mixed with 10 ml of scintillation fluid. Samples were counted in a Phillips liquid scintillation spectrometer. The radioactive incubation medium was prepared by adding 0.5 ml to 10 ml of scintillation fluid, mixed and counted.

Another set of tissues was identically tested. Wet weight was determined after gentle blotting and dry weight after the tissue was dried in an oven at 150°C for 24 h and allowed to cool in a dessicator.

Inulin space was determined as follows.

$$ECS = \frac{\dfrac{\text{cpm } ^{3}H \text{ in total tissue water}}{\text{cpm } ^{3}H/\text{ml medium}}}{\text{wet tissue weight}} \times 100$$

VII. IONIC ANALYSIS

A. DETERMINATION OF POTASSIUM, SODIUM AND WATER CONTENT

A lidded platinum crucible was weighed without and with a tissue strip, placed in an oven at 80°C for 24 h, removed and allowed to cool in a dessicator and reweighed. This procedure was repeated till a constant weight was obtained. The crucibles with lid on were then placed in an incinerator at 200°C for 2 h, then the temperature increased to 400°C for 2 h and finally the temperature increased to 600°C for 24 h. The gradual increase in temperature reduces spluttering of tissue. The crucibles were placed in a dessicator to cool, then the residue was dissolved in 1.0 ml of concentrated nitric acid. The resulting solution was then appropriately diluted with glass-distilled water and the sodium and potassium concentrations were determined by a Perkin Elmer atomic absorption spectrophotometer.

B. INTRACELLULAR CONCENTRATIONS OF POTASSIUM AND SODIUM

From the experimental values of total sodium and potassium content, intracellular concentrations of these ions were calculated per litre cell water by the equation of Boyles et al., (1941).

$$Ci = \frac{Ct - Co \times E}{1 - (E + D)}$$

Ci and Co are the intracellular and extracellular concentrations of an ion species, Ct the total content of the tissue, E = extracellular space and D = dry weight/wet weight ratio.

C. DETERMINATION OF TOTAL TISSUE CALCIUM AND MAGNESIUM

A 0.2 ml aliquot of the nitric acid solution obtained when tissues were prepared for potassium and sodium analysis was diluted with

1% lanthanum chloride in 5% hydrochloric acid to a volume of 2 ml. Lanthanum chloride was used to prevent interference by tissue phosphates in the determinations. Tissue calcium and magnesium were then determined by atomic absorption spectrophotometry. The instrument was adjusted with a blank solution of 1% lanthanum chloride in 5% hydrochloric acid and all standards for calcium and magnesium were also prepared in lanthanum chloride-hydrochloric acid solution.

VIII. PREPARATION OF MICROELECTRODES

The glass used for preparing microelectrodes was a high quality pyrex glass tubing, O.D. 1.2-1.5 mm, which was cut into 6 cm lengths, the ends flame polished and thoroughly cleaned before use by soaking in acid solution, rinsed with double distilled water, soaked in an alcohol-acetone mixture, then dried and stored in a vacuum dessicator. Electrodes were prepared with glass fibres by the method of Tasaki et al. (1968) and pulled on a David Kopf 700 C vertical pipette puller. Electrodes were then stored dry, tip up, in a covered plexiglass holder and filled immediately before use by injection of the electrolyte solution into the pipette via a 2 inch 27 gauge hypodermic needle. The electrolyte solution used was 3 M potassium chloride solution which was prepared by dissolving potassium chloride in glass distilled water and the solution then filtered through a 100 mμ millipore filter under slight negative pressure.

Electrode resistance and tip potentials were measured by a Grass P 16 microelectrode D.C. amplifier. Electrodes were discarded if the resistance was less than 50 MΩ or their tip potential was greater than 5 mV.

IX. CONDUCTION VELOCITY

Conduction velocity was determined by penetration of cells 1 or 2 cm apart with two microelectrodes. Unless otherwise stated all values reported in this thesis were obtained from responses travelling from ovarian to cervical end of the preparation. Conduction velocities were measured as shown in Fig. 1. The action potential in the upper portion of the figure was the first action potential recorded from the electrode placed near the ovarian end of the tissue and the action potential in the lower part of the figure was the first action potential recorded from another electrode placed near the cervical end of the preparation. The distance between the electrodes and the time interval between these potentials were measured, and conduction velocity expressed in mm/sec.

Values of conduction velocities were obtained from spontaneous activity of the tissue or from responses produced by electrical stimulation via two platimum electrodes placed 1 mm apart near the ovarian end of the preparation (Punctate stimulation).

X. ELECTRON MICROSCOPY

A portion of uterus was quickly removed, immersed in cold fixative and sliced into 1 mm^3. The blocks were initially fixed for 1 h in 2% glutaraldehyde in phosphate buffer, pH 7.2, washed for 1 h in the phosphate buffer, and postfixed for 1 h in osmic acid (Millong, 1962). The tissues were stained *en bloc* in 2% aqueous uranyl acetate, dehydrated in alcohol and embedded in Epon (Luft, 1961). Sections were cut on a Reichert OMU 2 ultramicrotome, mounted on uncoated copper grids, and stained with Reynold's lead citrate (Sjöstrand, 1967). A Hitachi HS 8 electron microscope was used, operated at 50 KV.

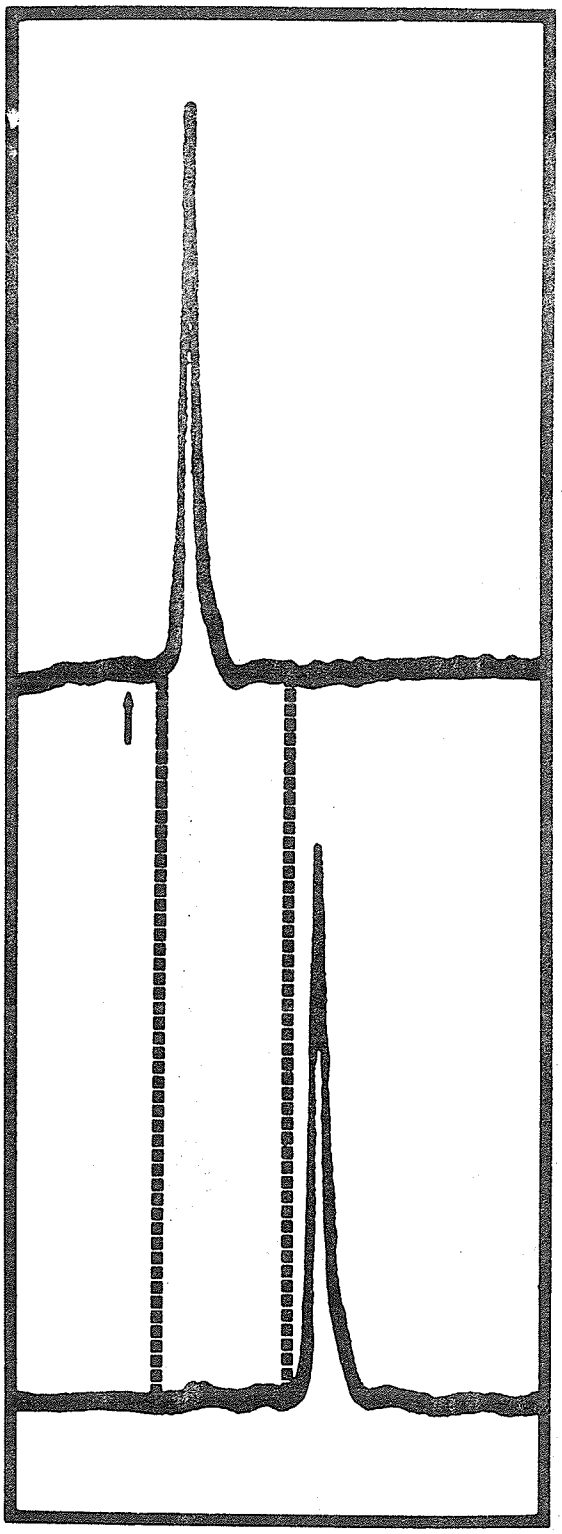

Fig. 1. Schema of determination of conduction velocities.

Action potentials were recorded from microelectrodes placed 2 cm apart in a uterine preparation. The value of conduction velocity was obtained by measurement of the time interval (indicated by the doted lines) between the potentials from calibrated oscilloscope sweep speeds. The arrow shows where an electrical stimulus was applied to the tissue.

XI. STATISTICAL ANALYSIS

Statistical significance was determined by the t-test for paired observations (Goldstein, 1964) in experiments where tests were done with a strip used as its own control. In comparison between different strips from the same or different rats statistical significance was determined by Student's t-test. All means are given with their standard errors. P values were obtained from a two tailed t table (Steel and Torrie, 1960).

XII. DRUGS AND EXPERIMENTAL MATERIALS

The compounds used in this study and the sources from where they were obtained are listed below. All solutions were made weight/volume in terms of the base unless otherwise specified. Stock solutions were stored at 4°C. Concentrations mentioned in the text are final concentrations in the bath fluid in g/ml. At no time was more than 1 ml of a testing solution added to the tissue bath.

Unless otherwise specified, stock solutions of the biogenic amines were made in 0.01 M HCl and other drugs in glass-distilled water. On the morning of use, the stock solutions were diluted as required with 0.9% sodium chloride solution.

TABLE 1

Acetylcholine chloride	Calbiochem
l-Adrenaline bitartrate	Mann Research Laboratories
Angiotensin amide	Ciba
Atropine sulphate	British Drug Houses
Caffeine	Calbiochem
Cocaine hydrochloride	British Drug Houses
5-Hydroxytryptamine creatinine sulphate	Calbiochem
Inulin Methoxy-^{3}H	New England Nuclear
l-Isoprenaline bitartrate	Winthrop Laboratories
Lanthanum chloride	Fisher
Ouabain	Nutritional Biochemical Corporation
Oxytocin	Sandoz
Papaverine	Smith, Kline & French
Phenoxybenzamine hydrochloride	Smith, Kline & French
Progesterone	Calbiochem
Reserpine	Ciba
Stilboestrol	British Drug Houses
Tetrodotoxin	Calbiochem

OTHER SOLUTIONS

Angiotensin amide (Hypertensin, Ciba) was prepared to the appropriate concentration from vials containing angiotensin amide, 2.5 mg, mannitol, 47.4 mg, and thimerosol, 0.1 mg, and the solution stored at 4°C.

Phenoxybenzamine hydrochloride (POB) (Smith, Kline and French) was kept as a stock solution containing 1 mg/ml in propylene glycol and 0.1 M HCl. Dilutions when required were made in 0.9% sodium chloride solution.

A stock solution of progesterone, 25 mg/ml, was made by dissolving progesterone in ethanol.

A stock solution of reserpine (Ciba) containing 5 mg of the base/ml was prepared for intraperitoneal injection; 100 mg reserpine was dissolved in a mixture of 2 ml glacial acetic acid, 2.5 ml propylene glycol, 2.5 ml ethanol and distilled water to 20 ml volume.

For stilboestrol appropriate dilutions were made in corn oil from ampoules containing stilboestrol, 5 mg/ml.

SECTION III

RESULTS

RESULTS

The relationship between membrane and myoplasmic activity is of special significance in spontaneously active smooth muscle, since factors affecting spontaneous membrane discharge can control contractile activity. Rat uterus is especially suited for study of these relationships in that the frequency and duration of contractions are usually governed by the frequency of action potential discharge, the duration of the train of action potentials and the total number of cells simultaneously and synchronously active.

There are reports that conflict with these findings and it appears that much of the disagreement is due to the use of uterine preparations from different strains of rats in different stages of pregnancy or under different hormone domination (Kao, 1958; Marshall, 1959; Goto, Kuriyama and Abe, 1960; Kuriyama, 1961).

Marshall (1959) and Kao (1961) reported that pacemaker cells of their preparations were not always restricted to any specific region of muscle, but could arise at random throughout the tissue. If such pacemaker activity occurs asynchronously, then membrane changes could occur during or after onset of contraction (Reynolds, 1949; Daniel, 1954; Jung, 1958).

Preliminary experiments were done to determine the characteristics of various uterine preparations and to find a preparation with a discrete pacemaker area and synchronously conducted action potentials. The term resting membrane potential (RMP) refers to the potential measured by an intracellular microelectrode relative to the extracellular fluid. The RMP has a negative sign but to conform with usual practice values of RMP are

given throughout this thesis without indicating their sign. Thus hyperpolarization refers to an increase in potential; depolarization refers to a decrease. The value of the RMP was taken as the maximum level of membrane polarization between trains of action potentials. In tissues where no action potentials occurred the RMP was taken as the maximum steady level of polarization on penetration of a cell by a microelectrode. Cells showing spontaneous slow depolarization followed by an action potential were taken to be pacemaker cells.

PARAMETERS OF RAT UTERINE PREPARATIONS

Values shown in Table 2 are the means ± SE of at least twenty determinations in each of six preparations from different animals.

IMMATURE ANIMALS

A. NO TREATMENT AND PROGESTERONE DOMINATED

Preparations from these animals were virtually inactive. They did not respond to stimulatory drugs, stretch or electrical stimulation. Resting membrane potential was around 35 mV.

B. OESTROGEN DOMINATED

The administration of oestrogen to immature animals raised the resting membrane potential to a mean value of 48 mV and tissues exhibited regular rhythmic contractile responses accompanied by trains of action potentials. A discrete pacemaker area was found near the ovarian end of the preparation and conduction velocity was constant over distances up to 2 cm.

C. OESTROGEN AND PROGESTERONE DOMINATED

When animals were treated with oestrogen and progesterone the value of the resting membrane potential was further increased to 58.3 mV.

TABLE 2

Parameters of various rat uterine preparations

Animal	Treatment	Resting Membrane Potential (mV)	Action Potential (mV)	Maximum Tension (g) per spontaneous contraction	Conduction Velocity mm sec.	Pacemaker Area
Immature	None	35	None	None	None	None
Immature	Oestrogen	48 ± 0.23	54 ± 0.42	5.9 ± 0.51	41.3 ± 1.7	Discrete
Immature	Progesterone	35	None	None	None	None
Immature	Oestrogen and Progesterone	58.3 ± 0.28	66.7 ± 0.32	3.8 ± 0.63	39.8 ± 2.2	Multiple
Mature	Preoestrus	48.3 ± 0.11	52.6 ± 0.11	5.7 ± 0.37	39.5 ± 2.4	Discrete
Mature	Dioestrus	49.6 ± 0.17	52.3 ± 0.19	5.2 ± 0.71	39.7 ± 1.8	Discrete
Mature	Oestrus	50.3 ± 0.21	56.5 ± 0.22	5.4 ± 0.79	41.1 ± 1.1	Discrete
Mature	Oestrogen	50.0 ± 0.23	54.8 ± 0.17	5.9 ± 0.55	47.8 ± 3.7	Discrete
Mature	Progesterone	59.1 ± 0.17	66.5 ± 0.23	4.1 ± 0.62	40.2 ± 7.1	Multiple
Mature	Oestrogen and Progesterone	61.2 ± 0.19	70.3 ± 0.21	3.9 ± 0.57	43.3 ± 6.4	Multiple
Pregnant	17 Day (A)	64.7 ± 0.19	72.3 ± 0.42	4.72 ± 0.17	67.1 ± 1.6	Multiple
Pregnant	17 Day (B)	60.2 ± 0.31	65.3 ± 0.37	4.95 ± 0.22	70.3 ± 2.1	Multiple
Pregnant	21 Day (A)	60.8 ± 0.42	69.2 ± 0.22	4.87 ± 0.18	58.3 ± 1.3	Discrete
Pregnant	21 Day (B)	50.3 ± 0.21	56.4 ± 0.32	5.31 ± 0.26	65.7 ± 2.0	Discrete

All values are the means ± SE of at least 20 determinations in each of 6 preparations from different animals.
(A) refers to preparations from interplacental sites
(B) refers to preparations from non-placental areas

This was significantly greater ($P < 0.01$) than that obtained from animals treated only with oestrogen. As shown in Fig. 2, the tissue gave frequent but irregular action potentials and contractile responses. Pacemaker cells could be found throughout the preparation. Conduction velocity could not always be measured, action potentials either not being recorded at the electrode near the cervical end or appearing before those recorded near the ovarian end. Values given in Table 2 may not be indicative of the true conduction velocity.

MATURE ANIMALS

A. OESTRUS CYCLE

Parameters of preparations obtained from mature rats in different stages of oestrus, did not differ markedly from each other or those of immature rats treated with oestrogen.

B. PROGESTERONE DOMINATED

In contrast to the immature rat dominated with progesterone, the mature rat treated with progesterone reacted like immature rats treated with oestrogen and progesterone.

C. OESTROGEN DOMINATED

Preparations from these rats gave values of measured parameters virtually identical to those of immature rats treated with oestrogen or mature rats in different stages of oestrus.

D. OESTROGEN AND PROGESTERONE DOMINATED

Results from these rats were similar to those obtained in immature rats treated with oestrogen and progesterone.

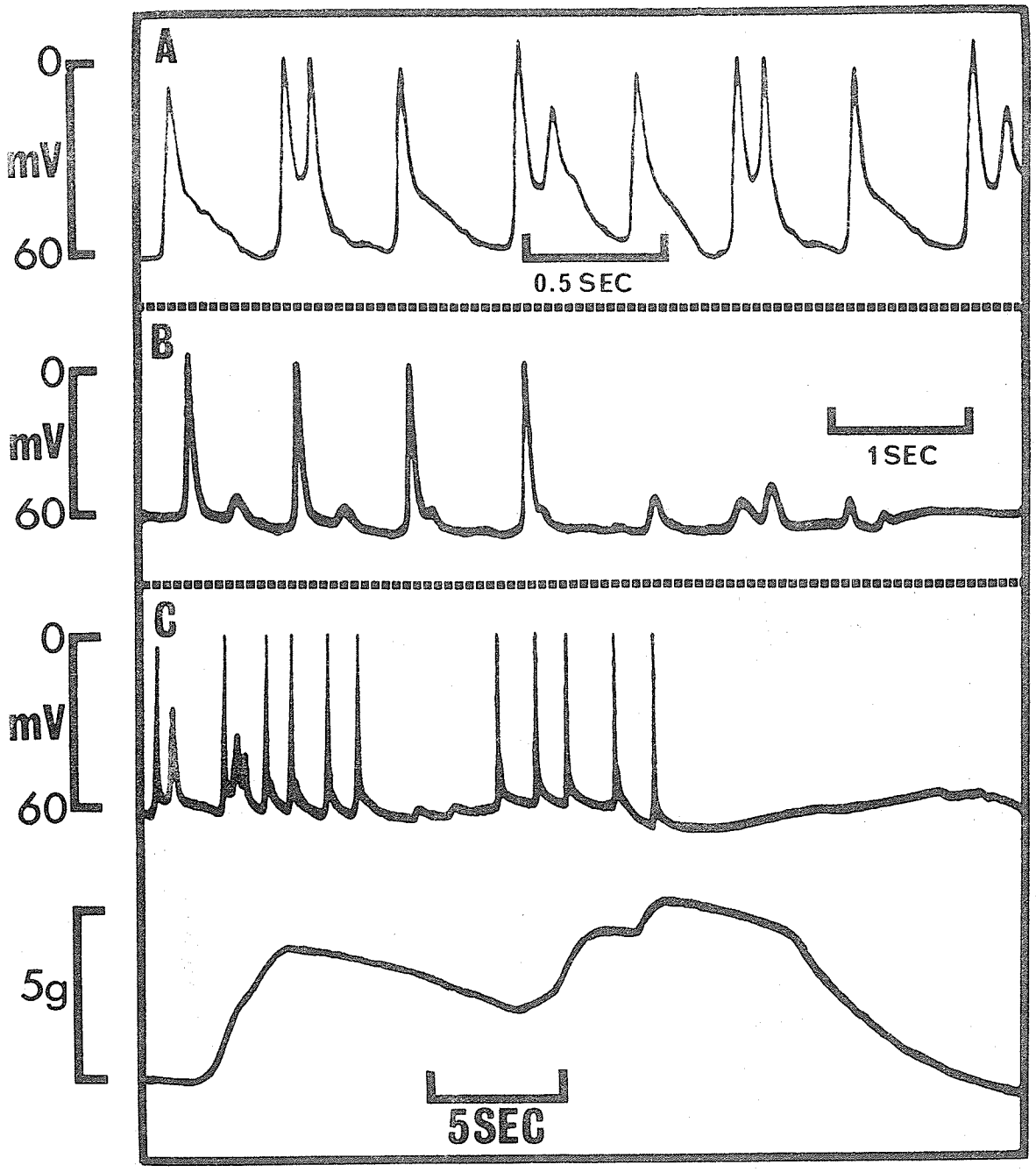

Fig. 2. Spontaneous contraction of uterus.

In A and B configuration of typical potentials seen in these preparations are shown on an expanded scale. In C membrane potentials and tension changes found in preparation from rat treated with oestrogen and progesterone.

PREGNANT ANIMALS

A. SEVENTEEN DAY PREGNANT

Two preparations were obtained from rats seventeen days pregnant (determined by vaginal smears).

1) Interplacental Strip

These preparations showed the greatest value of resting membrane potential and action potentials, and were similar to preparations from rats treated with oestrogens and progesterone in that contractile responses and action potentials were irregular and pacemaker activity was found throughout the tissue.

2) Non-placental Strip

These preparations were similar to interplacental strips except values of resting membrane potential and action potentials were lower ($P < 0.05$).

B. TWENTY-ONE DAY PREGNANT

Two preparations were obtained from rats twenty-one days pregnant (determined by vaginal smears).

Both interplacental and non-placental strips gave regular contractile responses and discrete pacemaker sites were found near the ovarian end of the tissue. Values of resting membrane and action potentials were such that values of interplacental strips were similar to those obtained from non-placental strips in the seventeen day pregnant rat, whereas values of non-placental strips were similar to those obtained from oestrogen treated rats.

The most striking finding was that conduction velocities were significantly faster ($P < 0.01$) in all preparations from pregnant rats

than in preparations from nonpregnant animals. We have reservations as to the actual value of conduction velocity in preparations showing multiple pacemaker sites.

During the experiments done to establish parameters of various uterine preparations we observed a number of intriguing phenomena.

CRITICAL MEMBRANE POLARIZATION

In a few preparations from immature rats treated with oestrogen, some cells showed a value of resting membrane potential (35 mV) similar to those found in untreated animals. When the tissue contracted spontaneously or was activated by drugs or electrical stimulation, hyperpolarization occurred before action potentials were seen. A typical record is shown in Fig. 3. Cells exhibiting this phenomenon were not pacemaker cells and were found only near the cervical end of the preparation. Action potentials appeared normal and membrane potential returned to the original resting potential between tissue responses.

In general, agents which depolarize the cell membrane lead to increased excitability. However, hyperpolarization resulted from treatment of immature rats with oestrogen, and this was accompanied by initiation of rhythmic contractile responses. This suggests that there may be a critical level of membrane polarization required before action potentials can be generated. In some preparations those cells near the cervical end of the uterus that had not reached this level of polarization apparently could be moved to the critical level by propagated impulses.

This explanation was tested in cells with a subcritical level of

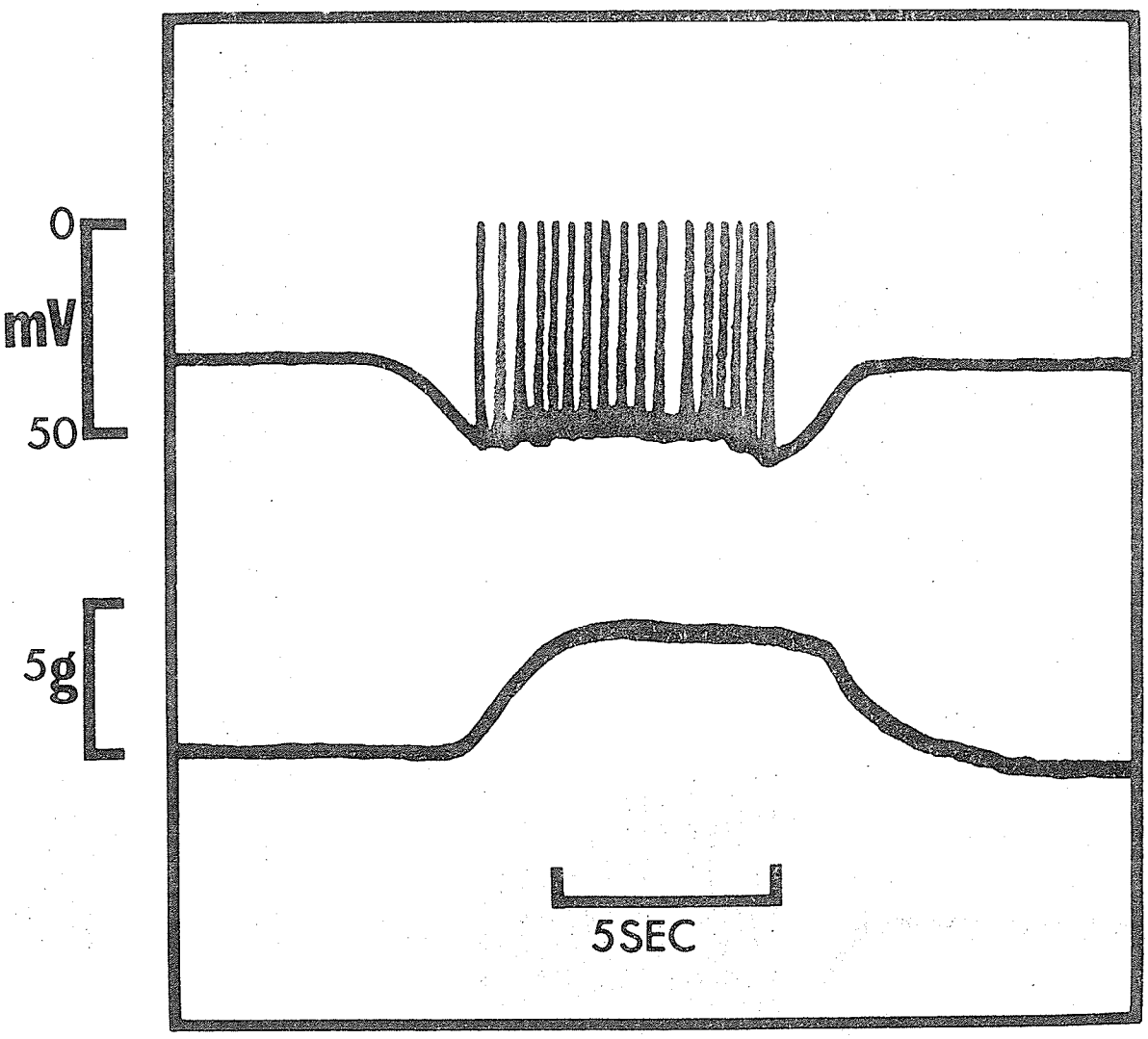

Fig. 3. Spontaneous contraction of uterus.

