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ASSOCIATION BETWEEN ION MOVEMENTS, ELECTRICAL EVENTS, AND THE ACTIONS OF DRUGS ON THE ISOLATED RAT MYOMETRIUM

by

© BARRIE JOHN HODGSON

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Association Between Ion Movements, Electrical Events, and the Actions of Drugs on the Isolated Rat Myometrium" submitted by Barrie John Hodgson in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ASSOCIATION BETWEEN ION MOVEMENTS, ELECTRICAL EVENTS, AND THE ACTIONS OF DRUGS ON THE ISOLATED RAT MYOMETRIUM

by

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ABSTRACT

The isolated longitudinal muscle of the pregnant rat provides a useful preparation for the study of drug action on ion movements. Although some indication of cellular damage, due to removal of the endometrial and circular muscle layers, is observed this is due to a proportion of inviable cells rather than a general decrease in the active properties of all cells.

Acetylcholine and noradrenaline, but not oxytocin, increase the passive permeability properties of the cell membrane to potassium, as indicated by induced movements of potassium-42 in normal tissues and in tissues depolarised by potassium chloride medium. Isoprenaline does not increase permeability to potassium at a time when it causes hyperpolarisation of the cell membrane. These results indicate that movements of potassium cannot be correlated with the primary site of drug action to change either membrane potential, or tension.

The effects of specific agents which antagonise the movements of calcium across membranes show that acetylcholine and potassium chloride cause contraction of the uterus, under the

conditions studied, by release of a sequestered source of calcium. When lanthanum is used to remove extracellular, bound calcium-45 and prevent outflow of cellular calcium-45, the relationship of calcium uptake and contractions is still obscure. Measurement of uptake of radioactive lanthanum into myometria and into cellular fractions indicates that intracellular penetration of lanthanum occurs. This questions the validity of assumptions used in 'lanthanum' technique for the measurement of cellular calcium.

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"In the middle of the flanks of woman lies the womb, a female viscus, closely resembling an animal, for it is moved of itself hither and thither in the flanks, also upwards in a direct line to below the cartilage of the thorax, and also obliquely either to the right or to the left, either to the liver or the spleen, and it likewise is subjected to prolapses downwards, and in a word is altogether erratic."

Aretaeus of Cappadocia

I. INTRODUCTION

I INTRODUCTION

IA Ionic distribution and membrane potential in smooth muscle cells

The ionic basis of the potential difference across the membrane of excitable tissues is based on the early work of Bernstein (1912), who considered that the resting cell membrane is selectively permeable only to potassium and that excitation results in the passive transfer of other ions such as sodium and chloride. The ionic theory of excitation has been extended to integrate ionic distribution profile, ionic fluxes and electrical events of excitable cells (Hodgkin and Rushton, 1946; Hodgkin, 1951; Hodgkin and Huxley, 1952). The acceptance of the membrane model has not been without exceptions (Ling, 1962; Troshin, 1966; Jones, 1970), but the driving forces across the cell membrane are the factors upon which most theories attempt to explain the resting potential of excitable cells. A fundamental feature is that the membrane is permeable to ions, in a varying degree. In all animal cells the interior of the cell contains relatively high concentrations of potassium and relatively low concentration of sodium, in comparison to the extracellular fluid. The electrochemical potential difference ($\Delta\mu$) is the sum of the electrical and concentration energy differences across the membrane for that