Hyperpolarization observed before action potentials occur in record from cell near cervical end of preparation from immature rat treated with stilboestrol.

membrane polarization. The cell was hyperpolarized by exposure to adrenaline 10^{-6} g/ml, or isoprenaline 3×10^{-7} g/ml. The tissue was then stimulated with acetylcholine 3×10^{-6} g/ml, and contractile and electrical responses were observed. No further hyperpolarization was noted before action potentials appeared. A typical record is shown in Fig. 4. Removal of adrenaline and isoprenaline returned the membrane potential to the original resting value and stimulation of the tissue produced responses similar to that shown in Fig. 3.

Because tissues treated with adrenaline and isoprenaline also became quiescent and showed a fall in resting tension, we tested the effect of 1×10^{-5} M papaverine. This treatment produced quiescence and a similar fall in resting tension but did not affect resting membrane potential. When the preparations were stimulated with acetylcholine 3×10^{-6} g/ml, contractile responses were obtained but the membrane potential again became hyperpolarized before action potentials occurred.

IMPROPER PENETRATION ARTIFACT

Occasionally in preparations from mature rats, cells showed low values of resting membrane potential and would also apparently become hyperpolarized when the tissue became active. However, action potentials began before or during the apparent hyperpolarization and the height of such potentials was always small (Fig. 5). The apparent hyperpolarization was concluded to be an artifact from cells improperly impaled by microelectrodes, since the initial change of potential upon penetration was slow, rather than the abrupt change noted when a proper penetration was done. In addition such recordings could never be maintained, the

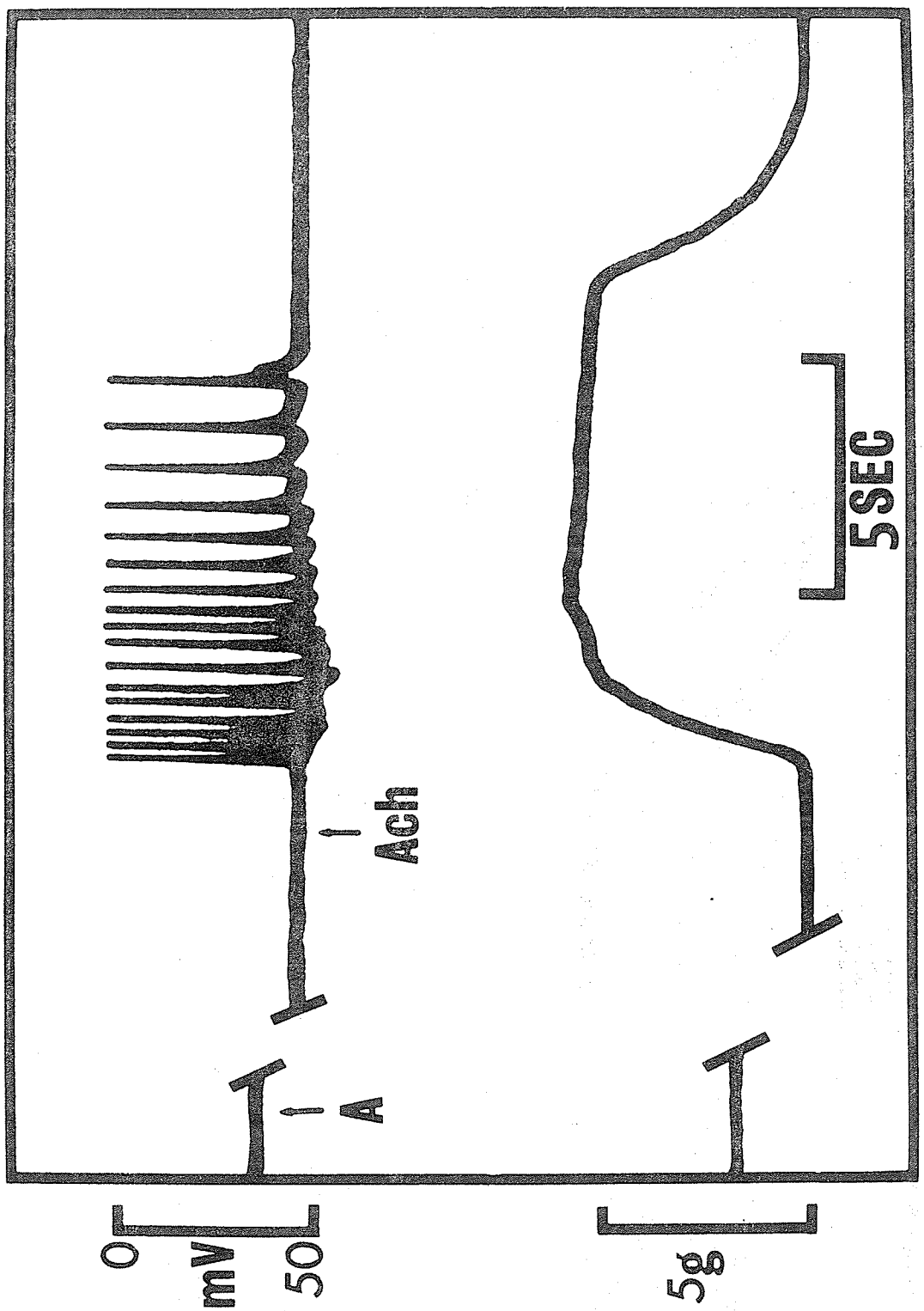

Fig. 4. Effect of adrenaline and acetylcholine on membrane polarization and tension in uterine preparation from immature rat treated with oestrogen.

At A adrenaline, 10^{-6} g/ml. was given and 90 sec later acetylcholine. 3×10^{-6} g/ml. was given at Ach.

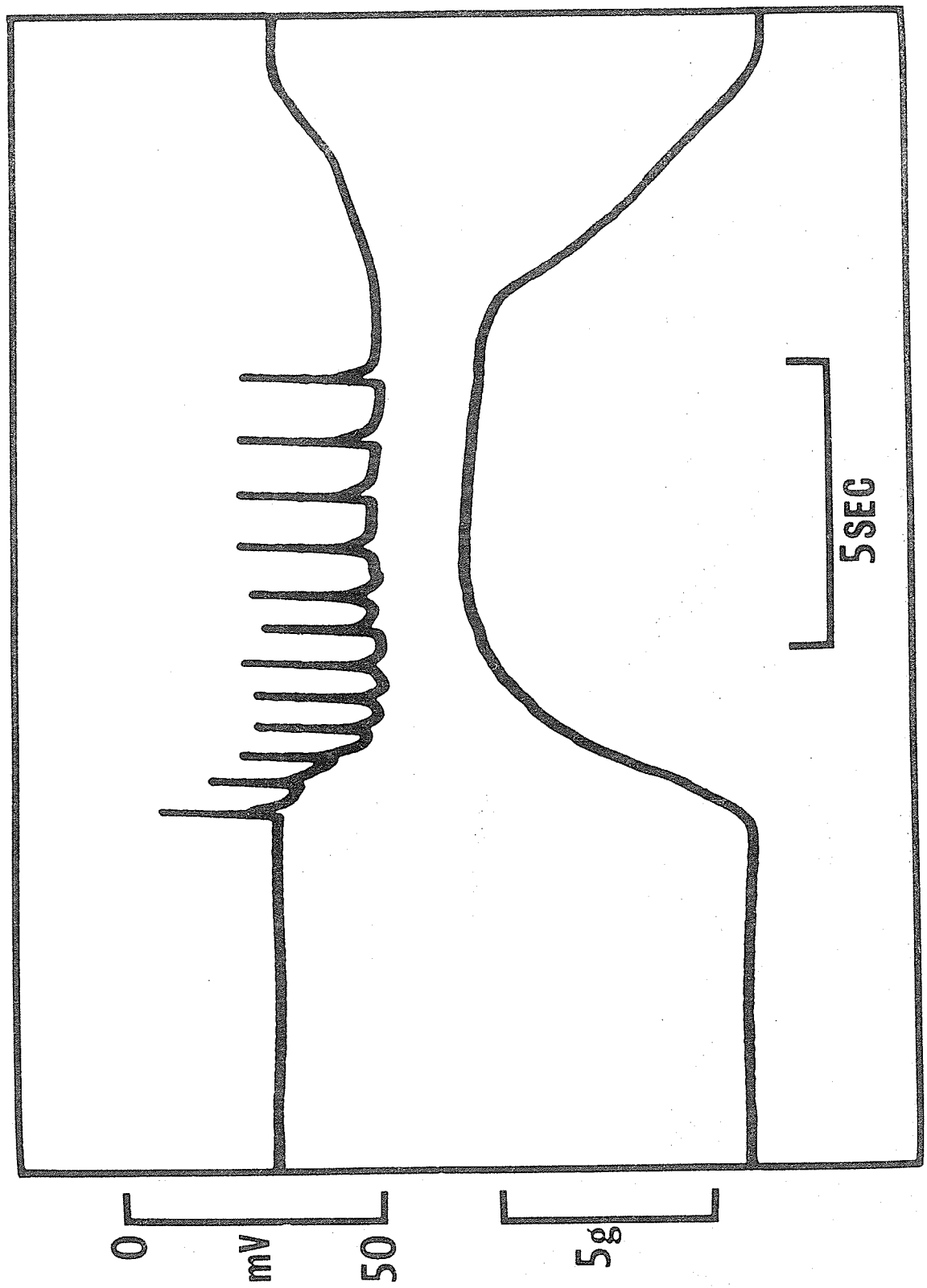

Fig. 5. Spontaneous contraction of uterus.
Record with damaged microelectrode (Tip resistance 5 MΩ)from a cell near the pacemaker site in uterus from a mature rat treated with stilboestrol.

electrode becoming dislodged from the cell in a short time. The hyperpolarization therefore appears to be due to a temporary improvement of penetration due to the muscle contraction.

A check of the microelectrode always showed a sharp decrease in resistance indicating the tip of the microelectrode had been damaged during penetration. Such recordings were obtained in any part of a preparation and could be produced by deliberately damaging the tip of a microelectrode.

ONSET OF ACTION POTENTIALS AND TENSION DEVELOPMENT

We were not surprised to find that asynchrony between membrane potentials and tension changes was the rule rather than the exception in tissues with multiple pacemaker areas; these results are in agreement with other workers (Daniel, 1957; Marshall, 1959). We were, however, disturbed to find in preliminary experiments that preparations from oestrogen treated animals which showed regular contractile responses, discrete pacemaker areas and constant conduction velocities produced recordings where start of action potentials occurred after onset of developed tension. A typical record is shown in Fig. 6.

This occurred only when the microelectrode was placed close to the cervical end of the preparation. The time between the onset of tension development and the onset of action potentials was directly proportional to the distance of the microelectrode from the pacemaker area, i.e. the time required for the propagated potential changes arising in the pacemaker area to activate cells near the cervical end of the tissue. When microelectrodes were placed in or near the pacemaker area, action potentials always preceded tension changes, as shown in Fig. 7.

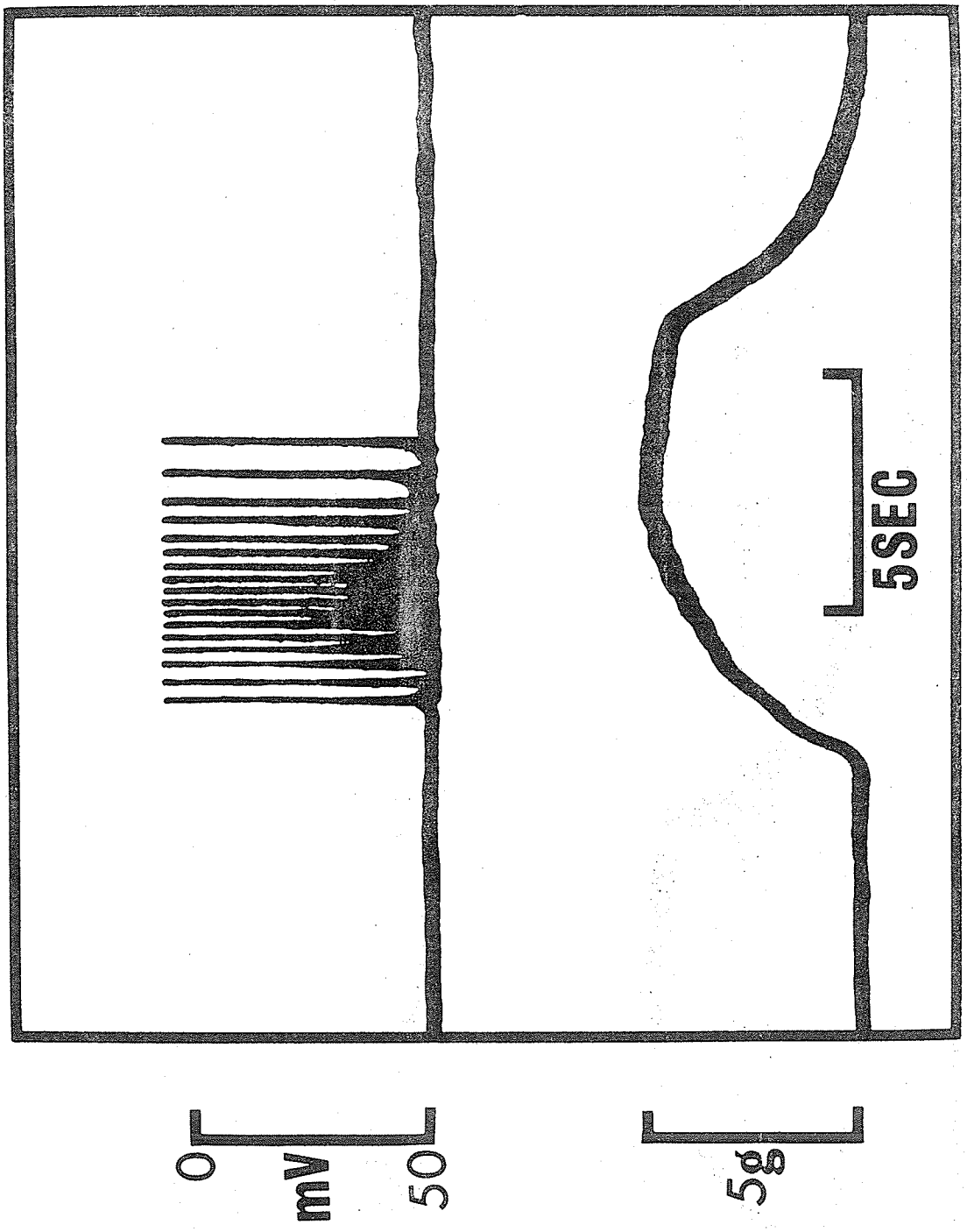

Fig. 6. Spontaneous contraction of uterus: Oestrogen treated rat.
Developed tension began before onset of action potentials recorded near cervical end of preparation.

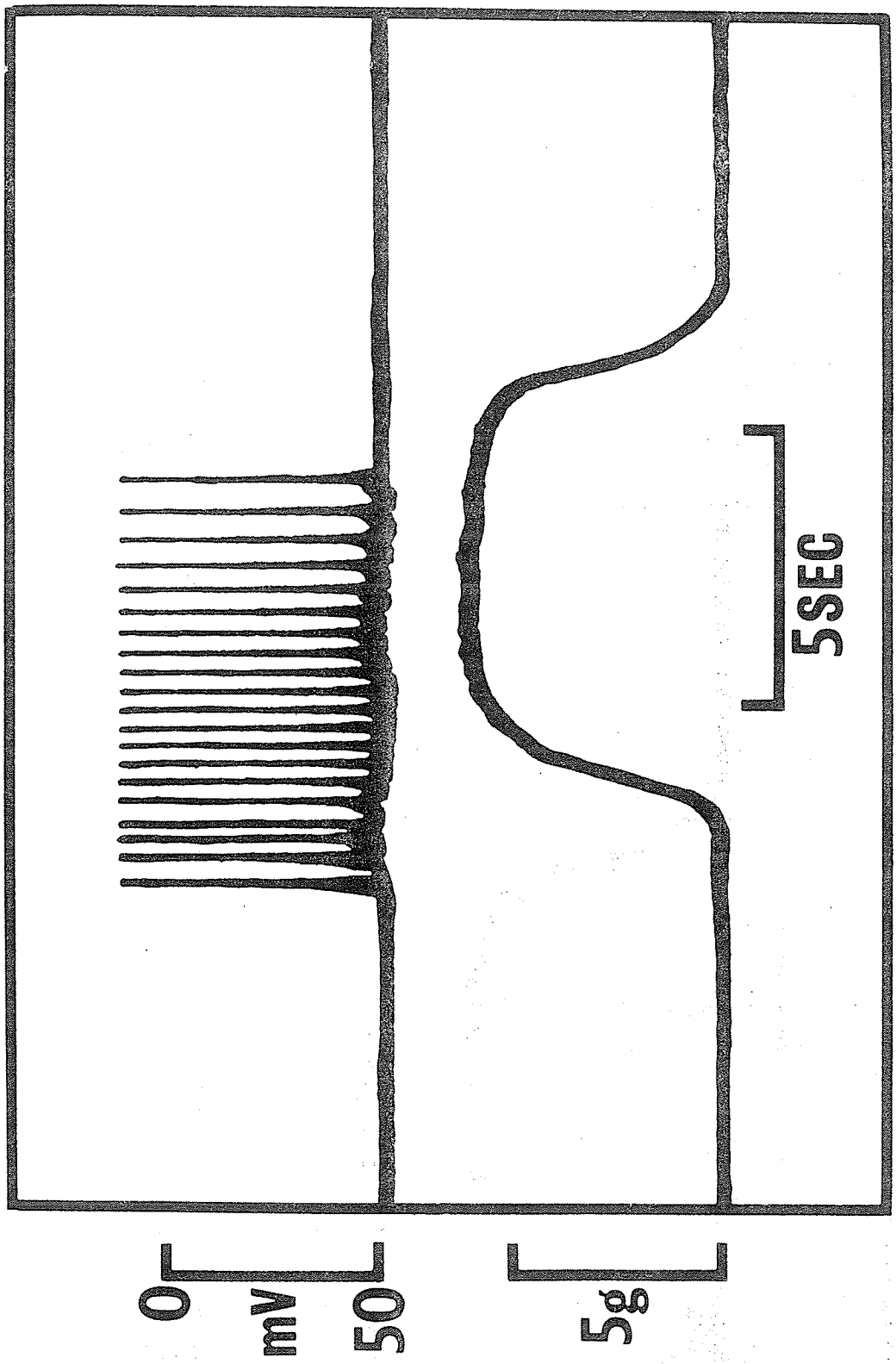

Fig. 7. Spontaneous contraction of uterus: Oestrogen treated rat.
Action potentials recorded near pacemaker area at ovarian end of preparation preceded onset of developed tension.

We found that action potentials in preparations from twenty-one day pregnant animals either preceded or were synchronous with tension changes. Because these preparations under 1 g resting tension were only 27-30 mm long and the conduction velocity was considerably greater than in preparations from oestrogen treated rats (Table 2), the electrodes had to be placed at the extreme cervical end before asynchrony could be observed. In all other experiments reported, the microelectrodes were placed within 1 cm of the pacemaker area, except in experiments to determine conduction velocities, when the furthest electrode was inserted 1 or 2 cm from the ovarian end of the tissue.

WATER, ELECTROLYTE CONTENT AND RESTING MEMBRANE POTENTIAL

The resting membrane potential varied between 35 and 65 mV in different uterine preparations (Table 2). In skeletal muscle and nerve, resting potential is close to the potassium equilibrium potential; in uterus, assuming an intracellular potassium concentration of 140 mM, the potassium equilibrium potential calculated by the Nernst equation is 91 mV (Daniel, 1961). The discrepancy between empirical and theoretical values for membrane potential in smooth muscle has been observed by many workers and the situation reviewed by Kuriyama (1961).

Many workers quickly remove portions of fresh tissue for ionic analysis and use these results to calculate values of membrane potential and compare these with measured values of membrane potential obtained in a tissue which has been in a physiological bathing fluid for at least one hour. We decided to analyse the same tissues for electrolyte content, extracellular space and water content, that were used for measurement of membrane potential.

Immature rats, untreated, or receiving progesterone alone, had significantly lower potassium content ($P < 0.05$) and greater sodium content ($P < 0.05$) than all other preparations. We found no significant difference in electrolyte content, extracellular space and water content between all other uterine preparations. The results of these analyses are shown in Tables 3, 4 and 5. The potassium equilibrium potential obtained from our data calculated by the Nernst equation was 90 mV.

TIGHTLY BOUND ELECTROLYTES

One possible explanation for discrepancies between calculated and empirical values of membrane potentials is that some of the ions present in tissues are not electrochemically free but firmly bound to tissue sites and play no role in concentration gradients across the cell membrane. We tested this possibility by placing uterine strips which had been used for measuring membrane potentials into tubes containing isotonic sucrose at pH 6.8 and storing these at 4°C for 20 days. Each day the fluid was changed three times for fresh cold isotonic sucrose and on the twentieth day, tissues were removed, blotted, and analysed for electrolyte and water content. The isotonic sucrose solution used for bathing the tissues was also analysed as a blank determination.

Results of these experiments are shown in Table 6. There was no significant change in water content of tissues compared with tissues analysed immediately on removal from the organ bath. All tissue calcium had disappeared, while approximately 16% of the magnesium remained. The most interesting finding was that tissues from oestrogen dominated and pregnant animals retained significantly more sodium and potassium

TABLE 3

Water and electrolyte content of uterus

Immature Rat	Water %	Extracellular Spaces (ml/kg)	mEq/kg Wet Weight			
			K	Na	Ca	Mg
No Treatment	73.1 ± 4.6	402 ± 8.7	64.3 ± 3.9	122 ± 5.7	4.8 ± 0.9	11.7 ± 1.7
Oestrogen Dominated	80.7 ± 3.3	383 ± 7.2	82.4 ± 4.2	69.2 ± 3.1	5.6 ± 1.6	12.9 ± 1.9
Progesterone Dominated	79.7 ± 3.8	403 ± 7.9	79.7 ± 4.6	77.6 ± 3.9	5.0 ± 1.4	12.1 ± 1.3
Oestrogen and Progesterone Dominated	79.3 ± 4.2	377 ± 5.3	85.6 ± 3.9	70.2 ± 2.4	5.8 ± 1.3	13.2 ± 2.0

Values shown are the mean ± SE of 12 determinations

TABLE 4

Water and electrolyte content of uterus

Mature Rat	Water %	Extracellular Spaces (ml/kg)	mEq/kg Wet Weight			
			K	Na	Ca	Mg
Preoestrus	79.1 ± 4.2	383 ± 13.2	80.6 ± 4.6	72.7 ± 3.9	5.4 ± 1.1	13.4 ± 1.4
Dioestrus	78.7 ± 3.6	364 ± 7.7	81.4 ± 3.3	71.3 ± 4.0	4.9 ± 1.4	11.9 ± 1.7
Oestrus	80.8 ± 2.7	377 ± 4.8	79.6 ± 4.2	68.7 ± 3.2	5.3 ± 0.8	12.3 ± 1.2
Oestrogen Dominated	78.3 ± 2.9	371 ± 6.9	83.6 ± 3.9	70.2 ± 2.9	5.4 ± 1.2	14.2 ± 1.8
Progesterone Dominated	80.2 ± 3.6	382 ± 7.9	86.4 ± 4.7	68.3 ± 3.6	5.2 ± 1.4	13.1 ± 1.1
Oestrogen and Progesterone Dominated	79.1 ± 4.7	366 ± 9.7	81.2 ± 2.2	69.1 ± 1.9	5.7 ± 1.8	12.6 ± 1.4

Values shown are the means ± SE of 14 determinations

TABLE 5

Water and electrolyte content of uterus

	Water %	Extracellular Spaces (ml/kg)	mEq/kg Wet Weight			
			K	Na	Ca	Mg
17 Day Pregnant						
Placental	79.6 ± 3.3	364 ± 7.1	81.9 ± 2.7	70.7 ± 3.3	4.7 ± 1.1	10.9 ± 1.3
Non-placental	78.3 ± 4.1	382 ± 8.4	82.6 ± 3.9	69.1 ± 2.7	5.6 ± 1.3	12.2 ± 1.2
21 Day Pregnant						
Placental	81.2 ± 4.3	386 ± 6.2	83.3 ± 4.0	71.1 ± 3.2	5.1 ± 0.9	12.6 ± 1.3
Non-placental	79.3 ± 2.6	365 ± 5.7	80.1 ± 2.9	70.1 ± 3.7	5.4 ± 1.3	13.7 ± 1.6

Values shown are the means ± SE of 20 determinations

TABLE 6

Residual electrolytes after soaking in isotonic sucrose at 4°C for 20 days

Mature Rat	mEq/kg Wet Weight				Water Content
	Na	K	Ca	Mg	
Oestrogen Dominated	21.2 ± 1.3	14.2 ± 2.2	0	1.37 ± 0.55	75.1 ± 3.2
Oestrogen and Progesterone Dominated	14.7 ± 1.2	3.7 ± 1.4	0	1.71 ± 0.41	73.8 ± 4.1
17 Day Pregnant	15.1 ± 1.4	3.9 ± 1.1	0	2.31 ± 0.31	78.6 ± 3.8
21 Day Pregnant	26.2 ± 1.4	16.4 ± 1.7	0	2.59 ± 0.28	76.3 ± 4.3

Values shown are mean ± SE of 6 determinations

($P < 0.05$) than tissues from oestrogen plus progesterone dominated and seventeen day pregnant animals.

STRETCH AND MEMBRANE POTENTIAL

When rhythmic smooth muscles are stretched the membrane potential response roughly parallels that occurring during a spontaneous contraction. "Excessive" stretch produces a maintained depolarization and spikes become slower and smaller (Goto and Woodbury, 1958).

Because we stretched our preparations until they maintained 1 g resting tension we might have caused some depolarization. This could explain some of the difference between empirical and theoretical values for membrane potential in uterus. We tested this possibility in two ways.

Resting membrane potential recordings were obtained in tissues prepared as usual except that resting tension was varied between 0.25, 0.5, 1.0, 1.5 and 2.0 g. Thirty minutes were allowed to elapse after a change of resting tension before membrane potentials were determined. No significant difference in resting membrane potential was found between preparations at different resting tensions. Resting potential at 0.25 g and 2.0 g were 49.7 ± 1.3 and 50.6 ± 1.6 mV respectively.

We measured resting membrane potentials of rat uterus in vivo, where the tissue should be under optimal conditions of length, tension and electrolyte gradients. Although we encountered some problems in using microelectrodes in vivo, namely that signal to noise ratio was less, and that in pregnant animals, foetuses often moved abruptly and dislodged or broke electrode tips, we were successful in obtaining values of resting membrane potentials and action potentials.

In the pregnant uterus we noted that single action potentials often occurred as well as trains of action potentials. Values obtained did not significantly differ from values obtained from comparable in vitro preparations. A typical record from a non-placental site of a twenty-one day pregnant animal is shown in Fig. 8.

CHOICE OF MAIN EXPERIMENTAL PREPARATION

We decided upon the non-placental strip from twenty-one day pregnant animals for most of the remaining experiments for the following reasons.

We found no difference in electrolyte, extracellular space or water content between the different preparations. Uterus from pregnant animals has advantages, in that cells show considerable hypertrophy compared with uterus from nonpregnant animals and we could maintain successful penetrations with microelectrodes over a longer period of time. The increased size of the uterus also allowed many strips for appropriate control experiments to be obtained from the same animal. The increased conduction velocity meant that synchronous activity of cells was more probable and preparations from the twenty-one day pregnant animal had a discrete pacemaker area. We decided on strips from non-placental sites because we would avoid possible interference from placental implantation. Unless otherwise stated all the following experiments were done on strips from non-placental sites from twenty-one day pregnant rats.

IONIC GRADIENTS AND RESTING MEMBRANE POTENTIAL

In experiments where tissues were kept in the organ bath for prolonged periods, over 7 hours, we found a net gain of sodium (Total Content,

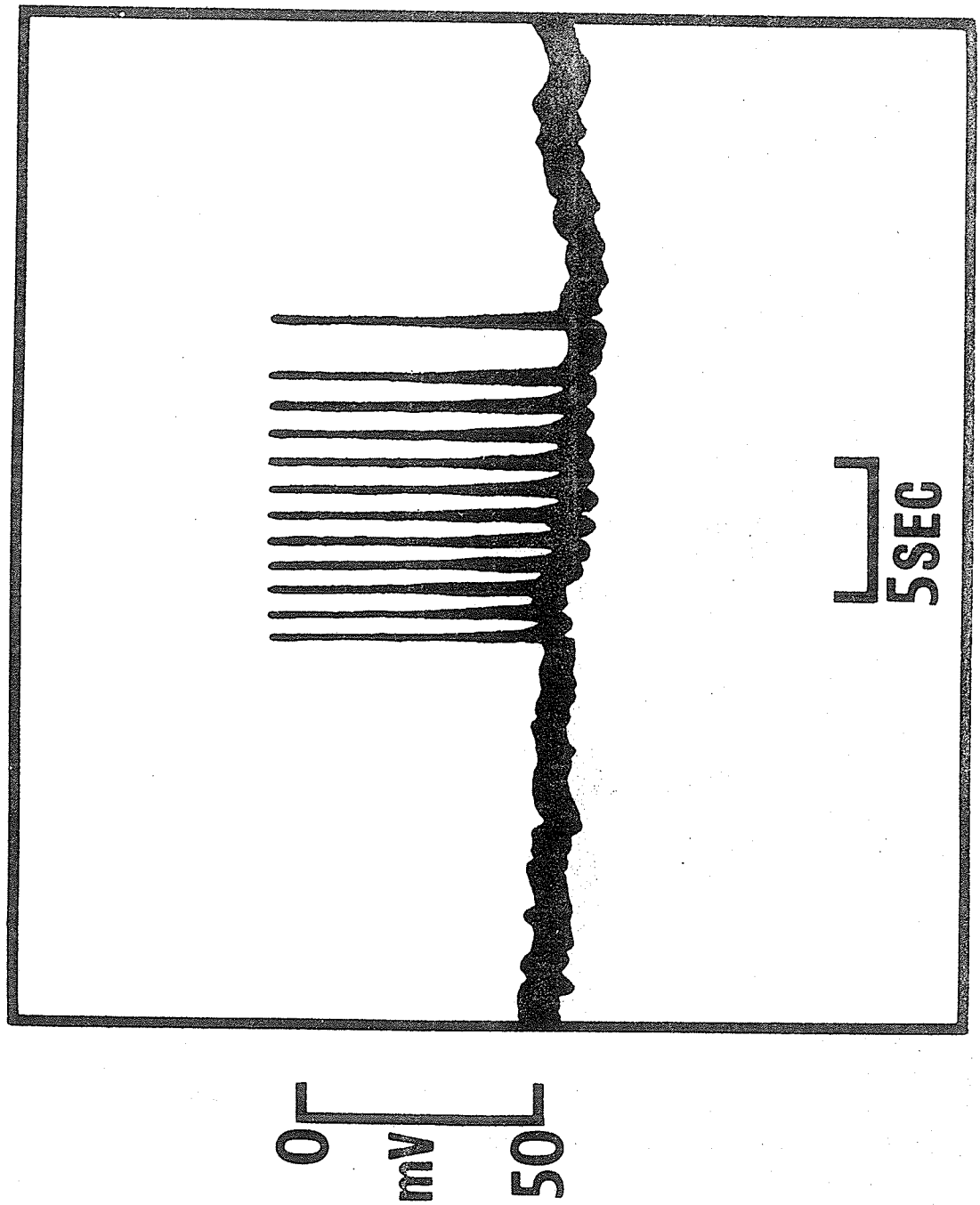

Fig. 8. Resting membrane potential and action potentials recorded in vivo at a non-placental site in uterus of a twenty-one day pregnant rat.

110 ± 4.8 mEq/kg wet weight, n=9) and a loss of potassium (Total Content, 52 ± 3.5 mEq/kg wet weight, n=9) although values of extracellular space and resting membrane potential remained unchanged. These tissues had received no other treatment and the bathing fluid had been changed every 10 minutes.

These results indicated that the relationship between membrane potential and sodium and potassium concentration gradients in rat uterus was not following the theoretical relationship, which predicts an inverse relationship between resting membrane potential and external electrolyte concentration. We therefore determined resting membrane potential in preparations exposed to varying external sodium and potassium concentrations. Before any determinations of resting membrane potential were made, the preparations were allowed to equilibrate for 30 minutes after the ionic composition of the bathing fluid was changed.

VARIATION OF EXTERNAL SODIUM CONCENTRATION

When the concentration of sodium in the bathing fluid was lowered, equivalent amounts of sucrose were added to maintain osmolarity. However, when external sodium concentration was raised, no compensation was made and the bathing fluid became hypertonic.

Experiments (8) to determine the effect of variation of external sodium concentration on membrane potential were not entirely successful. Excess external sodium (240 mM sodium) decreased the resting membrane potential from 50 to 38 mV, but sodium deficiency resulted in a fluctuating membrane potential, first increasing the membrane potential then gradually decreasing it. At 10 mM sodium the mean membrane potential

first increased from 50 to 58 mV and then slowly decreased to 42 mV over a 3 hour period.

VARIATION OF EXTERNAL POTASSIUM CONCENTRATION

When the concentration of potassium in the bathing fluid was decreased to less than 5 mM, equimolar concentrations of sodium chloride and sodium dihydrogen phosphate were substituted for potassium chloride and potassium dihydrogen phosphate respectively. When the external potassium concentration was increased above 5 mM, potassium chloride replaced equivalent concentrations of sodium chloride in the bathing medium.

Fig. 9 shows the means of at least 20 determinations of resting membrane potential at each potassium concentration in each of 6 preparations from different animals. A reduction of the potassium concentration to 1.25 mM increased the mean membrane potential from 50 to 64 mV. Higher concentrations of potassium decreased the membrane potential; at 160 mM the membrane was depolarized to 15 mV.

VARIATION OF EXTERNAL CALCIUM CONCENTRATION

Various workers have speculated about the role of calcium as a current carrier and regulator of membrane permeabilities in smooth muscle (Nonomura, Hotta and Ohashi, 1966; Bulbring and Tomita, 1969, 1970; Job, 1969; Kumamoto and Horn, 1970). We therefore decided to test the effects of varying external calcium concentrations on membrane potential. No attempt was made to compensate for changes in osmolarity in the bathing fluid when calcium was deficient or in excess. Concentrations of calcium greater than 10 mM could not be used because of precipitation of calcium salts.

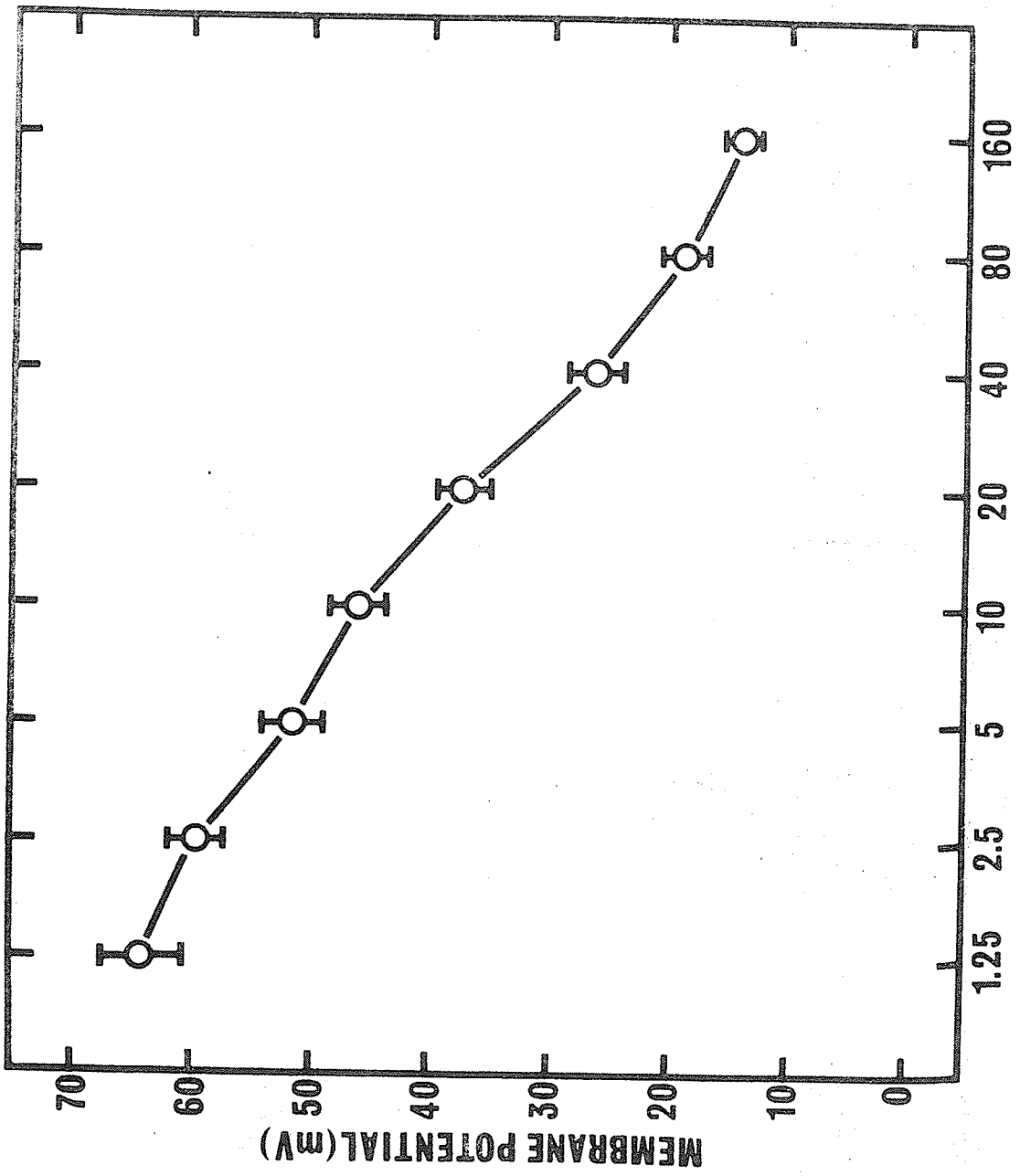

Fig. 9. The relation between resting membrane potential and logarithm of external potassium concentration in uterine muscle cells of twenty-one day pregnant rat.

When the external calcium concentration was decreased to 0.3 mM (about one tenth of normal) membrane potential decreased from 50 to 42 mV. Excess calcium, 10 mM, increased membrane potential from 50 to 67 mV. Fig. 10 shows the means of at least 20 determinations at each calcium concentration in each of 6 preparations from different animals.

EFFECT OF TETRODOTOXIN

Although tetrodotoxin is known to block sodium conductance specifically in many tissues, it apparently does not do so in smooth muscle (Kuriyama et al., 1966). We tested the effect of tetrodotoxin in concentrations up to 10^{-5} g/ml in 10 preparations from different animals. Tetrodotoxin had no effect on spontaneous contractions, resting membrane potential or action potentials (Fig. 11).

We had now determined many basic parameters and properties of our preparation and therefore decided to start testing the effects of procedures which produce subsensitivity and supersensitivity, thus modifying the responsiveness of the smooth muscle.

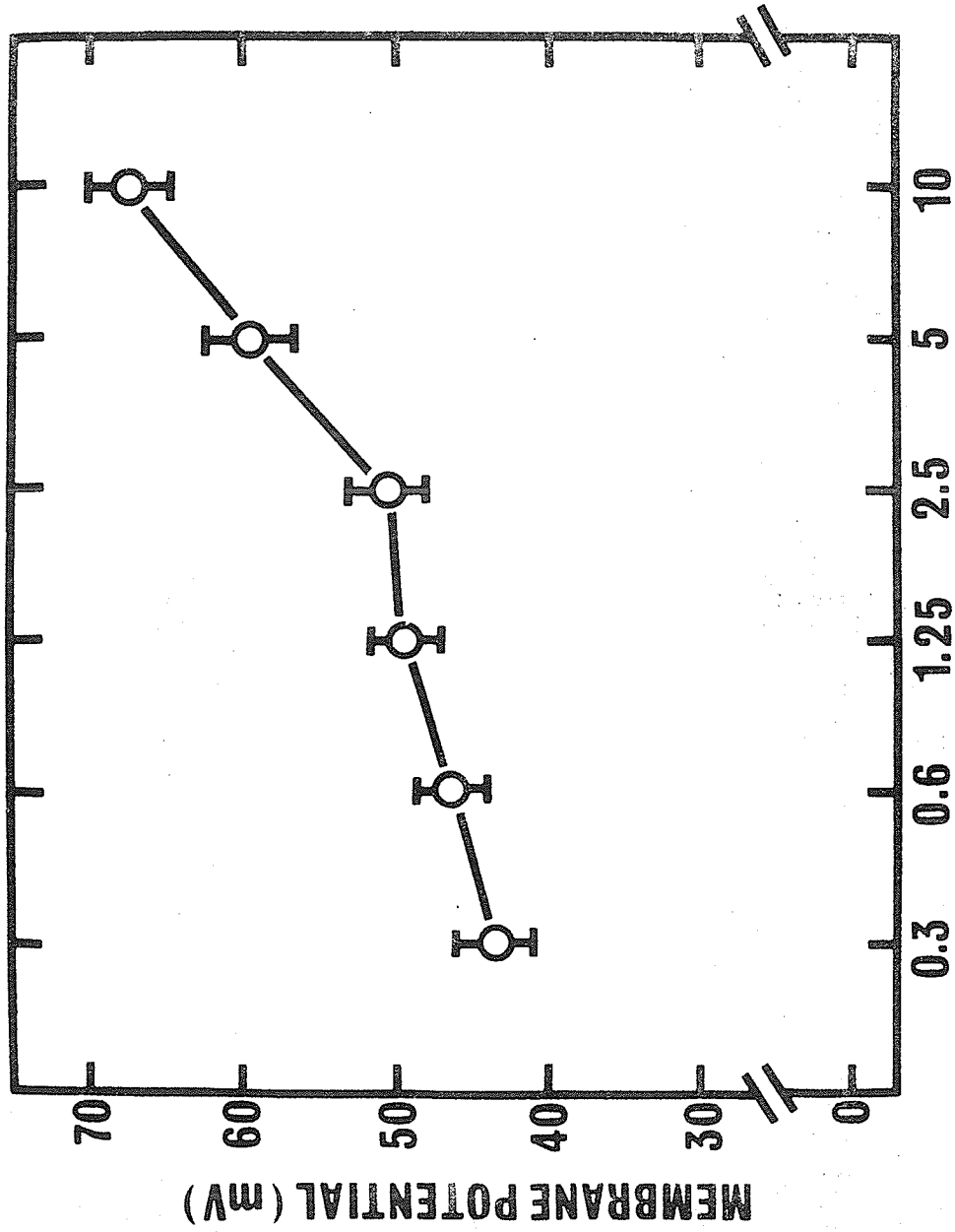

Fig. 10. The relation between membrane potential and logarithm of external calcium concentration in uterine muscle cells of twenty-one day pregnant rat.

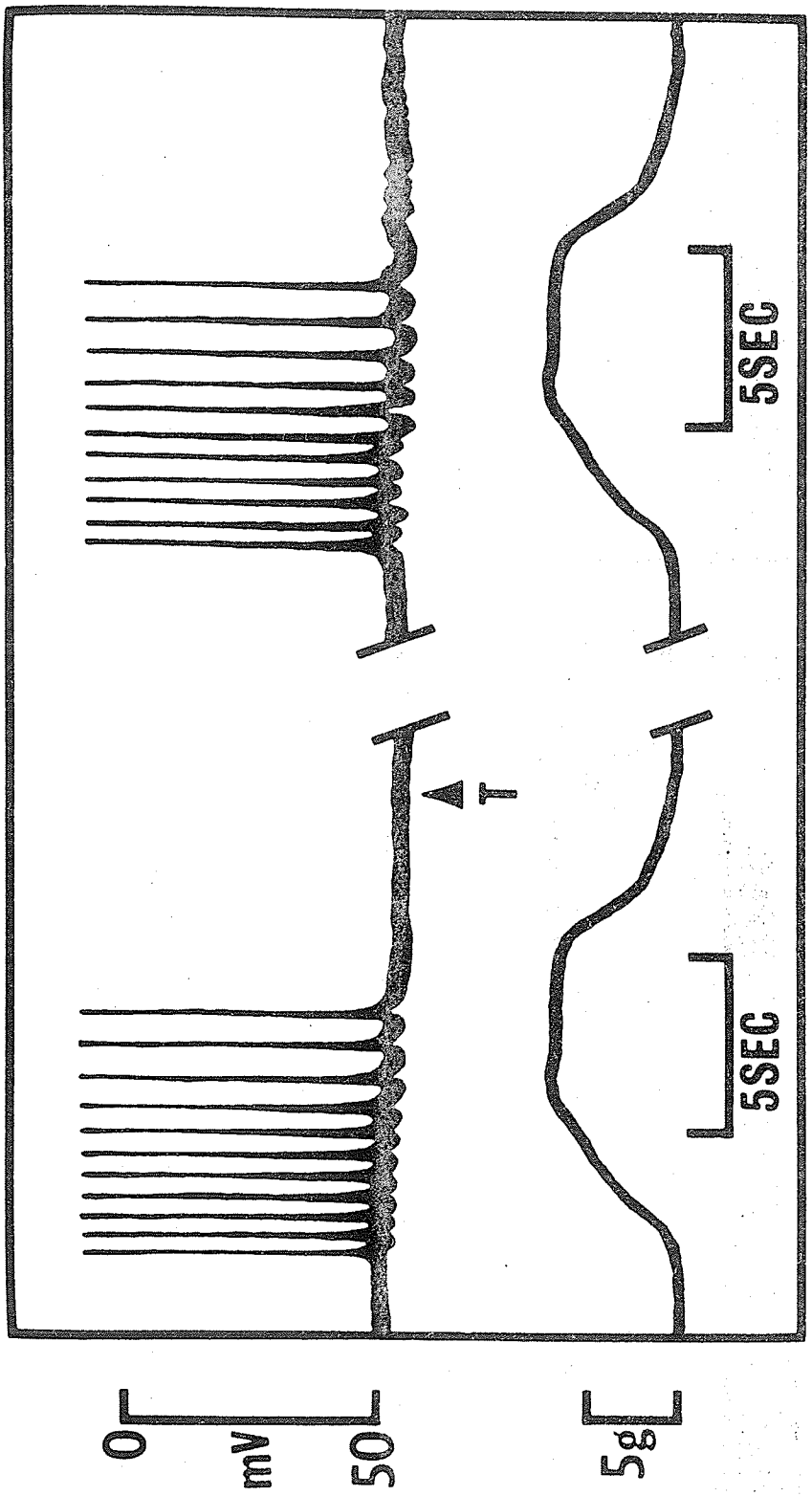

Fig. 11. Spontaneous contractions of uterus: Twenty-one day pregnant rat. Tetrodotoxin 10^{-5} g/ml given at T had no effect on rhythmicity, resting membrane potential or action potentials.

SUBSENSITIVITY

DESENSITIZATION: SPECIFIC AND UNSPECIFIC

When a tissue is exposed to a high concentration of an agonist, it may rapidly become insensitive to drugs. If the insensitivity is limited to the desensitizing agent itself or to the class of drugs to which this agent belongs, then the desensitization is considered to be specific (Feldberg and Schilf, 1930; Barsoum and Gaddum, 1935; Innes, 1962a, 1962b). On the other hand, if the tissue becomes insensitive to several groups of drugs, the desensitization is considered unspecific. Unspecific desensitization may occur with high concentrations of a drug which causes specific desensitization when used in lower concentrations (Cantoni and Eastman, 1946).

Specific desensitization is generally considered to be a receptor phenomenon, not due to interference with contractile mechanisms, because tissues respond normally to agonists acting through receptors other than those on which the desensitizing agent acts. Unspecific desensitization does not appear to be a receptor phenomenon, but may be related to the contractile mechanism, perhaps due to loss of intracellular potassium (Paton, 1961).

SPECIFIC DESENSITIZATION

Preliminary experiments showed that the uterus would respond to acetylcholine, angiotensin, or 5-hydroxytryptamine 10^{-7} to 10^{-6} g/ml. The electrical and mechanical recordings of responses to these agonists were similar to those from a spontaneous contraction, and we could not

distinguish between responses produced by these agents. Usually the responses obtained at 10^{-6} g/ml were of longer duration than a spontaneous contraction. A typical record of a response to acetylcholine 10^{-6} g/ml is shown in Fig. 12.

Preparations which were quiescent between spontaneous contractions or drug induced responses were stimulated mechanically by changes of bathing fluid if we changed the fluid by draining and refilling the organ bath. This response due to stretching of the tissue is referred to as a washout response. No such response occurred when the bathing fluid was changed by an overflow procedure. A typical record of a washout response is shown in Fig. 13. Concentrations of the agonists from 3×10^{-6} to 10^{-4} g/ml produced sustained contraction and a marked depolarization. A typical record of a response to acetylcholine 10^{-4} g/ml is shown in Fig. 14.

Specific desensitization of uterus was done using the technique of Innes (1962a). The uterus was repeatedly exposed to a concentration 10^{-6} g/ml, of one agonist, until no response was elicited by this agent. This usually required an exposure time of one hour. With this agonist still present in the bath the other agonists still produced normal responses. For example, after acetylcholine had remained in the bath for one hour and no longer caused a contraction, angiotensin or 5-hydroxytryptamine still produced completely normal responses. This procedure could be done with any two of the agonists in series, when the third agonist still produced a normal response. This is shown in Fig. 15 where the preparation was desensitized to both acetylcholine, 10^{-6} g/ml, and angiotensin, 10^{-6} g/ml; 5-hydroxytryptamine, 10^{-6} g/ml, still evoked a normal response. A standard washout response was still obtained.

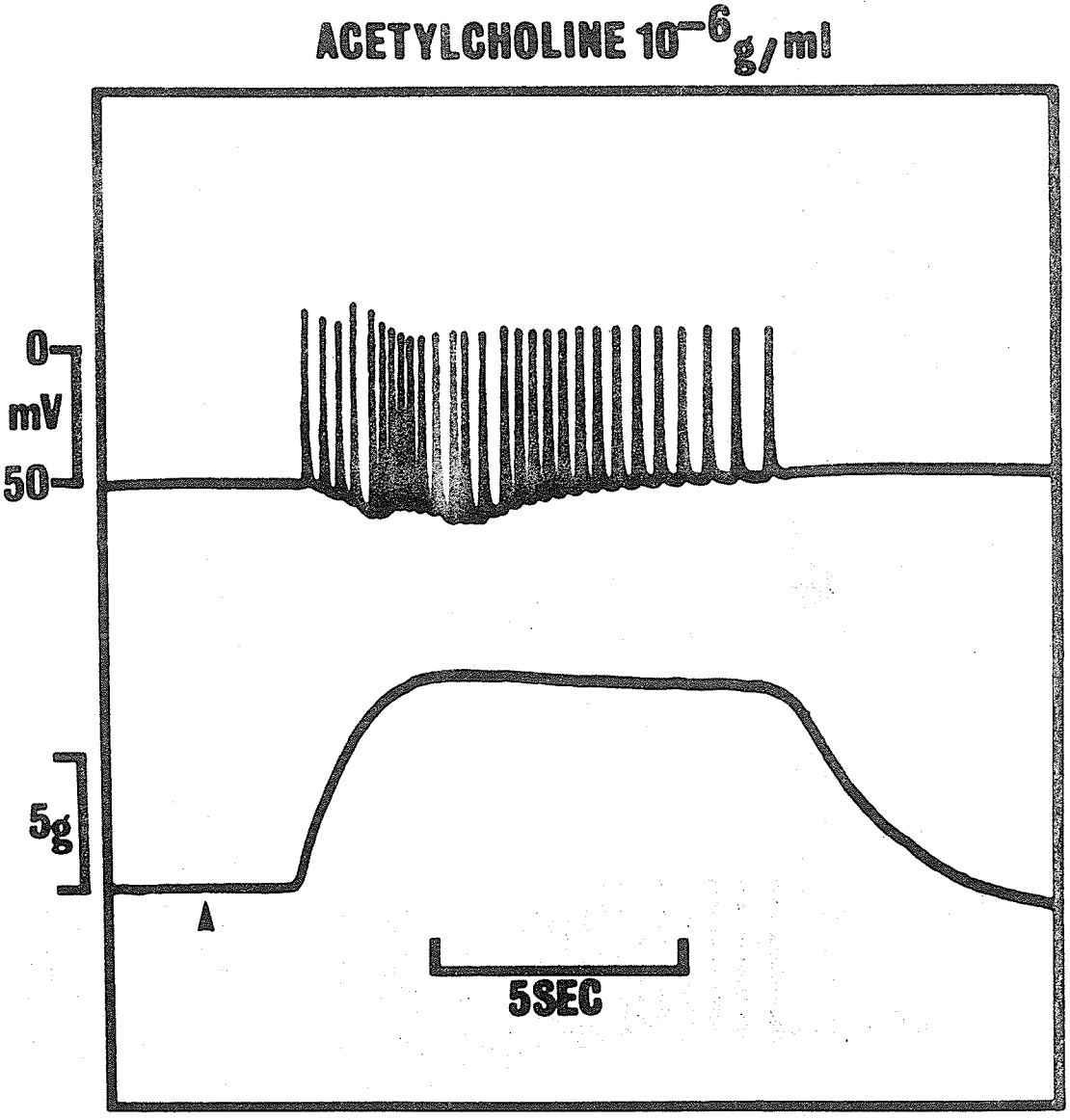

Fig. 12. Effect of acetylcholine on membrane polarization and tension in rat uterus.

Acetylcholine, 10^{-6} g/ml, was added to the bathing fluid at ▲.

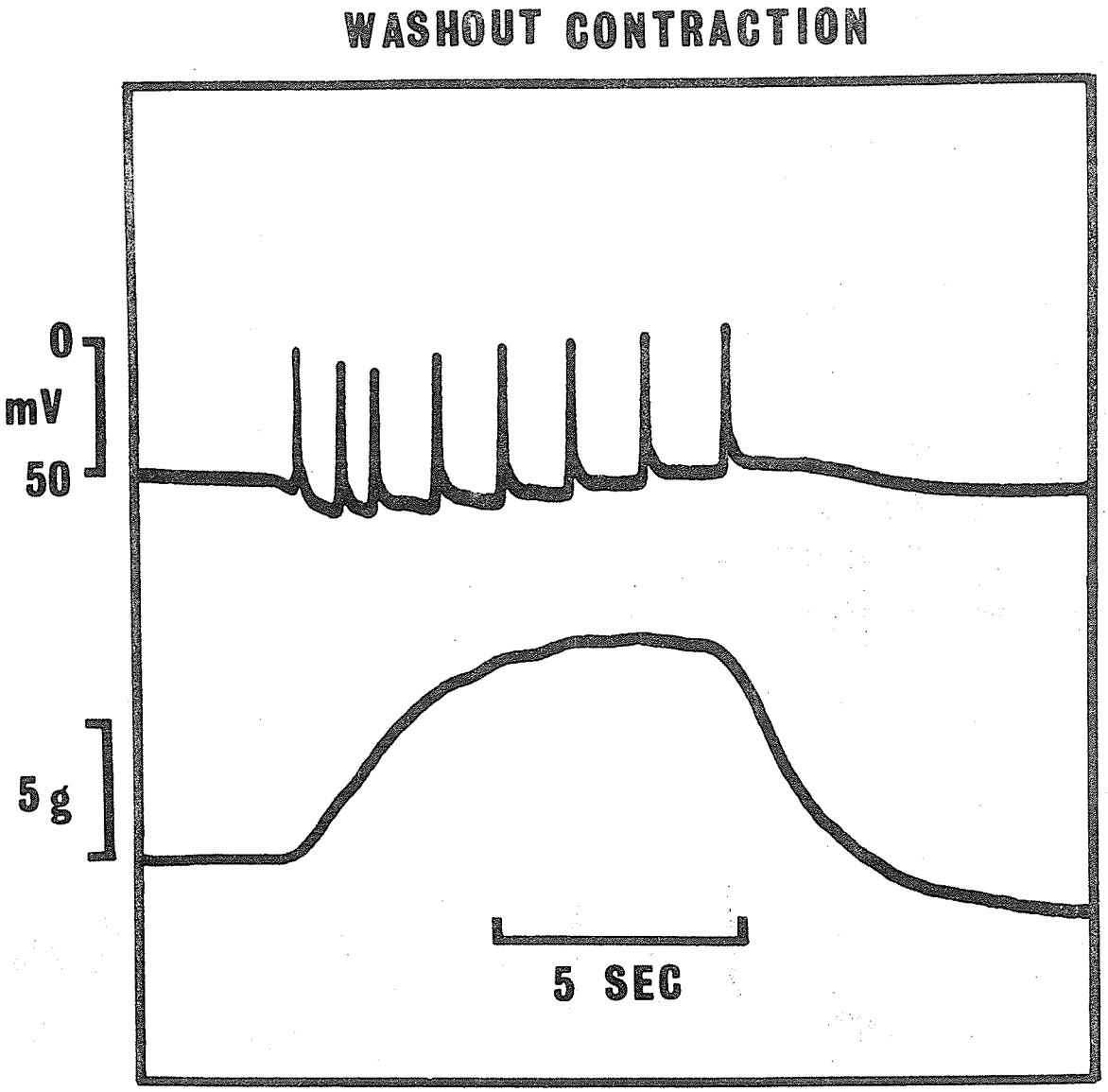

Fig. 13. Response of the rat uterus to washout.

Changes in electrical and mechanical activity of the rat uterus after the organ bath is drained and refilled.

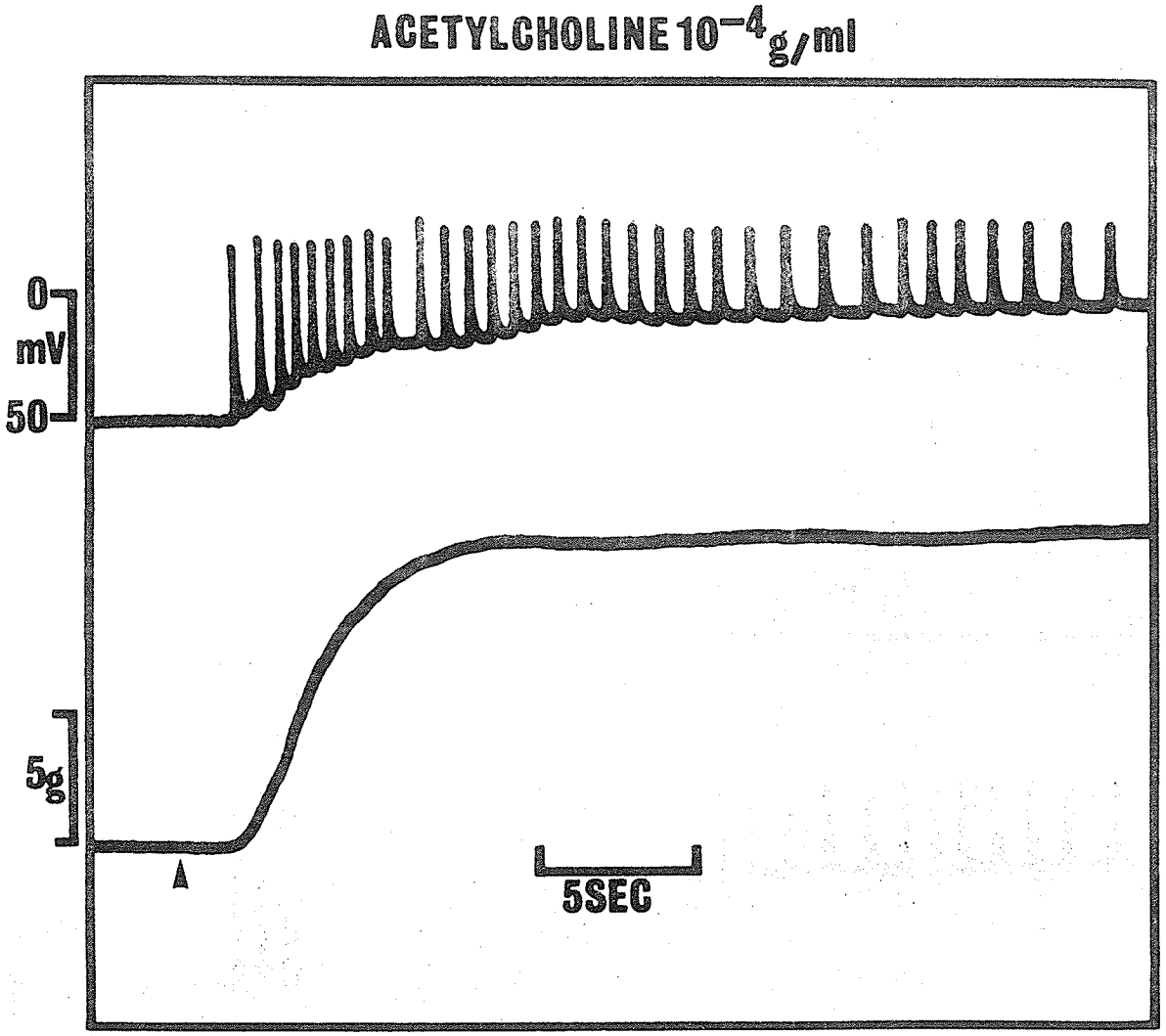

Fig. 14. Effect of acetylcholine on membrane polarization and tension in rat uterus.

Acetylcholine, 10^{-4} g/ml, added at ▲ produced depolarization of cell membrane and a sustained contractile response.

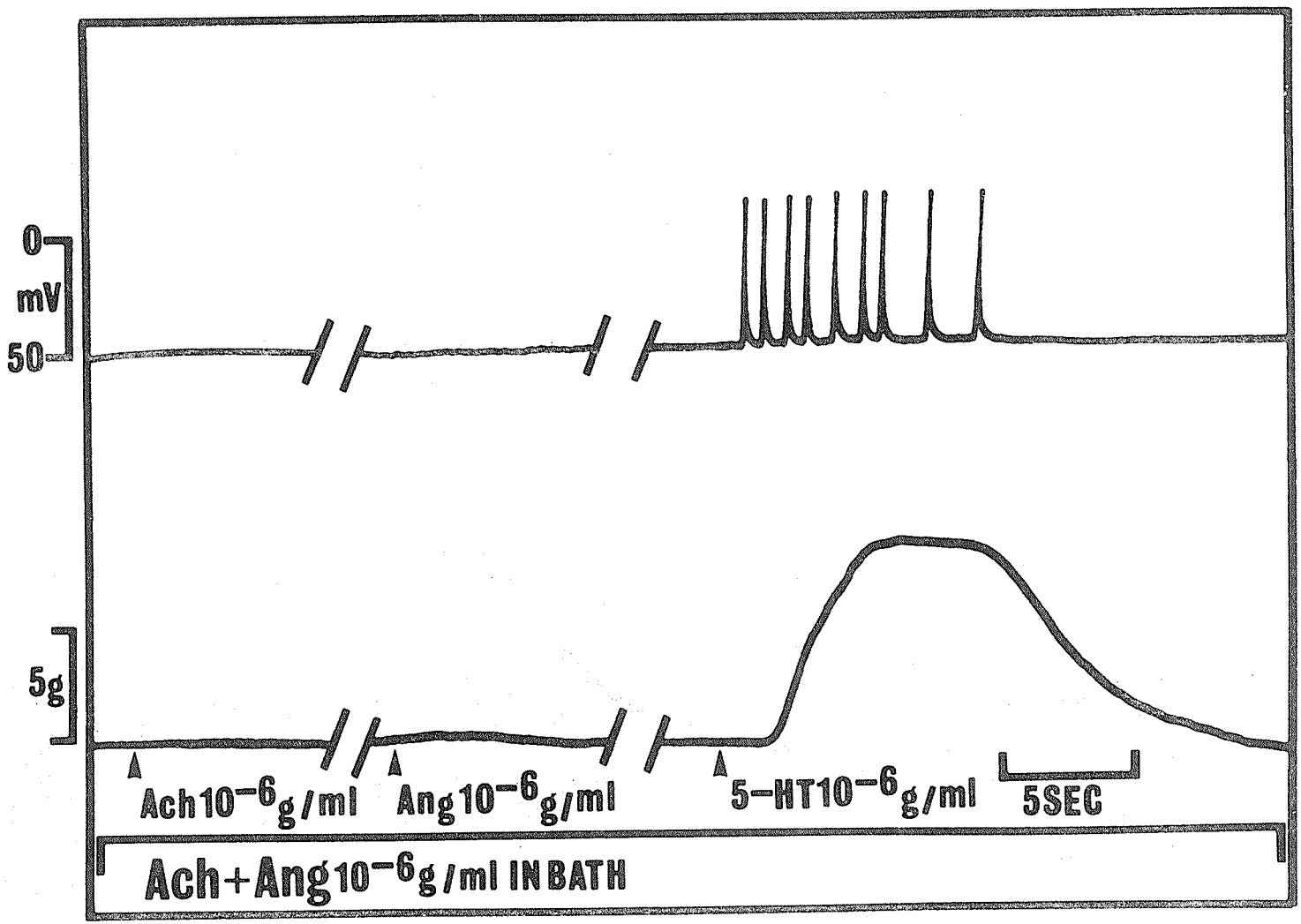

Fig. 15. Specific desensitization.

Rat uterus after prolonged exposure to acetylcholine and angiotensin, 10^{-6} g/ml, no longer responded to these agents. Addition of 5-hydroxytryptamine, 10^{-6} g/ml, with these agents still present, produced normal electrical and mechanical responses.

When the tissue was desensitized to all three agents, the tissue remained quiescent. Repeated tests showed there was no change in the value of the resting membrane potential. However, washout still caused a contraction, but this was not accompanied by action potentials, and there was little or no change in resting membrane potential. When the washout procedure was repeated 5-7 minutes later, the contraction occurred again, accompanied this time by small action potentials. A typical record of these events is shown in Fig. 16. These experiments were done in a series of 36 preparations from different animals. The sequence of agonists used to produce desensitization was rotated, all sequences gave identical results.

UNSPECIFIC DESENSITIZATION

Unspecific desensitization of uterus was done by repeated exposure of the tissue to a high concentration (10^{-4} g/ml) of any one of the agonists, until it no longer elicited a response. The response initially obtained was always similar to that shown in Fig. 14. Two patterns of response were then observed.

The contraction slowly decayed, action potentials disappeared and the tissue stayed quiescent; membrane potential remained at 10 to 15 mV.

The sustained contraction rapidly returned to baseline and then frequent contractions occurred, gradually becoming smaller until they finally stopped. Small action potentials accompanied the contractions. These slowly became less frequent, then stopped, and the membrane potential stayed at 10 to 15 mV. Whichever sequence followed the initial

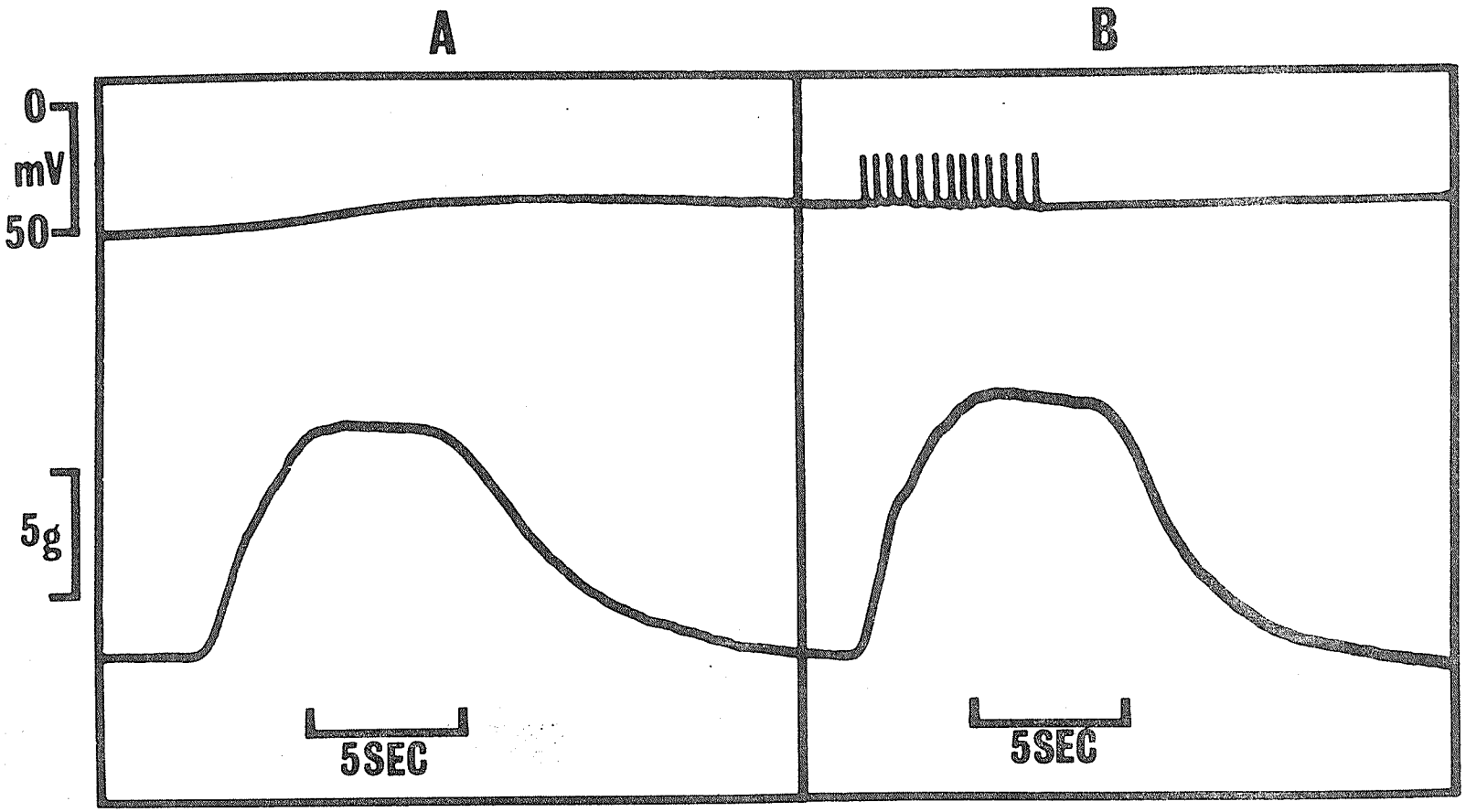

Fig. 16. Washout response of rat uterus specifically desensitized to acetylcholine, angiotensin and 5-hydroxytryptamine.

A. After prolonged exposure to all three agents, washout produced a contractile response unaccompanied by electrical activity.

B. Washout response of same tissue 7 minutes after removal of the drugs from the bathing fluid; a contractile response is now associated with small action potentials.

response to the agonist, the usual exposure time from adding the agonist to quiescence was 90 minutes.

Muscles desensitized unspecifically did not respond to either of the other agonists and gave no washout response. When the drug was removed from the bathing fluid the tissue showed complete recovery in 5 minutes and gave normal responses to any of the agonists tested and to the washout procedure.

The experiments described above were done in 12 preparations, 4 for each of the agonists, acetylcholine, angiotensin and 5-hydroxytryptamine were equally tested; only one agonist was used as the desensitizing agent on any preparation.

CAFFEINE

Caffeine causes skeletal muscle to contract by a process probably involving the release of calcium ions (Bianchi, 1961; Frank, 1962) and stimulates cardiac muscle apparently by a similar mechanism (Gubareff and Sleator, 1965). Caffeine inhibits spontaneous and drug induced responses in smooth muscle (Feinstein, 1966; Mitznegg, Hach and Heim, 1970). Somlyo and Somlyo (1968a) reported that mesenteric vein strips stimulated by noradrenaline showed depolarization and increased action potential frequency. Caffeine abolished spontaneous and noradrenaline-induced action potentials but did not prevent noradrenaline from producing depolarization or tension development. Because we had shown a dissociation between potential changes and tension development by specific desensitization (p. 68) we decided to study the effects of caffeine in uterine smooth muscle.

EFFECTS OF CAFFEINE

Caffeine, 1 mM, increased spontaneous activity of the uterus but did not alter resting membrane potential (Fig. 17). Caffeine, 5 mM, decreased the spontaneous activity of the uterus until it became quiescent. The time to produce quiescence after addition of 5 mM caffeine to the bathing fluid was 11 ± 0.32 minutes in a series of 8 experiments with preparations from different animals. As spontaneous activity decreased, resting membrane potential was unchanged but abnormal potentials often occurred (Fig. 18).

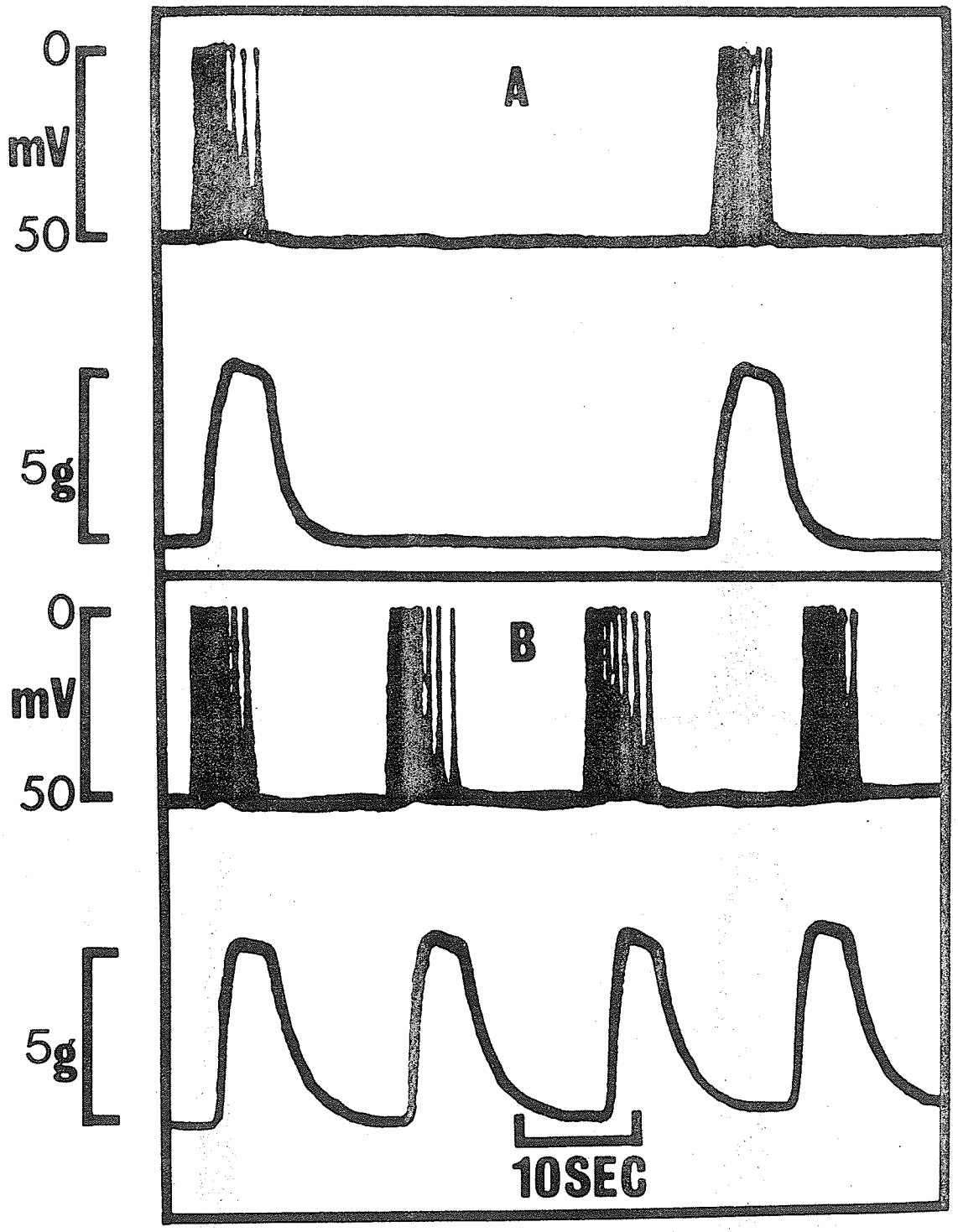

Fig. 17. Effect of caffeine on spontaneous activity of rat uterus.

A. Spontaneous electrical and mechanical activity of uterus, no caffeine present.
B. Caffeine, 1 mM, present in the bathing fluid increased spontaneous activity of the uterus.

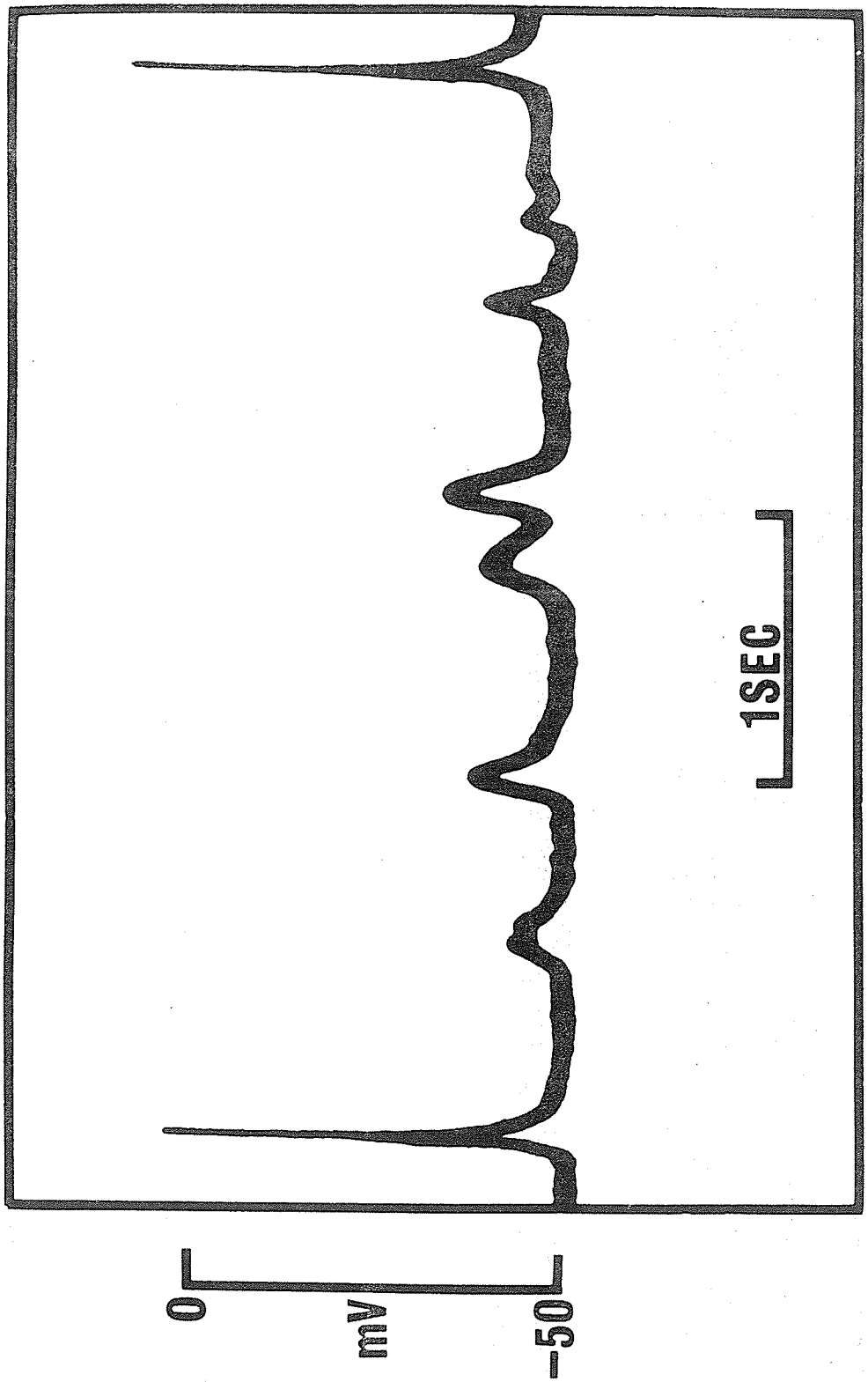

Fig. 18. Effect of caffeine on membrane polarization of rat uterus.
5 mM caffeine in the bathing fluid produced varied irregular membrane potentials.

EFFECT OF 5 mM CAFFEINE ON RESPONSES TO AGONISTS

Preparations inhibited by 5 mM caffeine and stimulated by acetylcholine, angiotensin or 5-hydroxytryptamine no longer showed the usual potential and tension changes. The muscle then gave graded contractile responses associated with a graded membrane depolarization. Caffeine generally increased the doses of agonists required to stimulate the preparation. Fig. 19 illustrates an experiment where acetylcholine, 10^{-6} g/ml, elicited the usual electrical and mechanical responses. In the presence of caffeine, 5 mM, this dose of acetylcholine had no effect, and doses of 10^{-5} and 10^{-4} g/ml were required.

CAFFEINE, ELECTRICAL STIMULATION AND WASHOUT RESPONSE

Tissues quiescent in the presence of 5 mM caffeine did not give a washout response and did not respond to punctate stimulation, and so we could not determine conduction velocity. When the tissue was stimulated via field electrodes a single spike was observed in the two electrodes placed in the tissue to determine conduction velocity. We found that a single spike could be recorded from an electrode placed within 2 mm of punctate electrodes.

These findings, that the tissue would not respond to stretch, conduction velocity could not be determined, and agonists produced graded contractile responses and depolarization strongly suggested that the preparation was behaving as a multi-unit smooth muscle. When caffeine was removed from the bathing fluid the tissue recovered withing 3 minutes and showed spontaneous electrical and mechanical activity and standard responses to all the usual stimuli.

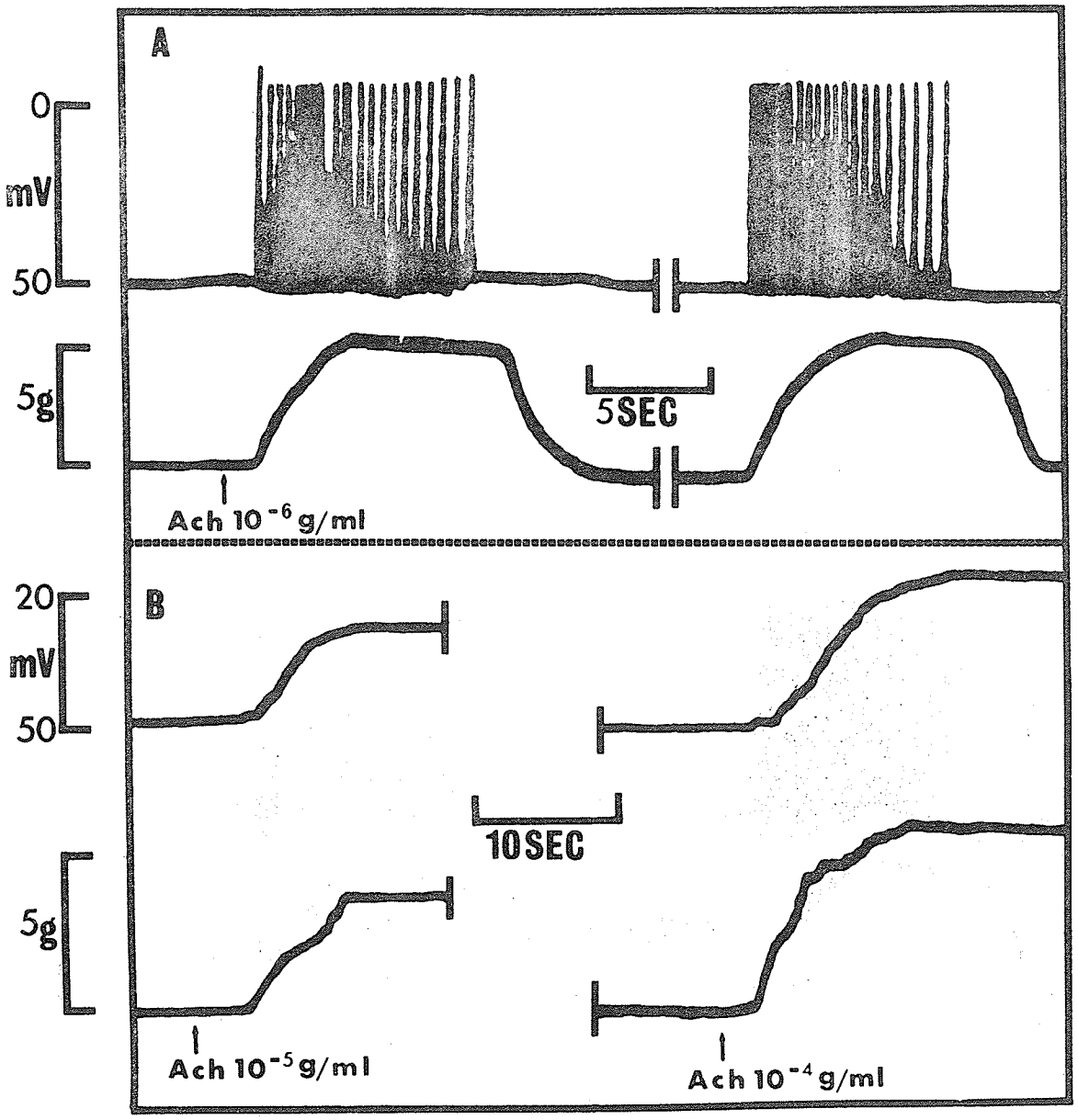

Fig. 19. Effect of caffeine on electrical and mechanical responses of rat uterus to acetylcholine.

A. Responses of uterus to acetylcholine, 10^{-6} g/ml, no caffeine in the bathing fluid.

B. Graded contractile and membrane responses to acetylcholine, 10^{-5} and 10^{-4} g/ml, 5 mM caffeine present in the bathing fluid.

EFFECT OF 5 mM CAFFEINE ON EXTRACELLULAR SPACE

Several authors (Dewey and Barr, 1962; Rhodin, 1967; Henderson, Duchar and Daniel, 1971) suggested that the major difference between single and multi-unit smooth muscles are areas of close cellular contact found in single-unit smooth muscle. We decided to test if caffeine produced its effects by altering these close junctions, and reasoned that we could expect to find changes in the tissue extracellular space. Accordingly we determined extracellular space in preparations treated in the following ways: (a) immediately on killing the animal (b) tissues bathed in normal Krebs-Henseleit solution (c) tissues with 5 mM caffeine present in the bathing fluid, and (d) tissues exposed to 5 mM caffeine, then placed in normal Krebs-Henseleit solution until spontaneous activity returned.

The results of these experiments are shown in Table 7. The means ± SE were obtained from determinations on tissues from 10 rats. Treatment with 5 mM caffeine significantly increased ($P < 0.01$) the extracellular space, compared with values from the control preparations. After removal of caffeine and recovery of spontaneous activity, tissue extracellular space did not significantly differ from values of control tissues.

ELECTRON MICROSCOPE EXAMINATION OF THE UTERUS

We decided to check whether we could find a histological correlate of this increase in extracellular space in tissues treated with 5 mM caffeine, and appropriately treated tissues were prepared for electron microscopy (p. 28). Typical results of experiments in tissues from 3 animals are shown in Figs. 20, 21, 22 and 23, and agreed with the results of extracellcellular space determinations, that caffeine disrupted areas of invagina-

TABLE 7

Determination of Extracellular Spaces

Tissue Treatment	Extracellular Spaces	Number of Determinations
Freshly Excised	39 ± 1.2%	10
Bath Controls	38.7 ± 1.7%	10
5 mM Caffeine	54.3 ± 1.8%	10
5 mM Caffeine + wash out to recovery	40.1 ± 1.3%	10
5mM Caffeine + 5 mM Calcium	39 ± 1.9%	6
5 mM Caffeine + 10^{-4} M Ouabain	38.3 ± 1.4%	6
5 mM Caffeine + Low Sodium	37.8 ± 1.6%	5

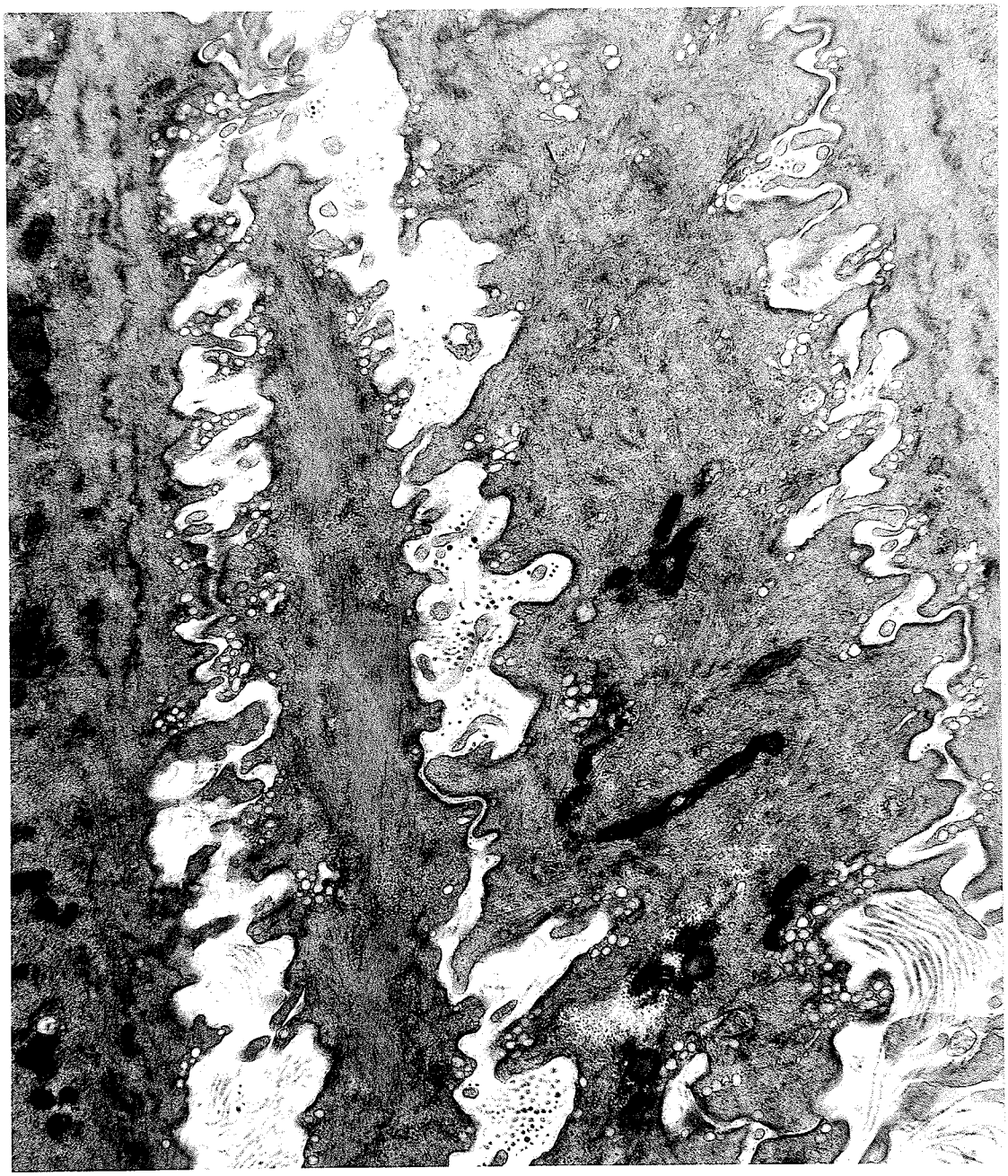

Fig. 20. Electronmicrograph of rat uterus (x 17,300). Uterus 1 h in Krebs-Henseleit solution.

Note areas of close cellular contact, membrane interdigitation and cytoplasmic vesicles.

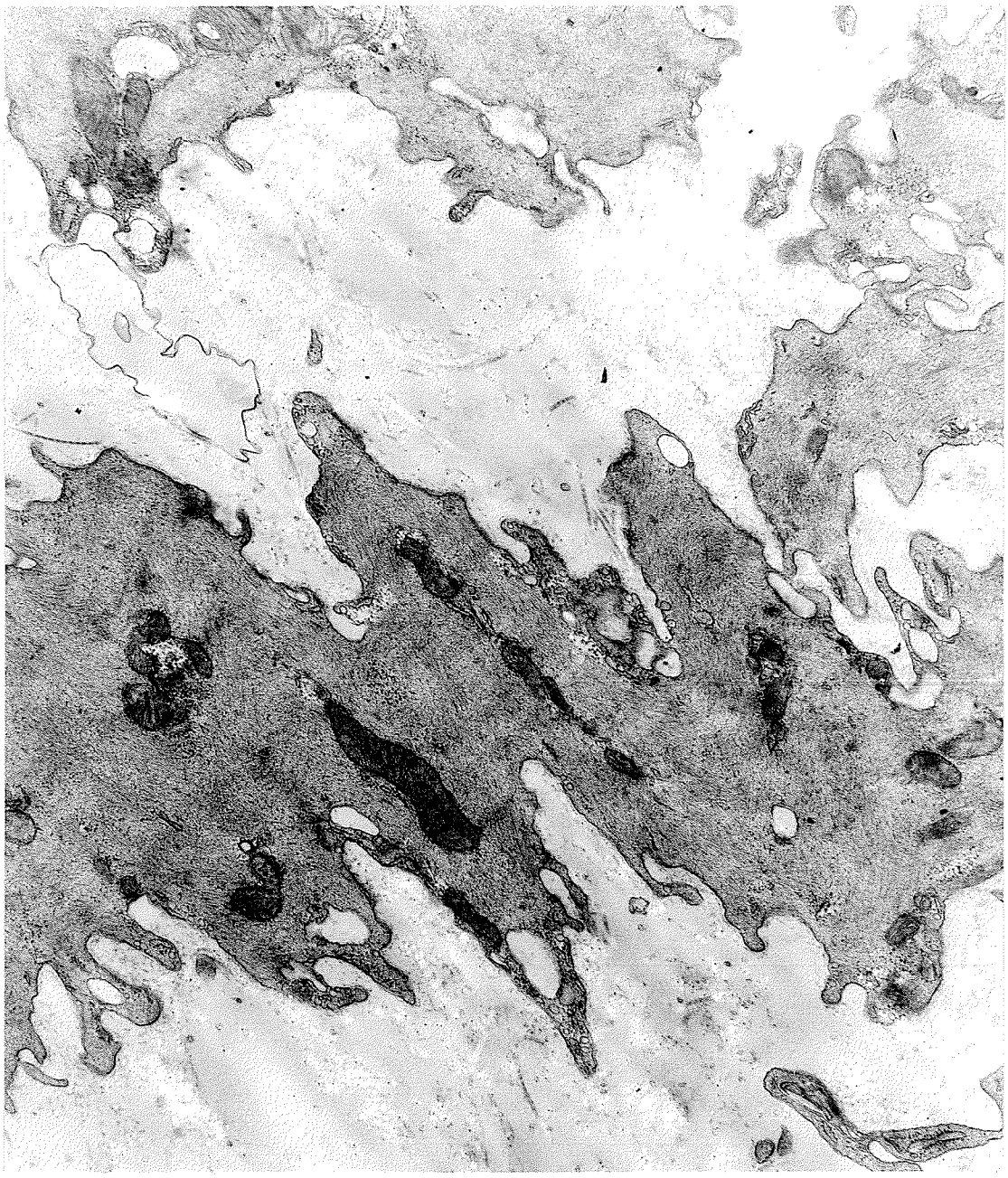

Fig. 21. Electronmicrograph of rat uterus (x 17,300). Uterus 1 h in Krebs-Henseleit solution containing 5 mM caffeine.

Separation of cells is clearly shown and cytoplasmic vesicles are few in numbers.

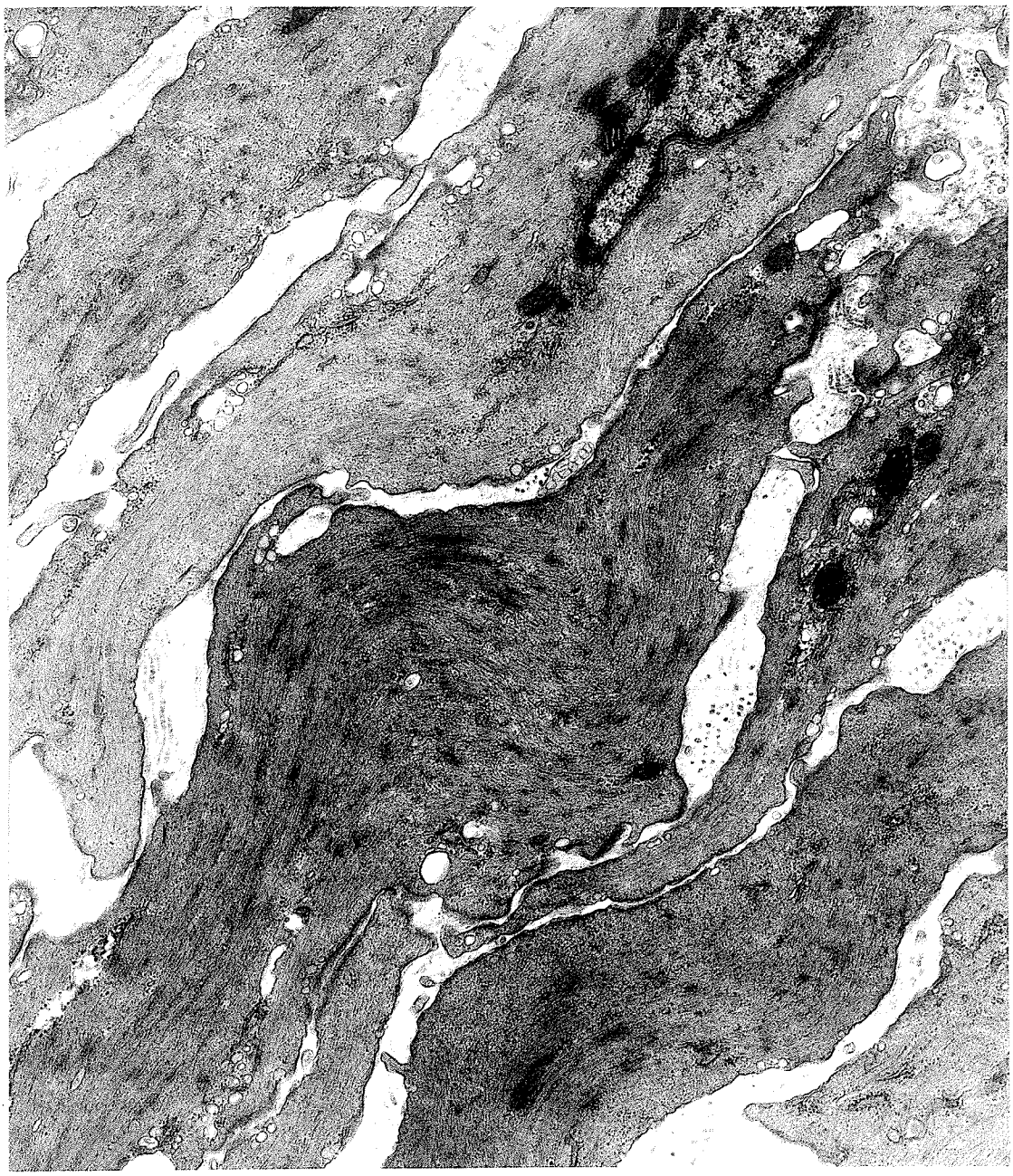

Fig. 22. Electronmicrograph of rat uterus (x 17,300). Uterus 1 h in Krebs-Henseleit solution containing 5 mM caffeine.

Areas of close cellular contact, interdigitation and numbers of cytoplasmic vesicles are markedly reduced.

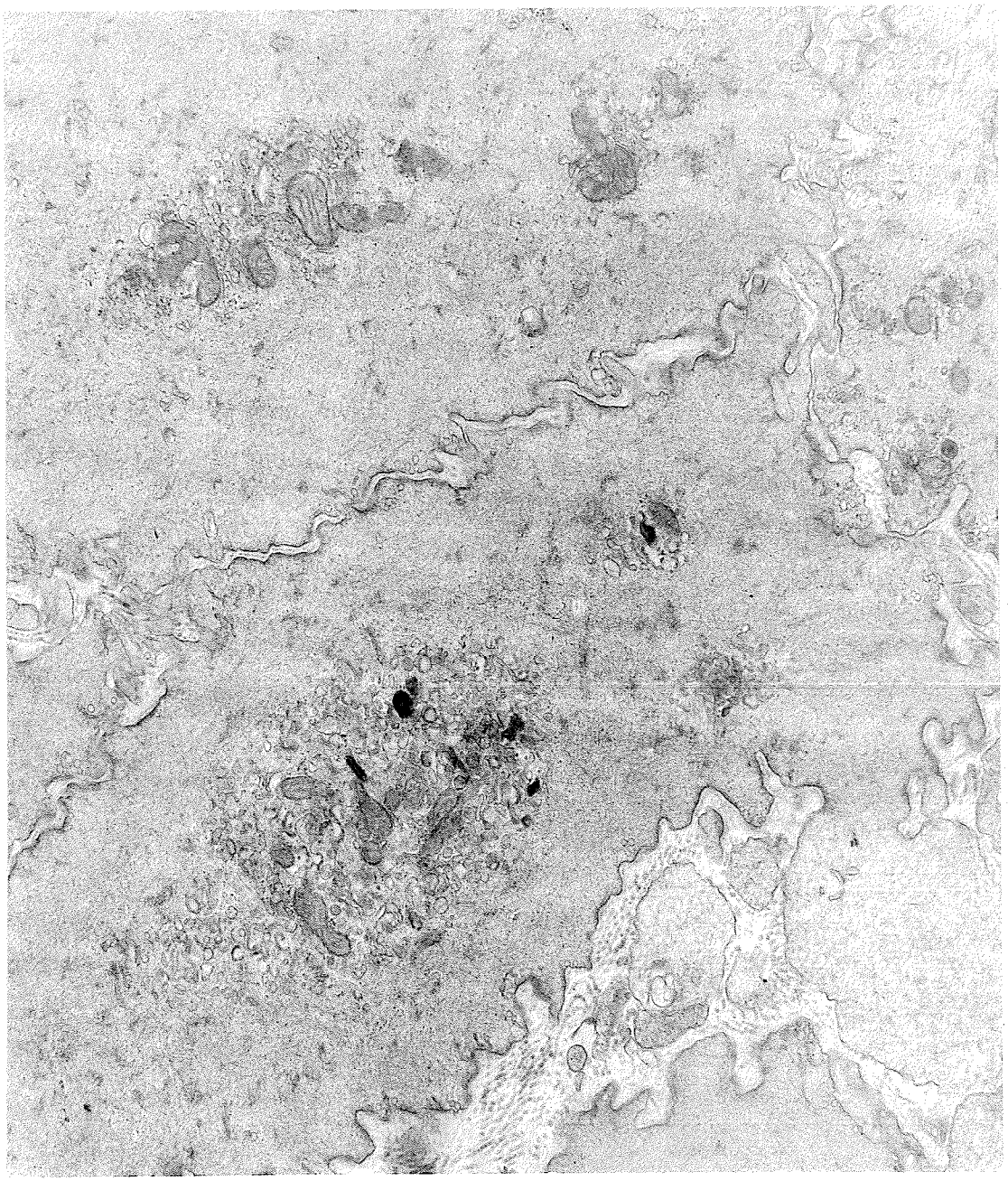

Fig. 23. Electronmicrograph of rat uterus (x 17,300). Uterus 1 h in Krebs-Henseleit solution containing 5 mM caffeine.

Caffeine removed and tissue kept in bath till automaticity returned. Areas of close cellular contact, membrane interdigitation and cytoplasmic vesicles have returned.

tions and close cellular contact. A further finding was that associated with this was a reduction in the number of vesicles normally found in the cell cytoplasm.

CAFFEINE, CALCIUM AND CYCLIC AMP

There are two main hypotheses on how caffeine inhibits smooth muscle.

A. INHIBITION OF PHOSPHODIESTERASE

Caffeine inhibits the enzyme phosphodiesterase and the resultant accumulation of cyclic AMP relaxes the smooth muscle. Adrenaline by stimulation of adenyl cyclase and papaverine by inhibition of phosphodiesterase also increase cellular levels of cyclic AMP. In our previous experiments (p. 39) with these agents we did not find the dissociation between action potentials and tension changes found in caffeine treated preparations. Because we had used a different uterine preparation in the earlier experiments, these were repeated on our standard preparation from twenty-one day pregnant rats. In this preparation as in the uterus from oestrogen-treated immature rats no dissociation was found between action potentials and tension development.

B. CALCIUM AND CAFFEINE

Caffeine may also reduce the calcium available to the contractile mechanism (Diamond and Marshall, 1969a, 1969b; Somlyo and Somlyo, 1970; Sunano, 1970; Harbon and Clause, 1971; Pfaffman, McFarland and Crow, 1971). Accordingly we tested the effects of raising the extracellular concentration of calcium on tissues which had been inhibited by 5 mM caffeine. With 5 mM caffeine in the bathing fluid the normal calcium chloride con-

centration of the bathing fluid was doubled (2.5 to 5 mM) by pipetting calcium chloride solution into the organ bath. Within 3 minutes preparations again showed action potentials and contractions. In all 6 preparations tested the increased calcium concentration did not change the resting membrane potential as would have been expected from the results of our previous experiments (Fig. 10), and the extracellular spaces did not significantly differ from controls (Table 7).

CAFFEINE, CALCIUM AND SODIUM

Niedergerke and Luttgau (1957) proposed that sodium competes with calcium for binding sites in smooth muscle, and competition between ions for cellular sites has been extensively studied by many workers (Bohr and Goulet, 1961; Waugh, 1962; Marshall, 1963; Goodford, 1966; Sparrow, 1969). Bohr, Seidel and Sobieski (1969) postulated that a sodium-calcium exchange mechanism exists in vascular smooth muscle which couples sodium efflux to calcium influx. Thus a muscle with an increased intracellular sodium concentration would also show an increase in calcium influx. They also suggested that calcium entry to cells is regulated by sodium competition as suggested by Neidergerke (1963) and Goodford (1966).

We reasoned that if interference with calcium was the main mechanism of action of caffeine in inhibition of smooth muscle, we might reverse the effects by increasing intracellular sodium or decreasing extracellular sodium.

INTRACELLULAR SODIUM

Ouabain is known to inhibit the sodium-potassium pump by inhibition

of sodium-potassium transport-ATPase (Skou, 1957; Dunham and Glynn, 1961) and thus increase intracellular sodium concentration. This has been shown to occur in rat uterus and other tissues (Daniel, 1964; Glynn, 1964; Casteels, 1966) and we therefore decided to determine the action of ouabain on caffeine-treated tissues.

Uterine strips were treated with 5 mM caffeine until completely quiescent and then exposed to ouabain, 10^{-4} M. During the next 12 minutes the resting membrane potential showed a gradual depolarization (50 to 12 mV); resting tension was unchanged. The muscle suddenly exhibited electrical and mechanical activity; membrane potential abruptly returned to 50 mV and action potentials appeared synchronously with the beginning of tension development (Fig. 24).

In the 6 experiments where we tested the effect of oubaine on caffeine inhibition of uterus, reversal of membrane potential preceded the start of action potentials and tension by 272 ± 27.9 msec. The extracellular spaces of the preparations treated with caffeine plus ouabain were not significantly different from controls (Table 7).

EXTRACELLULAR SODIUM

In 5 experiments uterine strips were treated with 5 mM caffeine until quiescent. The bathing fluid was then changed to a low (25 mM) sodium solution (p. 24) which also contained 5 mM caffeine. After 9 ± 0.42 minutes exposure to the low sodium bathing fluid, and with no preceding potential or tension changes, electrical and mechanical activity of the tissue returned. The extracellular spaces of these tissues did not significantly differ from controls (Table 7).

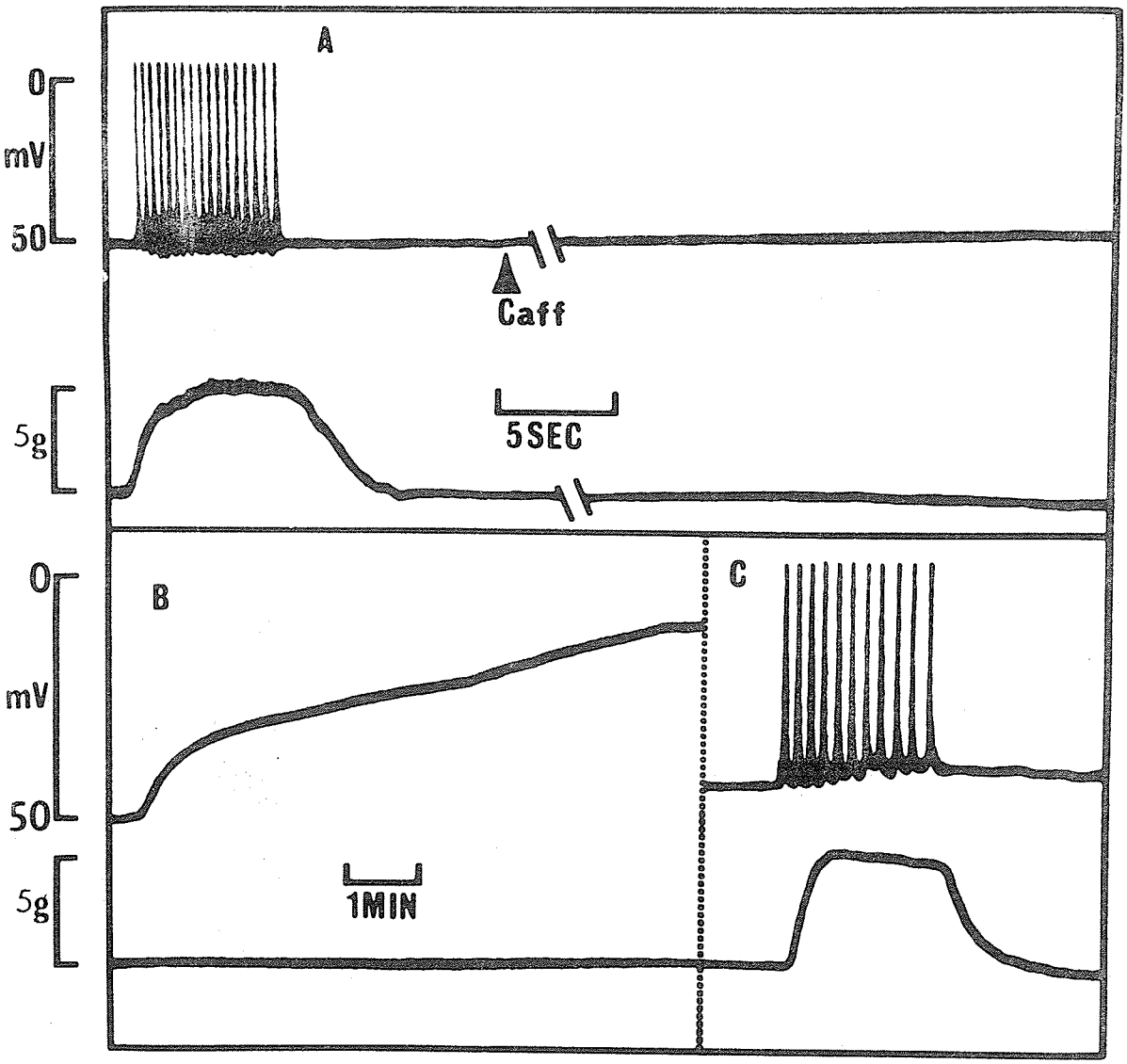

Fig. 24. Effect of ouabain on rat uterus treated with caffeine.

A. Rhythmic responses of uterus suppressed by 5 mM caffeine added at Caff.

B. 10^{-4} M ouabain, added to the bathing fluid produced a slow depolarization with no change in resting tension.

C. With 10^{-4} M ouabain still present, a sudden spontaneous repolarization is followed by resumption of rhythmic responses.

SUPERSENSITIVITY

The subject of supersensitivity has been extensively reviewed from time to time (Cannon and Rosenblueth, 1949; Furchgott, 1955; Emmelin, 1961; Trendelenburg, 1963 and 1966) and various hypotheses have been put forward to account for observed supersensitivity, though in general these have not withstood the test of critical experimentation.

Many pharmacological agents can produce supersensitivity and this study was limited to two agents, cocaine and reserpine.

(a) Cocaine's ability to cause supersensitivity was first reported by Frölich and Loewi (1910), but its mechanisms of action were still described by Furchgott (1955) as the "cocaine paradox". Cocaine has the advantage of producing supersensitivity when given acutely in vivo or in vitro, and potentiates responses to catecholamines in many effector organs in most species. However, there is not yet agreement on the ability of cocaine to potentiate responses to other classes of agonists (Rosenblueth, 1932; Tsai et al., 1968).

(b) Reserpine depletes tissue stores of catecholamines and 5-hydroxytryptamine and produces supersensitivity in various organs and species. Though a controversy exists as to the time of treatment required, most authors now agree that reserpine causes an unspecific supersensitivity (Innes, 1960; Trendelenburg and Weiner, 1962; Schmidt and Fleming, 1963; Green and Fleming, 1968; Kalsner and Nickerson, 1969; Davidson and Innes, 1970).

There are few reports on the effects of cocaine and reserpine in spontaneous rhythmic smooth muscles, and we decided to test the effects of these agents on modifying the responsiveness of spontaneously active rat uterus.

EFFECT OF COCAINE ON UTERUS

Preliminary experiments determined that the concentration of cocaine which effectively modified spontaneous uterine activity was 3×10^{-5} g/ml. Accordingly in 12 preparations from different animals we determined the frequency and duration of spontaneous contractions, maximum tension developed, resting membrane potential, conduction velocity and threshold electrical stimulus (punctate stimulation, single stimulus) before and in the presence of cocaine, 3×10^{-5} g/ml.

Cocaine did not change the maximum tension developed but significantly increased ($P < 0.01$) the duration of spontaneous contractions compared to control values (11.3 ± 0.9 to 23.4 ± 1.2 sec). The values stated are the means ± SE of at least 100 spontaneous contractions in each of 15 preparations. Typical effects of cocaine are shown in Fig. 25. Cocaine did not alter the resting membrane potential but changed the excitability of the tissue in that the value of threshold electrical stimulus was reduced from 23 ± 1.2 V.200 msec to 10 ± 0.9 V.200 msec ($P < 0.05$) and conduction velocity was increased from 74.2 ± 2.1 to 104.7 ± 1.7 mm/sec ($P < 0.05$). In most preparations a discrete pacemaker area remained; in others (20% of total preparations tested) we recorded irregular contractions and potentials (Fig. 26) and in these preparations multiple pacemaker areas could be detected.

The irregular contractions and multiple pacemaker area found in some preparations in the presence of cocaine were similar to those shown by preparations from rats seventeen days pregnant. Preparations from rats twenty-one days pregnant quickly lose spontaneous activity when placed in calcium-free Krebs-Henseleit compared with preparations from

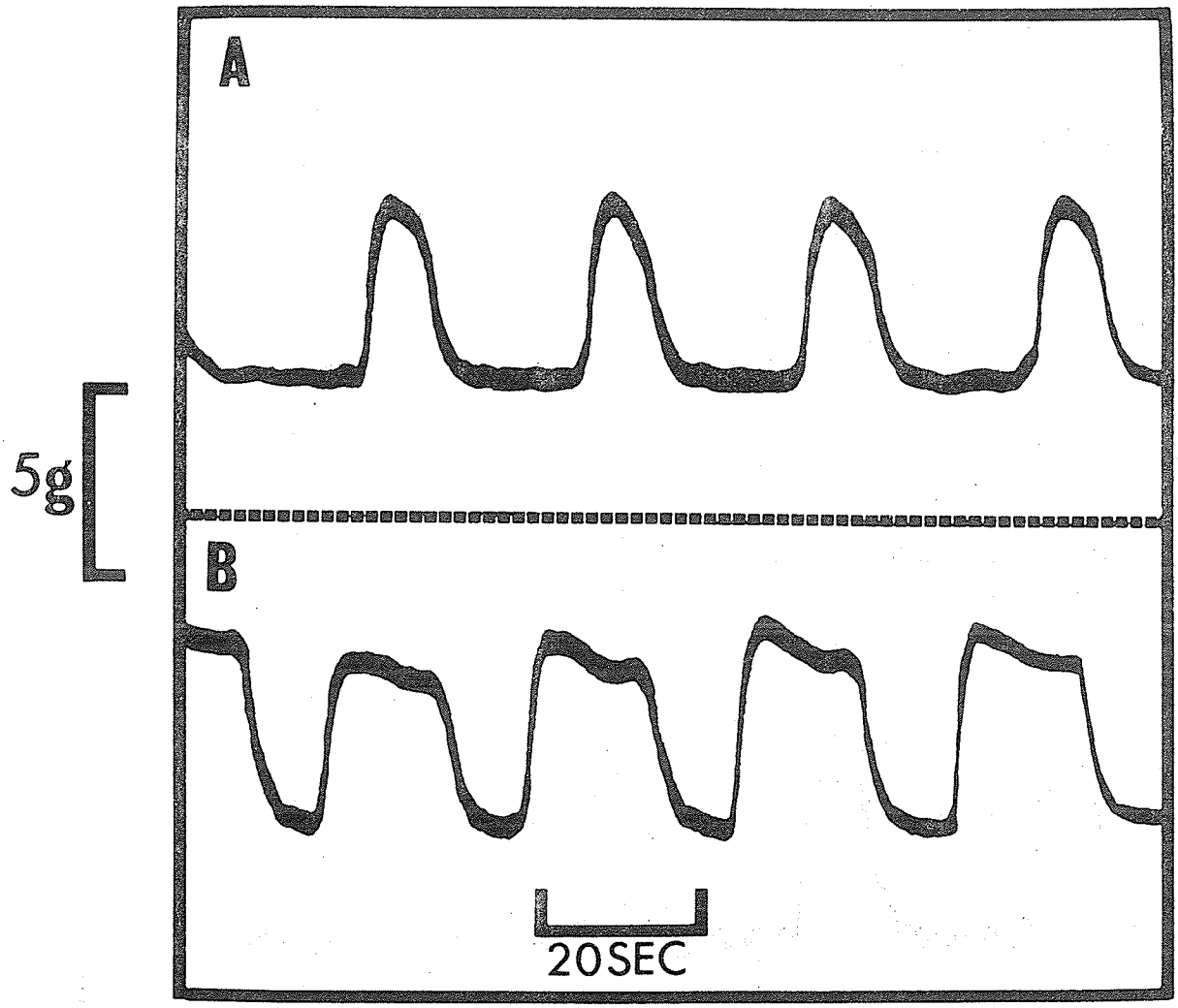

Fig. 25. Effect of cocaine on spontaneous contractions of rat uterus. Cocaine, 3×10^{-5} g/ml, in the bathing fluid increased duration of spontaneous contractions.

A. Spontaneous contractions in the absence of cocaine.
B. Spontaneous contractions in the presence of cocaine.

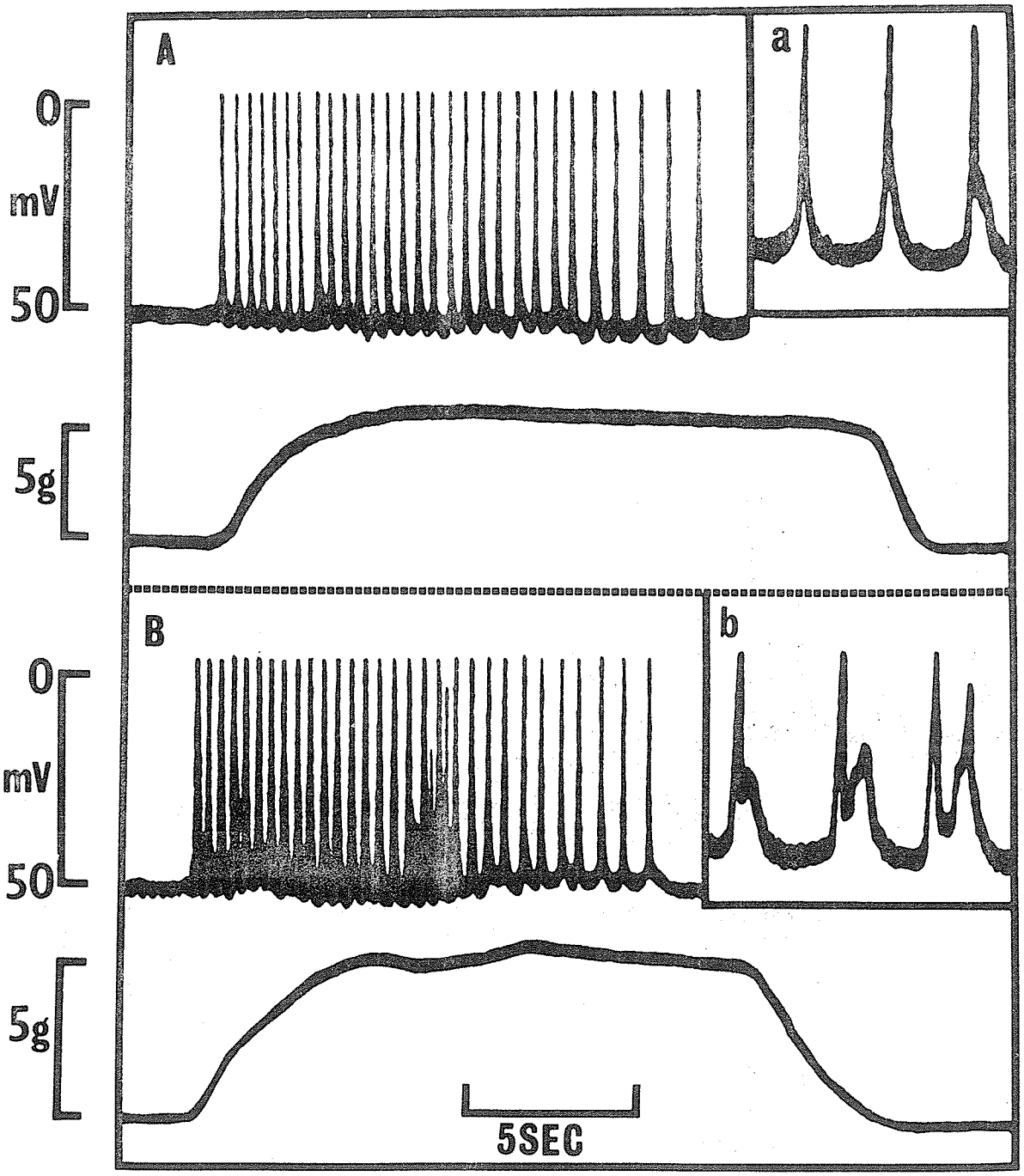

Fig. 26. Effect of cocaine on electrical and mechanical phenomena in spontaneous contractions of rat uterus. Spontaneous contractions of uterus with cocaine, 3×10^{-5} g/ml, in the bathing fluid.

A. A preparation showing regular electrical and mechanical activity.

B. A preparation showing irregular electrical and mechanical activity. Inserts a and b show action potentials on an expanded scale.

rats seventeen days pregnant (Marshall and Csapo, 1961). In our uterine preparation from rats twenty-one days pregnant we determined the time to quiescence in calcium-free bathing fluid, before and in the presence of cocaine, 3×10^{-5} g/ml.

In all preparations cocaine significantly increased ($P < 0.05$) the time to quiescence produced by placing preparations in calcium-free bathing fluid. Results are shown in Table 8.

CAFFEINE AND COCAINE

Because we had shown that doubling the calcium concentration in the bathing fluid reversed caffeine-induced quiescence and that cocaine increased the time to quiescence of preparations in a calcium free bathing medium, we decided to test possible interactions between caffeine and cocaine.

In 9 experiments we determined the ability of 5 mM caffeine to produce quiescence in uterine preparations before and in the presence of cocaine, 3×10^{-5} g/ml. Caffeine again produced quiescence in tissues not treated with cocaine but had no visible effect on tissues in the presence of cocaine, 3×10^{-5} g/ml, even when the concentration of caffeine in the bathing fluid was increased to 15 mM. We also determined the time required for 5 mM caffeine to inhibit spontaneous activity in preparations from animals seventeen days pregnant or when such preparations were placed in a calcium-free bathing medium. Times to quiescence, produced by 5 mM caffeine and calcium-free bathing medium were 37.4 ± 1.5 and 30.9 ± 1.3 minutes respectively. These values are significantly greater ($P < 0.01$) than those obtained in preparations from animals

TABLE 8

Determination of time to produce quiescence in rat uterus

	Time to quiescence (min)			
	Pregnant 21 day	Pregnant 21 day + Cocaine 3 x 10^{-5}g/ml	Pregnant 17 day	Number of Determinations
Calcium-free	6.2 ± 0.7	18.9 ± 0.36	30.9 ± 1.3	9
5 mM Caffeine	11 ± 0.27	No quiescence produced	37.4 ± 1.5	12

twenty-one days pregnant and treated in the same manner. Results of these experiments are shown in Table 8.

In 6 preparations cocaine, 3×10^{-5} g/ml, did not reverse the inhibition produced by 5 mM caffeine. Moreover, spontaneous activity of the preparations quickly returned after washout of both drugs, but the pattern of activity observed was characteristic of tissues treated with cocaine, i.e. increased duration of spontaneous contractions. However, the irregular contractile or membrane phenomena, indicative of multipacemaker areas, did not occur.

EFFECT OF RESERPINE PRETREATMENT ON UTERUS

The effect of reserpine could not be determined within a single preparation because pretreatment of the animal is required to produce supersensitivity. We therefore determined the frequency and duration of spontaneous contractions, maximum tension developed, resting membrane potential, conduction velocity and threshold electrical stimulus (punctate stimulation, single stimulus) in 10 preparations from rats given reserpine (1 mg/kg 24 h before the experiment) and compared these with values obtained from an equal number of preparations from untreated rats.

A comparison of values determined in preparations from untreated rats showed that treatment with reserpine significantly increased ($P < 0.05$) the maximum tension developed by spontaneous contractions (4.86 ± 0.22 to 7.73 ± 0.34 g) and the frequency of contractions ($P < 0.01$) as shown by the decrease in time interval between these (16.2 ± 0.6 to 5.2 ± 0.4 sec). The duration of spontaneous contractions was slightly reduced but this was not significant. Values stated are the means ± SE of at least 100 spon-

taneous contractions in each of 10 preparations from treated and untreated animals. Fig. 27 shows the typical effect of reserpine.

Reserpine treatment did not alter the value of resting membrane potential, but the responsiveness of the preparation was strikingly altered. The threshold electrical stimulus compared to that obtained in preparations from untreated animals was reduced from 30.1 ± 1.9 V.200 msec to 0.82 ± 0.14 V.200 msec ($P < 0.001$) and conduction velocity was increased from 72.0 ± 2.3 to 138 ± 2.8 mm/sec ($P < 0.01$). The values stated are the means ± SE of at least 10 determinations in each of 10 preparations from treated and untreated animals. Determinations of time to quiescence produced by 5 mM caffeine or calcium-free bathing medium were not significantly different from those obtained in preparations from untreated animals.

The changes in maximum tension development and other parameters might have been due to changes in excitability and tone of tissues caused by alteration of normal mediator influences; reserpine is known to deplete tissues of their normal catecholamine content (Burn and Rand, 1958) and increase acetylcholine content (Malhotra and Das, 1962; Green, Fleming and Schmidt, 1968).

We therefore tested the effect of atropine and tetrodotoxin which should abolish nervous influences (Narahashi, Moore and Scott, 1964) on the spontaneous activity of preparations from animals pretreated with reserpine. Neither atropine, 10^{-7} g/ml, nor tetrodotoxin, 10^{-5} g/ml, singly or in combination, had any appreciable effect on the spontaneous activity of the preparations.

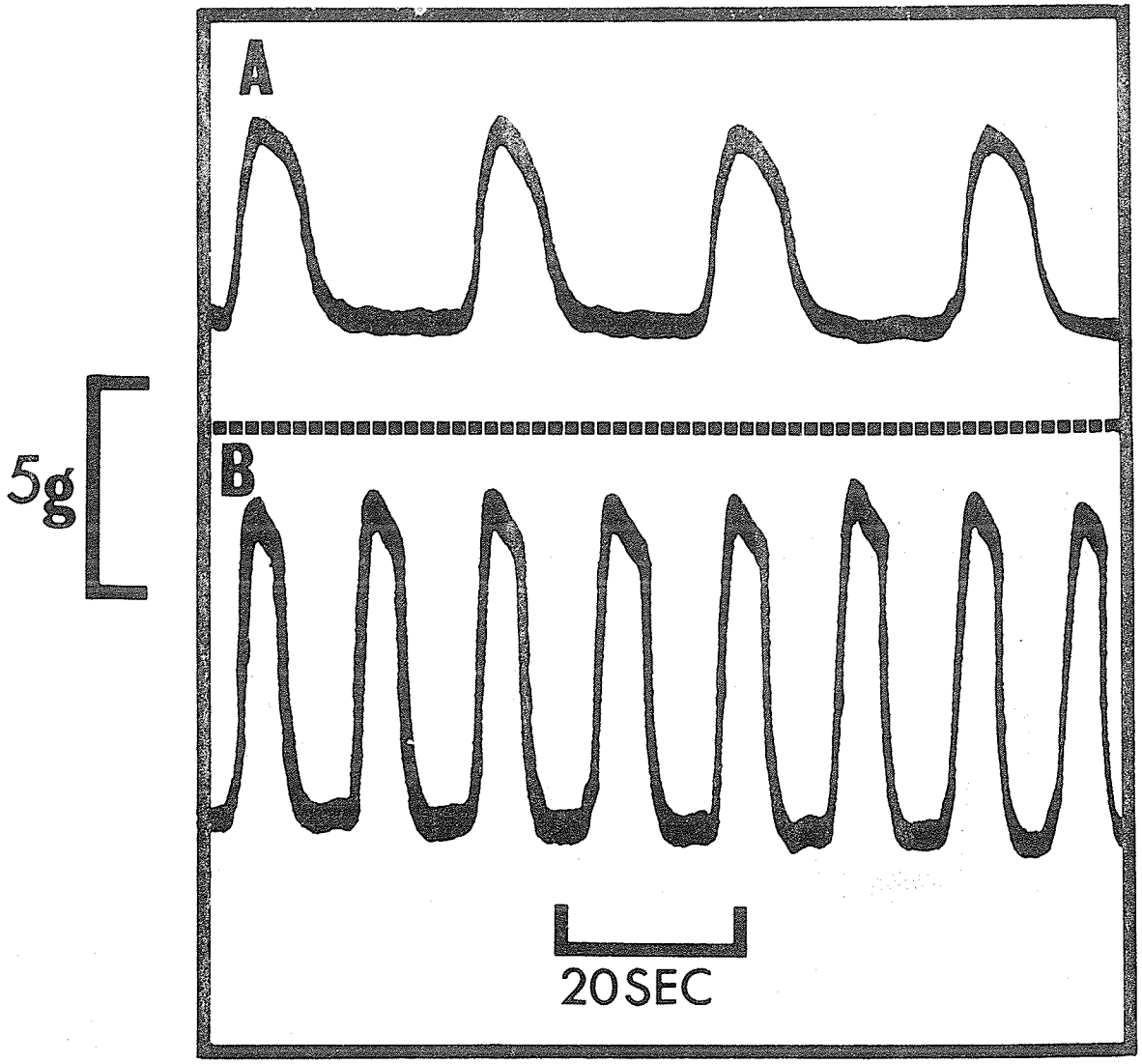

Fig. 27. Effect of reserpine treatment on spontaneous contractions in rat uterus.

Contractions of uterus: A from a rat without reserpine treatment, B from a rat treated with reserpine (1 mg/kg).

PACEMAKER AREAS

None of the preparations from animals treated with reserpine showed any evidence of multiple pacemaker areas, but on probing the discrete pacemaker area we could apparently detect an increase in the number of active pacemaker cells within the area. The usual pacemaker area in preparations from twenty-one day pregnant rats lay very close to the ovarian end of the tissue and pacemaker cells could be detected only in that area which appeared to be usually about 2 mm square. The size or location of the area did not appear to be altered by reserpine treatment but the number of pacemaker cells found was increased. Experiments showed that we usually detected in an untreated preparation 6 active pacemaker cells for every 3 h probing in the pacemaker area. In preparations from reserpine treated preparations we usually detected 30 active pacemaker cells for every 3 h probing in the pacemaker area. We thought that one possible explanation was the frequency that cells changed from pacemaker to non-pacemaker and vice versa had been increased by reserpine treatment.

A typical record of a non-pacemaker cell in the pacemaker area is shown in Fig. 28 A. An active pacemaker cell in the pacemaker area with the slope of the prepotential directly proportional to the frequency of firing of the cell is shown in Fig. 28 B and C. Attempts to hold a pacemaker cell until it changed to a non-pacemaker cell showed that the length of time that an active pacemaker cell remained as such in preparations from untreated and reserpine treated animals was usually longer than we could successfully hold cells with the microelectrode (average holding time 20 minutes, range 3 to 33 minutes). Figs. 29 and

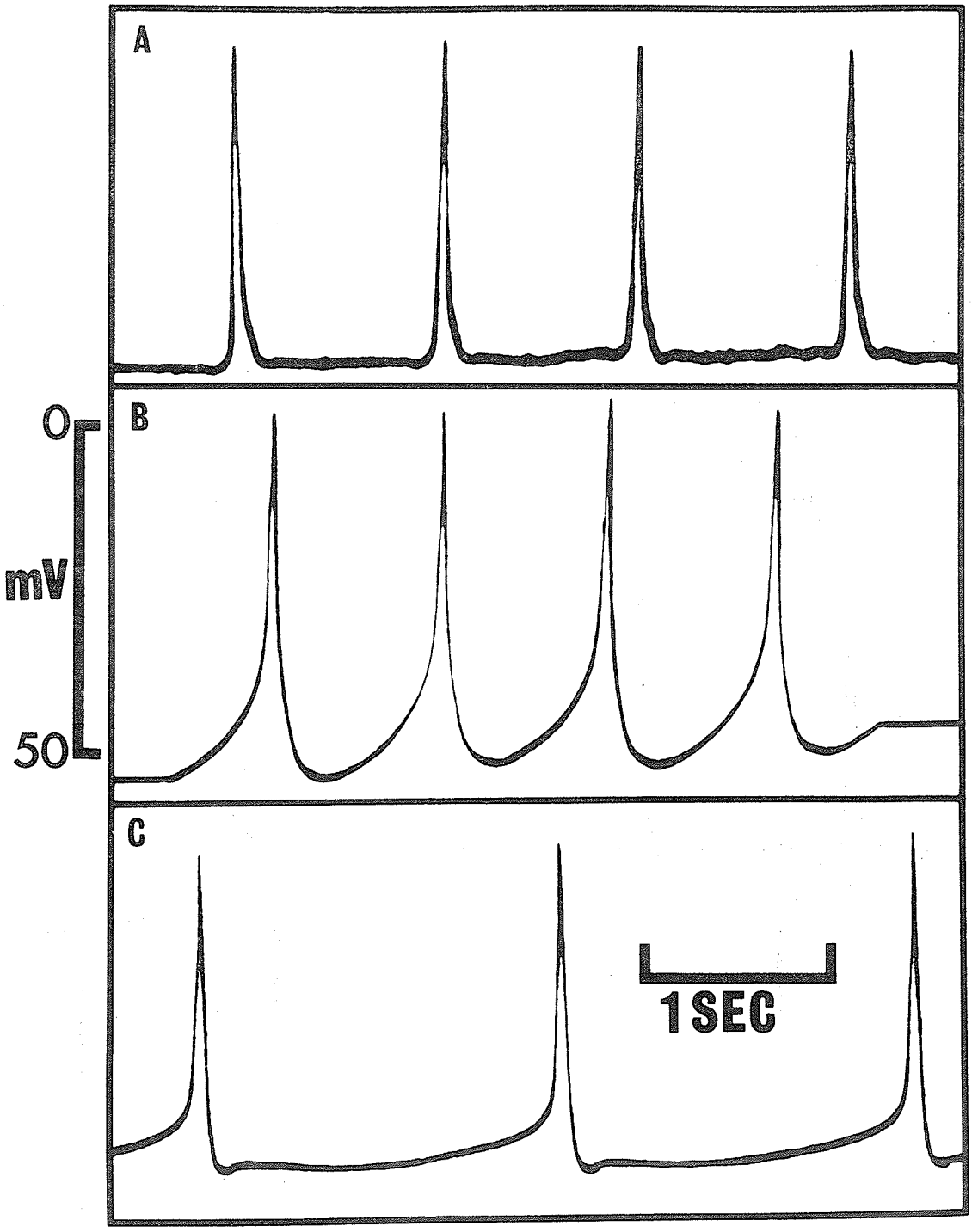

Fig. 28. Action potentials of rat uterus.

A. Typical action potentials from cell of rat uterus.
B. and C. Typical action potentials from the same pacemaker cell of rat uterus, showing the relationship between the slope of prepotential and frequency of action potentials.

30 show records of cells changing from pacemaker to non-pacemaker and vice versa. These events, however, are not easy to detect, as we have only been able to record these on 3 occasions despite repeated and painstaking efforts. The transition from non-pacemaker to pacemaker was quite abrupt with no gradual prepotential changes (Fig. 30). While the change from pacemaker to non-pacemaker shown in Fig. 29 also occurs abruptly between two action potentials we are not certain if the smaller action potential seen at the time of change is a significant or regular event or a recording artifact.

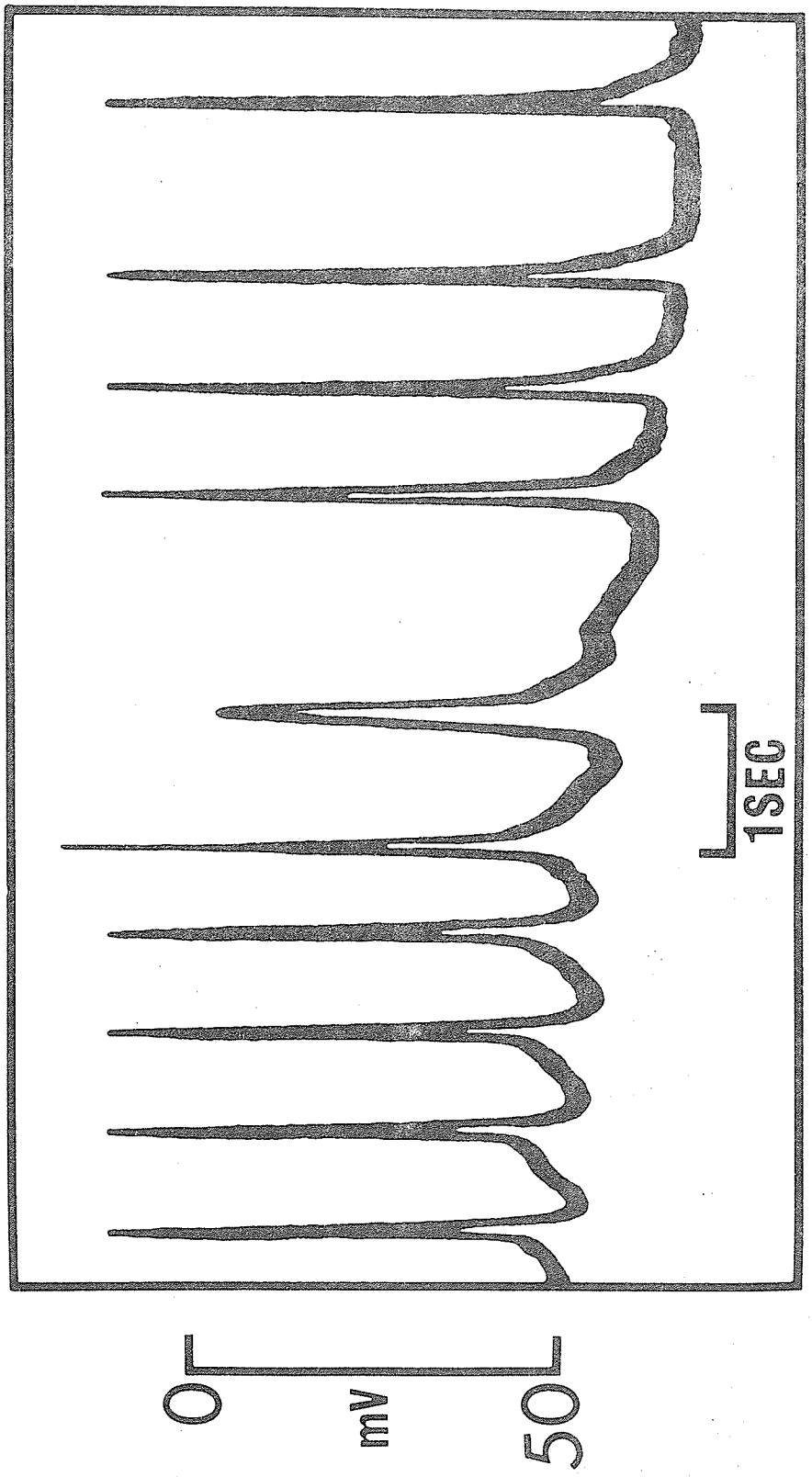

Fig. 29. Action potentials of rat uterus.

Action potentials showing prepotentials typical of pacemaker cell suddenly change to action potentials typical of cell not acting as a pacemaker.

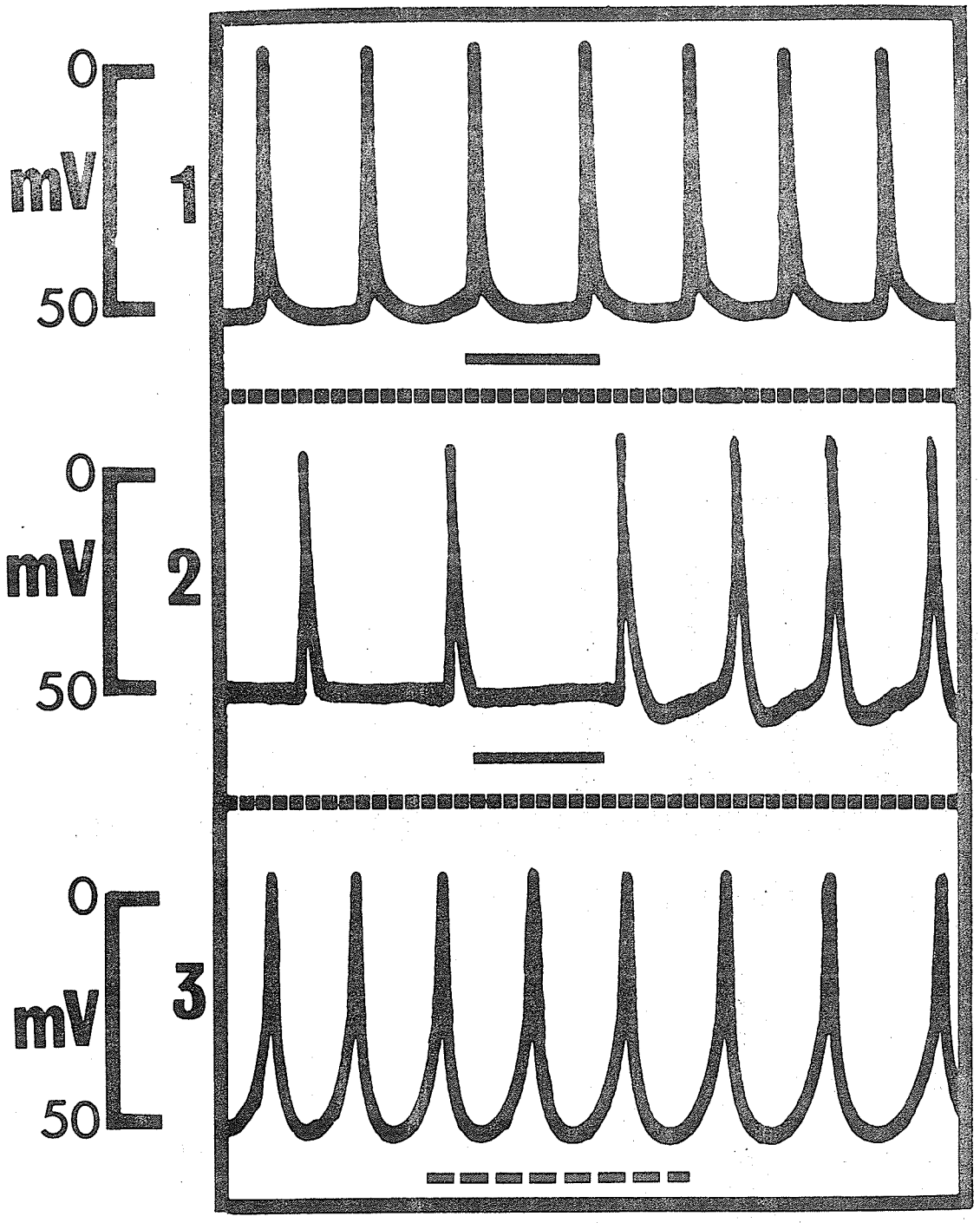

Fig. 30. Action potentials of rat uterus. Recordings are from the same cell in rat uterus.

1. Typical action potential suddenly changes in 2 to action potential characteristic of those recorded from pacemaker cell.
3. Action potentials from the same cell still shows pacemaker cell characteristics.

Time Scale ——— = 1 sec - - - = 1 sec.

SECTION IV

DISCUSSION AND CONCLUSIONS

DISCUSSION AND CONCLUSIONS

Changes in endocrine condition of the living animal can effect chemical or physical changes in smooth muscle cells of the uterus which are reflected in the behaviour of *in vitro* preparations. This study was done on smooth muscle of rat uterus. We began by examining some basic properties of rat uterus under various hormonal influences so that we could choose a suitable preparation to determine changes that occurred after procedures modifying excitability and contractility.

EFFECTS OF OESTROGENS

Preliminary experiments agreed with the conclusions of Bozler (1948) and Csapo (1955) that uterine preparations from immature animals exhibit no spontaneous activity and have a low resting membrane potential (35 mV). The administration of oestrogen to such an animal raises the resting membrane potential to a critical level, around 50 mV, where spontaneous discharge of action potentials begin and regular rhythmic contraction of smooth muscle occurs.

Allen and Doisy (1923) first showed that substances, later identified as oestrogens, present in liquor folliculi, when injected into immature mice brought on oestrus; the uterus became enlarged, hyperaemic and showed powerful spontaneous contractions. A significant advance in the understanding of the mode of action of oestrogens occurred when Glascock and Hoekstra (1959) and Jensen and Jacobson (1962) showed that, when physiological doses of oestrogens were given to immature female rats the uterus showed greater and prolonged retention of oestrogens compared

to so called non-target organs. Toft and Gorski (1966) demonstrated the presence of a soluble protein component of uterine homogenates which was capable of specifically binding oestradiol. These oestrogen receptors have been demonstrated in the uterus of many species (Maurer and Chalkley, 1967; Gorski et al., 1968).

The concentration of unoccupied high affinity oestrogen receptor sites in rat uterus has been studied and shown to vary in a cyclic manner through the 4 day oestrus cycle. The lowest concentrations were found on the day of oestrus and rose to a maximum during late dioestrus (Feherty et al., 1970). The binding characteristics of the receptor isolated from different species are similar; oestrogens are bound in the order, oestradiol, oestrone and oestrol with decreasing affinity. In human myometrium a cytoplasmic receptor with a high affinity for oestradiol has been reported (Wyss et al., 1968; Mester et al., 1970). A study on variation in concentrations of human oestrogen receptors in normal and pathological states could well be clinically important.

While the exact mechanisms of action of oestrogens are still not understood recent workers have demonstrated in rat uterus that the oestrogen-receptor complex migrates to the cell nucleus and there is an increase in the amount of phospholipids, RNA synthesis and protein synthesis (Aizawa and Mueller, 1961; Noteboom and Gorski, 1963; Lafreniere and Singhal, 1970; Weihs, Truhlsen and Banerjee, 1970; Giannopolous and Gorski, 1971a, 1971b). Histological examination shows that in uteri of immature animals treated with oestrogens there is an increase in the size of smooth muscle cells, and some interdigitations of opposing cell membranes with areas of close contact. The number of ribosomes increases

as does the amount of rough endoplasmic reticulum (Ross and Klebanoff, 1967; Bo, Odor and Rothrock, 1968).

RELATION BETWEEN ELECTRICAL AND MECHANICAL ACTIVITY

Some early workers postulated that smooth muscle cells behaved as separate units and spread of excitation was within an interstitial network (Keith, 1915; Tiegs, 1925; Ambache, 1947) while Rosenblueth (1936) suggested excitation by diffusion of a transmitter substance. The accepted view at present is that smooth muscle can be divided into two classes, multi-unit where excitation is probably produced by neurohumoral transmitter release and single-unit where excitation most likely proceeds from cell to cell, and that conduction by an intercellular network is unlikely (Bozler, 1938, 1941, 1948; Prosser and Sperelakis, 1956; Daniel and Singh, 1958; Dewey and Barr, 1962).

Bulbring (1955) and Landa, West and Thiersch (1959) reported that tension was always directly proportional to spike frequency, but their preparations were quite short. Reports which conflict with these findings are confusing in that preparations varied in size and were obtained from different tissues under varied hormonal influences.

Many workers now agree with Daniel (1957) that asynchrony will only be seen in preparations where activity is governed by multiple pacemaker cells firing irregularly or where there is a conduction block between different parts of a preparation. The results of our experiments in preparations from animals treated with oestrogens and progesterone or seventeen days pregnant also confirm the suggestion by Daniel. In these preparations we could detect multiple pacemaker areas whose activities

were often out of phase, and spontaneous contractions were usually irregular and asynchronous with recorded action potentials.

The observation that action potentials began after the onset of developed tension in some preparations from oestrogen-treated animals was unexpected. Our results clearly showed that preparations from oestrogen dominated animals exhibited regular smooth contractions, simple spike potentials and a discrete pacemaker area near the ovarian end of the preparation. The results of further experiments indicated that the apparent asynchrony could only be observed in potential recordings from cells at the cervical end of the preparation. The anomaly was due to the time required for a propagated impulse arising in the pacemaker area to activate cells near the cervical end. Further evidence to support this explanation was that asynchrony between action potentials and developed tension was not seen if microelectrodes were impaling cells near the pacemaker area. Preparations from twenty-one day pregnant animals also showed regular contractions, simple spikes and a similarly placed discrete pacemaker area, but conduction velocity in these preparations was almost twice as fast as that in preparations from non-pregnant animals. A very slight asynchrony could be detected if electrodes were placed at the extreme cervical end of the preparation.

The membrane potential of oestrogen-dominated uterine muscle cells is usually close to the threshold for spontaneous discharge, resulting in bursts of action potentials which produce regular rhythmic contractions. The results obtained in a few preparations showed that after oestrogen treatment, in some cells the resting membrane potential had not changed from that usually recorded in immature animals. When

activated by pacemaker impulses or other stimuli, these cells would become hyperpolarized before action potentials occurred. These findings combined with the fact that oestrogen treatment hyperpolarizes the membrane potential of the immature rat uterus (35 to 50 mV) strongly suggested that there was a critical firing zone of membrane potential and that the effect of a stimulus on uterine cell membrane depended on the existing level of membrane potential.

Results of experiments with adrenaline and papaverine confirmed this and also suggested that the lowest critical membrane potential was 45 mV. Cells which already showed critical membrane potential and were further hyperpolarized by adrenaline did not require repolarization to the same level before action potentials occurred. This is further confirmed by the findings that action potentials without preceeding depolarization can occur in cells from a normal resting membrane potential of 65 mV. The results indicated a zone of critical membrane potential for initiation of action potentials from 45-65 mV. Additional evidence which lends support for this view comes from the effects of stimulation by large doses of agonists which depolarized the membrane potential beyond the critical firing zone. Here smaller action potentials than usual appeared, followed by cessation of spike discharge.

PROGESTERONE

Corner and Allen (1929) prepared extracts of sow corpora lutea which maintained pregnancy in ovariectomized rabbits, and in a uterus primed with oestrogens induced an endometrium suitable for implantation of a blastocyt. The hormone isolated from these extracts was called

progesterone. Recent workers have shown there is a specific progestogen binding site in the cytoplasm of uterine cells. Primary stimulation of the uterus by oestrogen is required before progestogens can produce their effects, among which are inhibition and stimulation of synthesis of various enzymes (Hodgen and Falk, 1971; McGuire and DeDella, 1971; Pepe and Yochim, 1971a, 1971b and 1971c).

Our results indicate that progesterone alone had no effect on the immature uterus, but in mature animals or immature animals primed with oestrogen, treatment with progesterone raised the membrane potential to 65 mV. In such preparations we could detect multiple asynchronously firing pacemaker areas associated with frequent but irregular contractions and action potentials. These results are in agreement with reports of earlier workers (Marshall, 1959; Daniel, 1960; Csapo, 1961). We also found that conduction velocity could not always be determined. This was probably due to impulses from one or more of the pacemaker areas producing a conduction block, cells being refractory to the potential initiated by electrical stimulation. The values we did obtain suggest that progesterone did not significantly change conduction velocity from that measured in preparations from animals dominated only by oestrogens.

PREGNANCY

We agree with the results of Marshall (1962) that preparations from seventeen day pregnant animals behaved similarly to those from animals treated with oestrogens and progesterone. The preparations from rats twenty-one days pregnant behaved like those from animals dominated with oestrogen except that areas near placental implantation sites showed

a membrane potential 10 mV higher. Wichmann (1967) showed that the rat uterus has only a limited number of specific binding sites for progesterone and that those near the placenta become saturated quite early in pregnancy. The percentage saturation in myometrium is a function of distance from the placenta. Nearer term no significant difference can be found between the progesterone concentrations in any part of the myometrium. This indicates that the uterus changes from an asymmetrical system in early pregnancy towards a more symmetrical system. Similar results have been reported in the human (Runnebaum and Zander, 1971).

Our findings that conduction velocity in pregnant uterus was twice that determined in uterus from non-pregnant animals might be explained on the basis of increased size of cells. The cells of pregnant uterus show considerable hypertrophy, roughly doubling in size (Woodbury and McIntyre, 1954). However we appreciate that other possibilities such as an increase in cellular interaction or coupling could also explain these findings.

IONIC CONTENT AND MEMBRANE POTENTIAL

Treatment with progesterone alone had no effect on the ionic content of immature rat uterus, but treatment with oestrogen caused a gain of potassium and loss of sodium. While most workers have reported changes in potassium content after treatment of immature rats with oestrogens, the magnitude of the increase varies considerably, and some workers report no changes (Cole, 1950; Horvath, 1954; Kalman, 1957; Daniel, 1958; Kao, 1961). The results of determinations of ionic content, water content, and extracellular space in all preparations except

untreated or progesterone treated immature animals showed no significant difference.

The potassium equilibrium potential obtained from our data, calculated by the Nernst equation was 90 mV, while the measured membrane potentials in preparations from animals under different hormonal influences or stages of pregnancy varied between 50 to 65 mV. This discrepancy between measured and theoretical values has been observed by many workers and the situation was reviewed by Kuriyama (1961). Because all our measured values were obtained from tissues under comparable environmental conditions, we looked for possible explanations for the differences in determined resting membrane potentials and the discrepancies between calculated and empirical values.

DAMAGE TO CELL MEMBRANE

The penetration of a cell membrane by a microelectrode with tip diameters of 0.1 to 0.5 μ could result in damage to the cell membrane so that leakage of ions occurred and erroneous estimates of resting membrane potential would be obtained. However we would have expected that such cells would not have been able to maintain a steady membrane potential under such conditions and we were able to record stable resting membrane potentials for as long as 33 minutes. We also reasoned that damage would be proportional to cell size. There was no significant difference in cell size from animals at the different stages of pregnancy, yet 15 mV potential differences were recorded between such preparations. We therefore rejected cell damage by microelectrodes as an explanation of the discrepancies.

BOUND ELECTROLYTES

The results of experiments in which tissues were bathed in isotonic sucrose solution at 4°C for twenty days clearly showed that a substantial percentage of ions in the uterus are tightly bound. Under these conditions; metabolic processes should have been inactivated and all cell ions should have been removed by diffusion. Residual ions detected could not be expected to be electrochemically free and were an obvious source of error in calculations of membrane potential. A finding of particular interest was that tissues from oestrogen dominated and twenty-one day pregnant animals contained significantly more sodium and potassium than tissues from oestrogen plus progesterone dominated and seventeen day pregnant animals. These findings were further supportive evidence that preparations from twenty-one day pregnant animals are mainly under oestrogen influences, while preparations from seventeen day pregnant animals are mainly under progesterone domination. We were also surprised to find that although all calcium ions had been removed some 16% of magnesium content remained. We speculate that at least part of the remaining magnesium ions are bound to enzymatic sites, e.g. ATP-ases.

The differences between residual sodium and potassium contents obviously reflect important alterations produced by progesterone in the nature of binding sites. At present we cannot reasonably speculate on the nature of these changes or the sites at which they occur. We also calculated that whichever way we assumed these tightly bound electrolytes to be distributed, and we have no evidence to justify placing them at any particular cellular or extracellular site, we still could not explain discrepancies in empirical and theoretical values of membrane potential.

Harris and Palmer (1970) reported that mucopolysaccharides in arterial walls can act as cation binding sites. Other workers (Garrahan, Villamil and Zadunaisky, 1965; Friedman, Gustafson and Friedman, 1968) suggested that such areas in arterial segments might well act as extracellular compartments with cation concentrations higher than that of extracellular fluid.

The situation which would change the theoretical value of resting membrane potential in uterus nearest to the empirical value of the potassium equilibrium potential would be one where all the residual potassium ions were extracellular. Accordingly we assumed that all the residual potassium was present in uterus in some extracellular compartment and then recalculated our values of the potassium equilibrium potential. The values obtained for oestrogen dominated, twenty-one day pregnant and oestrogen plus progesterone, seventeen day pregnant uteri were 83.5 and 81.1 mV respectively. These values still deviate 33.5 and 26.1 mV from the theoretical value.

STRETCH AND MEMBRANE POTENTIAL

Excessive stretch produces a maintained depolarization (Goto and Woodbury, 1958) and could have explained some of the differences between our empirical values and theoretical values of membrane potential. Results of our experiments in which we varied the resting tension of the tissue clearly showed that we were not excessively stretching our preparations.

IN VIVO MEMBRANE POTENTIALS

The findings that resting membrane potentials of rat uterus

measured in vivo were not significantly different from those obtained from comparable in vitro preparations was gratifying but puzzling. The uterus in vivo should have been under optimal conditions of length, tension and electrolyte gradients. We could detect no difference in values of membrane potentials obtained when we used different agents to anaesthetize animals. However, in the in vivo preparation single action potentials often occurred as well as trains of action potentials. Single action potentials rarely occurred in preparations in vitro.

There are few reports with which we can compare our in vivo data, although Daniel, Honour and Bogoch (1960) studied the electrical activity of dog small intestine in vivo with intracellular recordings and noted that the resting membrane potential was similar to that recorded in other intestinal preparations in vitro.

IONS AND MEMBRANE POTENTIALS CONTINUED

The results of another series of experiments where tissues kept in organ baths in Krebs-Henseleit solution for prolonged periods gained sodium and lost potassium but did not show any changes in resting membrane potential. This evidence added to the evidence that we had already gathered forced us to reassess the whole situation.

The ionic hypothesis states that the resting potential of excitable cells results from the distribution of ions across a selectively permeable cell membrane and the potassium concentration gradient has been considered responsible for the resting membrane potential of muscle cells (Hodgkin, 1951).

Daniel and co-workers (1962) reported that sodium fluxes in

smooth muscle are many times faster than those of potassium and they also reported (1969, 1970, 1971) the existence of an electrogenic sodium pump in rat uterine smooth muscle. Buck and Goodford (1966) also reported that in guinea-pig taenia coli the quiescent smooth muscle cell membrane is more permeable to sodium than the skeletal muscle membrane. Casteels (1971) showed that in guinea-pig taenia coli chloride was not passively distributed, but can move into cells against the electrochemical potential, apparently in association with potassium ions. Casteels, Droogman and Hendrickx (1971) produced evidence that an electrogenic sodium pump was present in guinea-pig taenia coli and might play a part in the maintenance of the resting membrane potential.

These reports, which mainly appeared after we had done many of our early experiments, clearly indicated that control of membrane potentials in smooth muscle was far more complex than that of skeletal muscle.

We also felt that attempts to recalculate the theoretical value of resting membrane potential by the use of the Goldman (1943) equation, where permeability of other ions can be taken into account, would be utterly futile. There are several reasons for this conclusion. Preparations we used contained endometrium and myometrium, and dissection of endometrium from myometrium causes downhill ion movements (Daniel, 1964; van Breeman, Daniel and van Breeman, 1966). Determinations of extracellular space are at best only an approximation of the true value, and so calculations of myometrial ionic distribution based on total tissue ionic content might well be completely erroneous. Also we have no values of the permeabilities of ions involved in regulation of membrane potential. The situation is even more complex when the possibility of

structured cell water is considered. Many workers suggest that the asymmetrical distribution of sodium and potassium between the cell cytoplasm and the extracellular fluid might be a consequence of the "ice likeness" of cell water (Odeblade, Bhar and Lindstrom, 1956; Bratton, Hopkins and Weinberg, 1965; Fritz and Swift, 1967; Ling, 1967; Cerbon, 1967; Cope, 1969; Hazelwood, Nichols and Chamberlain, 1969; Hazelwood et al., 1971; Wiggins, 1971; Zierler, 1971). The evidence for the existence of structured cell water is increasing, and we predict that soon biological workers will no longer be able to ignore this. We cannot be sure what this will mean in terms of the popular ionic hypothesis but feel confident that acceptance of these facts will stimulate much needed research and uncover new facts about mechanisms of all living cells.

However we decided that whatever the mechanisms were that regulated membrane potential we should investigate and obtain some information on which of the main cations present in the bathing fluid did play a role in determining the level of membrane potential. These and all subsequent experiments unless otherwise explicitly stated were done on our final preparation of choice, the non-placental strip from twenty-one day pregnant animals.

VARIATION OF POTASSIUM IN BATHING FLUID

The results of experiments in which we varied the concentration of potassium ions in the extracellular fluid strongly suggest that potassium ions do play a role in determining the level of resting membrane potential in uterine smooth muscle. The membrane potential varied with

the logarithm of external potassium concentration in a linear fashion, only deviating at either very low or high external concentrations of potassium. According to the Nernst equation a ten-fold change in external potassium concentration should produce a 60 mV change in membrane potential. Values from our experiments were only 34 mV, and agree with those obtained by other workers using smooth muscle (Holman, 1958; Goto and Csapo, 1959; Jung, 1959).

VARIATION OF SODIUM IN BATHING FLUID

Varying the sodium concentration in the bathing fluid produced an unstable membrane reflected by a resting membrane potential that varied with time. We are not certain whether this indicates that sodium is bound to the cell membrane on sites that stabilize the membrane or that changes in external sodium concentration alters binding and permeability of other ions, thus producing instability.

VARIATION OF CALCIUM IN BATHING FLUID

Although there are postulates that calcium by binding at certain membrane sites may act as a membrane stabilizer and regulate permeability of other ions (Daniel, 1964; von Hagen and Hurwitz, 1967) we were surprised that resting membrane potential could be varied quite markedly by alteration of the external calcium concentration. A reduction of the external calcium concentration did not markedly change the resting membrane potential until a ten-fold change had been achieved. However even slight increases in the external calcium concentration would markedly increase the value of the resting membrane potential. Unfortunately we could not follow these changes above a concentration of 10 mM calcium,

because calcium phosphate and carbonate precipitates were formed. We recorded the value of membrane potential at 10 mM calcium concentration, though we could visibly detect some slight percipitation.

SUBSENSITIVITY

SPECIFIC AND UNSPECIFIC DESENSITIZATION

The technique of specific desensitization has mainly been used to identify which receptors, drugs combine with to produce their effects (Barsoum and Gaddum, 1935; Innes, 1962a, 1962b). Most workers who investigated the phenomena of desensitization produced an unspecific desensitization by exposing tissues to a large concentration of an agonist. They then washed out the large concentration of drug and studied the responsiveness of the tissue to various agonists during recovery. They could observe specific desensitization at some part of the recovery period. However, we question the validity of evaluating mechanisms involved in specific desensitization in a tissue recovering from unspecific desensitization.

The results of our experiments show that continued exposure of the uterus can result in loss of responsiveness to that particular agent. With the desensitizing agent still present, the tissue can still respond normally to other agonists. In such a tissue, resting membrane potential was unchanged and a normal stretch response could be elicited by our washout procedure. We believe that we are the first to have shown that a tissue can be specifically desensitized to two different types of drugs, used in series. The tissues still had a normal resting membrane potential and gave a normal washout response. When the tissue was desensitized to three different agents, resting membrane potential was normal. The tissue gave a contractile response to washout, but this was not associated with any electrical membrane changes. We feel

that the tissue was still specifically desensitized in the light of results of our experiments on unspecific desensitization. Tissues which were desensitized to large concentrations of any one agonist, would not respond to other agonists or washout. These tissues also showed depolarization of the cell membrane; resting membrane potential was now about 10 mV.

The results of our preliminary experiments clearly showed that spontaneous or drug-induced contractions were mediated by action potentials. We now had a situation where contractile response was dissociated from cellular electrical activity. Various authors have reported that drugs could still produce contractile responses in a tissue depolarized by high concentrations of potassium (Evans, Schild and Thesleff, 1958; Robertson, 1960; Waugh, 1962a, 1962b). These authors failed to appreciate that contractile responses to drugs in a tissue which is already depolarized are not necessarily dissociated from the requirement of depolarization. Other workers have reported a dissociation between electrical and mechanical activity in tissues. Axelsson and Bulbring (1960) showed that removal of glucose from the bathing fluid would abolish tension changes in guinea-pig taenia coli while electrical activity persisted. Axelsson (1961) reported that substitution of sodium in the bathing medium by lithium, hydrazine or choline would also abolish tension changes in taenia coli while electrical activity still remained. Kuribayashi (1969) demonstrated that electrical stimulation could elicit action potentials in smooth muscle of guinea-pig taenia coli when high concentrations of manganese or zinc were present but dit not cause contraction.

These reports are similar in that they all show that mechanical events can be suppressed while electrical activity remains. We feel that in these cases either the link between excitation and contraction has been broken or the contractile mechanism itself has been inactivated. Matthews and Sutter (1967) showed that ouabain would abolish requirement of action potentials for drug-induced contractile responses in rabbit anterior mesenteric vein. Because ouabain also depolarized the tissue we again feel that they failed to show a complete dissociation between electrical and mechanical events. However, we have clearly shown in a system where the effective stimulus normally included action potentials and depolarization, that we could produce contractile responses in a normally polarized tissue and these were not associated with action potentials or membrane depolarization.

We were indeed challenged to find an explanation of how stretch can activate the contractile mechanism without accompanying electrical events. Burnstock and Prosser (1960) reported that a requirement for response to stretch in a variety of smooth muscle preparations was close cellular contacts. They showed that only single-unit smooth muscles will respond to stretch, although occasionally local electrical changes could be detected in renal vein, a multi-unit smooth muscle.

We reasoned that intracellular calcium probably activated the contractile mechanism as a flux of extracellular calcium would probably have been reflected by some potential change in the membrane. We would also agree that we have no other evidence to confirm this fact.

Cerbon (1964, 1965, 1967) analysed cell water and lipid by nuclear magnetic resonance and found mobilities of both moieties were

restricted. The alignment of lipid and water molecules in a membrane could well represent a minimal energy state and a change in the direction of the molecules could be associated with an energy change. Fergason and Brown (1967) postulated that changes in the arrangement of lipid molecules could result in the release of energy which in turn could be converted into mechanical energy, producing a torque wave which could transmit local changes.

The effects of stretch could be explained by such a mechanism and detection of such events promises to be an exciting challenge in the future.

CAFFEINE

Pickering (1893) first showed that caffeine activates the contractile activity of skeletal muscle. Axelsson and Thesleff (1958) showed that caffeine induced a contracture even in a completely depolarized muscle, while Bianchi (1961) and Frank (1962) showed that caffeine displaced calcium ions from intracellular binding sites to initiate contraction. In smooth muscle reports vary in that excitatory and inhibitory effects of caffeine can be obtained, depending on the concentration of caffeine used, temperature, and existing tone of the preparation (Feinstein, 1965; Somlyo and Somlyo, 1968a; Sunano, 1970; Pfaffman, McFarland and Crow, 1971).

Our results show that low concentrations of caffeine increased spontaneous activity of the uterus but did not alter resting membrane potential. High concentrations of caffeine decreased spontaneous activity until the uterus became quiescent; resting membrane potential was still unchanged.

When other agents, adrenaline or papaverine, which inhibited the uterus were antagonized by excitatory drugs, acetylcholine or angiotensin, rhythmic contractions and associated electrical activity appeared normal. Adrenaline and papaverine both increased cyclic AMP levels in cells by different actions. However, caffeine which also increases cyclic AMP levels completely altered the responsiveness of the uterus. Agents which normally stimulated the uterus to produce rhythmic all-or-none contractile responses, associated with action potentials. We feel that this clearly shows caffeine produced its effects by actions other than increasing cell content of cyclic AMP.

Most workers accept that multi-unit smooth muscles do not show spike potentials but give a graded depolarization in response to drugs (Keatinge, 1964; Somlyo and Somlyo, 1968a). We also noted that in the presence of caffeine, the uterus did not respond to stretch or give a conducted response to electrical stimulation. We reasoned that the uterus was now behaving as a multi-unit smooth muscle. We obtained evidence to confirm this from the results of our experiments on determination of extracellular spaces and the histological examination of caffeine treated tissues.

The finding that caffeine had disrupted close cellular contacts and areas of invaginations and that the uterus would no longer show rhythmicity supported the proposals of various workers that spread of excitation in single-unit smooth muscles occurs at close contact regions (Dewey and Barr, 1962; Rhodin, 1967; Barr, Berger and Dewey, 1968; Henderson, Duchar and Daniel, 1971). Some authors have suggested that excitation will spread only at areas of fusion of cells, usually called nexuses. Because we rarely see such regions in the uterus, we feel that excitation between smooth muscle cells is mainly at invaginations and other areas of close membrane apposition. Caffeine treatment had not permanently damaged cells, since washout of the caffeine readily restored normal activity to the uterus, and the estimated extracellular spaces and the histological appearance were also restored to normal.

We were intrigued to find that treatment with caffeine had drastically reduced the usual numbers of cytoplasmic vesicles. These vesicles have been suggested to be pinocytotic in origin and may play a role in movement of calcium and sodium across cell membranes. At present we do

not know if there is only one type of vesicle or if there are populations of vesicles with different properties. Results of other work showed that chronic treatment of rats with phenoxybenzamine induces automaticity in the in vitro vas deferens preparation from such animals (Davidson and Innes, unpublished data). Histological examination of these tissues showed a significant increase in size and numbers of cytoplasmic vesicles. We feel that cytoplasmic vesicles will be shown to play an important part in regulation of automaticity.

The finding that doubling the calcium content in the bathing fluid reversed the inhibitory effects of caffeine and restored the tissue to spontaneously active state agrees in part with proposals of workers who suggested that caffeine reduces the calcium available to the contractile mechanism (Diamond and Marshall, 1969a, 1969b; Somlyo and Somlyo, 1970; Sunano, 1970). We also noted that the resting membrane potential was unchanged by the higher calcium concentration in the bathing fluid. Doubling the calcium content of the extracellular fluid in untreated tissues usually increased resting membrane potential to about 60 mV. This indicated that caffeine probably depleted or displaced calcium from membrane or other cellular sites where calcium regulates membrane potential.

The role of calcium as a coupling factor in excitation-contraction is generally accepted. Other actions of calcium as a membrane stabilizer and hence in excitable membranes, a controlling factor of membrane permeability and active ionic conductances have been suggested by Frankenhaeuser and Hodgkin (1957) Koketsu (1965) Hagiwara (1966).

Various workers have proposed that sodium might play an important

role in regulation of calcium movements. Bohr, Brodie and Chou (1958) showed that a reduction in external sodium concentration potentiated drug responses in rabbit aorta. Briggs and Melvin (1961) observed that in rabbit aortic strips exposed to a low extracellular sodium concentration, calcium influx increased by 225% and a contracture was obtained. Goodford (1967) showed that when sodium was removed from the bathing fluid the calcium content of guinea-pig taenia coli increased. These findings are not peculiar to smooth muscle, since Niedergerke (1963) reported that in frog heart calcium influx increased when the sodium content of the bathing fluid was reduced by 50%.

Tuttle (1966) showed that ouabain potentiated responses to noradrenaline in isolated cat spleen strips, but suggested that blockade of uptake of noradrenaline might explain this effect of ouabain. Brinley (1968) reported that strophanthidine reduced sodium efflux by 80-90% and caused a contracture of barnacle muscle. Factors affecting ion movements were reviewed by Caldwell (1968) who concluded that the available evidence was consistent with the idea that inhibition of the active movement of potassium and sodium would cause an increase in calcium influx. Bohr, Seidel and Sobieski (1969) also showed that a low extracellular sodium, or treatment with ouabain potentiated responses to noradrenaline in rabbit mesenteric artery. They postulated that in smooth muscle there were at least three ionic exchange mechanisms.

a) A ouabain - sensitive pump which exchanges sodium for potassium, usually referred to as the sodium pump.

b) A sodium-calcium pump which moves calcium into the cell. This exchange can be accelerated either by increasing intracellular sodium by

poisoning the ouabain-sensitive sodium-potassium pump, or removal of external sodium which competes with calcium for a carrier at the cell surface.

c) A process is essential for the production of relaxation by removal of calcium from the contractile myofilaments. We must also add to these mechanisms the ouabain-sensitive electrogenic sodium pump which has been demonstrated in rat uterus and other tissues (Taylor, Paton and Daniel, 1969; Bose, 1971).

Batra and Daniel (1970) suggested that calcium influx in rat uterus is not directly coupled to sodium-potassium exchange but could be linked to the degree of depletion of metabolic energy; they postulated a structural element of the membrane which controls calcium permeability and requires ATP for maintenance but not for function. However, they agreed there was the possibility of a different transport system.

We found that exposure of the uterus to ouabain or a low sodium bathing fluid reversed caffeine-induced inhibition and restored electrical and mechanical rhythmic activity. These results and the findings that raising extracellular calcium concentration produced the same final effect strongly suggested that sodium and calcium movements in rat uterus are linked together.

These procedures also restored areas of close cellular contact in the caffeine-treated uterus; extracellular space determinations after these procedures returned to normal values. This suggested that calcium ions play an important role in the maintenance of areas of close cellular apposition. Further studies with other agents which interfere with calcium movements, chlorpromazine, lanthanum and manganese, should give us

more detailed knowledge of regulation of areas of close cellular contact in smooth muscle.

Exposure of the caffeine-treated uterus to low sodium bathing fluid did not result in a fluctuating membrane potential as seen in our earlier experiments (p. 84). This suggested that fluctuations in these previous experiments might be due to exchanges between calcium compartments in the membrane and cytoplasm produced by the sodium-calcium competitive carrier system. If caffeine had already displaced calcium from membrane and other sites then these fluctuations would not take place.

We were intrigued that in a tissue depolarized by ouabain we obtained a sudden restoration of resting membrane potential. We expected the sodium pump in rat uterus to be blocked by the concentration of ouabain we used (Daniel, 1963).

We suggest that calcium might now have been regulating membrane potential and acting as the main carrier of inward current during the action potentials.

Evidence for calcium spike electrogenesis has been shown in crayfish and barnacle muscle fibres (Fatt and Ginsborg, 1958; Hagiwara, 1966). Anderson, Ramon and Snyder (1971) developed an elegant double sucrose gap technique to study rat uterus in current clamp and voltage clamp experiments. In many tissues the sodium conductance channel is blocked by tetrodotoxin. However in uterus from oestrogen dominated rats, both calcium and sodium were required to activate a common conductance channel. This channel is insensitive to the blocking action of tetrodotoxin.

SUPERSENSITIVITY

Mechanisms of supersensitivity, though widely investigated, are poorly understood. The fact that most procedures which cause potentiation produce an unspecific type of supersensitivity strongly suggests that some fundamental change is produced in muscle cells beyond the level of the usual receptors for drugs. An obvious possibility is altered membrane permeability as suggested by Cannon (1939). Although Lenman (1965) demonstrated a consistent and significant decrease in the resting membrane potential of denervated skeletal muscle in mice, studies on the electrical properties of smooth muscle have so far been confined to characterising normal values of membrane potential and other such parameters.

Many pharmacological agents can produce supersensitivity, but this study was limited to two agents, cocaine and reserpine.

COCAINE

Various explanations have been put forward from time to time to account for the potentiating effect of cocaine, and these have been extensively reviewed (Furchgott, 1955; Trendelenburg, 1963, 1966). At present many workers believe that potentiation of catecholamines is due to blockade of neuronal uptake, although evidence that this is not the complete answer is growing (Maxwell, Wastila and Eckhard, 1966; Bevan and Verity, 1967; Kalsner and Nickerson, 1969; Davidson and Innes, 1970).

Davidson (1970) showed that cocaine can produce an unspecific supersensitivity and suggested a postsynaptic action of cocaine. Greenberg (1968 and Kasuya and Goto (1969) suggested that cocaine may alter

the utilization of calcium stores for contraction. Some alteration of a common pathway in the contractile process could explain the potentiation of responses to agonists which act on other receptors, for example, acetylcholine and histamine (Rosenblueth, 1932), 5-hydroxytryptamine (Innes and Kosterlitz, 1954).

RESERPINE

The supersensitivity which results from pretreatment of animals with reserpine resembles that caused by decentralization (preganglionic denervation) (Innes, 1960; Fleming and Trendelenburg, 1961; Westfall, 1970). Most authors now agree that reserpine causes an unspecific supersensitivity (Green and Fleming, 1968; Kalsner and Nickerson, 1969; Davidson, 1970) and suggest that postsynaptic changes are responsible.

Carrier et al. (1970) showed a decrease in calcium content of aorta, heart and liver of rabbits after treatment with reserpine. These authors treated animals with large doses of reserpine. In contrast, those workers who used a smaller dose regimen did not observe a reduction in tissue calcium content although these doses caused supersensitivity (Davidson, 1970; Hudgens and Harris, 1970). This suggests that these losses were toxic effects of reserpine not related to observed supersensitivity. Garret and Carrier (1971), using small doses of reserpine, found that responses of the rabbit aorta to noradrenaline tested in calcium-free Ringer's solution showed a reduced extracellular calcium dependence, suggesting an increased affinity for calcium in these tissues. They also suggested that changes in calcium utilization might explain the supersensitivity observed after treatment with reserpine. The results of

our experiments with cocaine and reserpine suggested that these agents acted on more than one site in uterine cells. The interpretations of these findings will be discussed along with relevant findings from other experiments.

TREATMENT WITH COCAINE

Our observation that treatment with cocaine significantly increased the duration of spontaneous contractions in rat uterus, suggested a delayed removal of calcium from the contractile elements. If more calcium had been made available to the contractile elements we would have expected an increase in the developed tension. This was not seen. Such an increase in tension can occur, since the uterus of rats pretreated with reserpine showed an increase in the maximum tension developed in the spontaneous contractions but without an increase in the duration of each contraction. We do not necessarily suggest that treatment with reserpine increased the amount of calcium made available to the contractile elements. Other possibilities such as improved cell to cell communication or altered reactivity of the contractile apparatus also have to be entertained.

Treatment with cocaine increased the time to quiescence of rat uterus exposed to a calcium-free bathing medium. When cocaine was present in the normal bathing medium, caffeine, which usually produced quiescence in uteri without cocaine, apparently by interfering with some cellular actions of calcium, no longer did so, even when higher doses of caffeine were used. These findings also suggested that cocaine altered the responsiveness of rat uterus via some calcium mechanism.

In contrast, uteri from twenty-one day pregnant rats pretreated with reserpine showed no alteration in the time to quiescence produced by caffeine or exposure to calcium-free medium. These results were not in accord with those of Garret and Carrier (1971). These authors used rabbit aorta, a multi-unit smooth muscle, and found that the time to loss of responsiveness to stimulating agents was longer in preparations from reserpine treated than from untreated rats. We tentatively suggest that this could mean different stores of calcium may be utilized for spontaneous contractions and drug induced responses. Results of experiments with tetrodotoxin and atropine indicated these agents did not change the increased automaticity of uterus found after reserpine treatment. This provides reasonable evidence that the increased activity of uterus after reserpine treatment was not due to increased activity of cholinergic nerves.

We noted some similarities between cocaine uterine preparations from twenty-one day pregnant rats and those obtained from seventeen day pregnant rats. Both preparations showed multiple pacemaker areas, irregular contractions and increased time to quiescence in calcium-free bathing fluid. However the similarity is not complete since the resting membrane potential of uterus from twenty-one day pregnant rat is lower than that of the seventeen day pregnant rat, and is not increased by cocaine, although it can be increased by high extracellular concentrations of calcium. These findings further indicated that there were various cellular sites where calcium had important regulatory function.

The results of experiments with caffeine showed that caffeine could suppress spiking in the uterus in response to stimulation by

agonists without abolishing the graded contractile responses. This suggested that caffeine did not prevent the movement of calcium to the contractile elements but, as discussed earlier (p. 120), interfered with cell membrane sites. Treatment with cocaine did not reverse caffeine-induced quiescence, but upon removal of both drugs spontaneous activity recurred in the pattern typical of tissues exposed to cocaine, i.e. increased duration of spontaneous contractions. However these preparations did not show any evidence of the multiple pacemaker areas with irregular membrane potentials and contractile responses which are also typical of cocaine treated tissues. We suggest that caffeine did not prevent cocaine from attaching to the site or sites where it delayed removal of calcium from the contractile elements. However, we believe that caffeine, by acting at membrane sites, had prevented cocaine from producing the multiple pacemaker areas, while it did not prevent cocaine from delaying removal of calcium from the contractile elements.

Treatment of the uterus with cocaine in the absence of caffeine induced multiple pacemaker areas and lowered the threshold value of electrical stimulation. These results show that cell membranes were more unstable and would become depolarized more readily, either spontaneously or in response to electrical stimulation. Uterus from rats pretreated with reserpine did not show multiple pacemaker areas but did show an apparent increase in the number of active pacemaker cells in the normal discrete pacemaker areas. This finding could explain the increased frequency of spontaneous contractions observed after treatment with reserpine.

The threshold of electrical stimulation was even more markedly

reduced by treatment with reserpine than by cocaine. Pretreatment with reserpine also had more effect than cocaine in increasing conduction velocity. Our experiments with caffeine showed that cellular communication was interrupted and that this effect of caffeine could be reversed by raising the concentration of calcium in the bathing fluid, by treatment with ouabain or by exposure to a low sodium bathing fluid, which we postulated would restore calcium to cells. We suggest that cocaine and reserpine increased intercellular communication and thus increased conduction velocity and increased excitability. Cocaine, by a separate action on some membrane site, induced multiple pacemaker areas, and possibly the resultant asynchrony might explain why reserpine had a greater effect than cocaine in altering conduction velocity and threshold values to electrical stimulation. Treatment of the uterus with calcium increased the resting membrane potential but did not induce multiple pacemaker areas. However, when the calcium concentration in the bathing fluid was increased to reverse inhibition of uterus produced by caffeine, spontaneous activity was restored but resting membrane potential was unchanged. These findings indicate that treatment with high calcium can restore cellular communication. The site at which high calcium concentration in the bathing fluid usually acted to increase resting membrane potential was apparently still affected by caffeine even with cocaine present so that no change of potential occurred. Further evidence of this membrane stabilizing effect of caffeine was shown by the results of experiments with low sodium bathing fluid. Uterus exposed to low sodium bathing fluid showed an unstable membrane, indicated by a resting membrane potential that varied with time. Uterus inhibited by caffeine, then exposed

to a low sodium bathing fluid, still in the presence of caffeine, regained rhythmicity but at no time showed a variable resting membrane potential. These results indicate that sodium and calcium both play a role in stabilizing cell membranes in rat uterus. We also suggest that this evidence supports the findings of Anderson, Ramon and Snyder (1971), who suggested that in rat uterus sodium and calcium were both necessary for a common conductance channel which carried inward current during action potentials.

GENERAL CONCLUSIONS AND SPECULATIONS

Smooth muscle continues to be an exciting challenge to the biological worker. Although many major advances have been made in the understanding of mechanisms controlling functions of smooth muscle, there are still large gaps in our knowledge. The variability in properties of smooth muscle is controlled by so many factors, e.g. hormonal state, age, and sex, that only comparative studies can be done. These facts are too often ignored by many workers in their eagerness to produce a general field theory of excitability and contractility applicable to smooth muscle.

We feel that Bozler deserves special recognition for his pioneering work in which he studied many aspects of smooth muscle and clarified many problems by categorizing smooth muscle into two divisions, multi-unit and single-unit. It is now evident that there are many smooth muscles whose properties lie between these two extremes, and a particular smooth muscle can behave as single-unit or multi-unit, depending on experimental conditions. However the initial stimulus provided by Bozler was really the start of a new era of research in smooth muscle. Recent technical advan-

ces in electrophysiology and electronmicroscopy have enabled research workers to begin to understand mechanisms of ionic movements and membrane functions. However, we are sure that combined, biochemical, electrophysiological and histological studies will be necessary to assess cellular functions. We trust that relationships between various cellular events will not be stated as causal until proven so.

We have confined this work to rat uterus and obviated some of the aforementioned problems. The use of a rhythmic smooth muscle allowed us to investigate electrical phenomena associated with excitation-contraction mechanisms. We have for the first time studied such events in smooth muscle with comparisons between untreated tissues and tissues where excitability and contractility were altered by procedures causing subsensitivity and supersensitivity.

The activity of rat uterus smooth muscle is normally controlled by conducted action potentials originating in pacemaker areas. The ability of uterus to give a coordinated contractile response depends on two main factors. The discreteness of pacemaker areas and intracellular communications. Our results indicate that both these factors are normally under hormonal influences. Oestrogens normally initiate synthesis of uterine cell proteins in such a way that cellular communication is established and maintained. Mechanisms of these actions of oestrogens are currently widely investigated, and we have already discussed relevant findings. The functions of progesterone in uterus appear to be such that during pregnancy the uterus will not be able to develop a coordinated contraction which might permaturely expel the foetus. Investigations on the effects of these hormones on other systems might well be fruitful.

Hypertension in women taking oral contraceptives may not only be due to fluid retention, but to effects of the hormones on vascular smooth muscle. Hypertension before the menopause is less frequent in women, and again suggests effects of female hormones on the cardiovascular system.

Since the start of this century calcium has been known to be an essential requirement for contractile processes. We believe on the basis of our experiments and the findings of many other workers that the actions of calcium are the key to understanding many cellular functions. Rasmussen (1970) reviewed the role of calcium in excitation-secretion, carbohydrate metabolism and many hormonal responses.

Various authors have proposed that there are multiple pools or stores of calcium involved in excitation-contraction processes in smooth muscle (Hinke, 1956; Daniel, 1963, 1965; Hurwitz, Joiner and von Hagen, 1967). These proposals were based mainly on results of experiments in which tissues exposed to a calcium-free medium showed a differential time to loss of responsiveness to different stimulatory agents. The results of our experiments with caffeine, desensitization, cocaine and reserpine, indicated that calcium, by different mechanisms or acting at different cellular sites, has a major role in the production of subsensitivity and supersensitivity.

We fully admit that there are gaps in our knowledge which we hope we can at least partially fill in the near future by carefully designed experiments. We suggest that there are different membrane sites which calcium acts upon and regulates resting membrane potential, membrane stability, and possibly on another site which controls entry of extracellular calcium to the cell cytoplasm. We propose that in the cell

cytoplasm there are source(s) of calcium which can release calcium to the contractile elements and sink(s) of calcium which can remove calcium from the contractile elements. Morphological correlates of these sink(s) and source(s) may be sarcoplasmic reticulum, mitochondria and cytoplasmic vesicles. We are aware that these sink(s) and source(s) may be identical entities which can release and recover calcium.

SECTION V

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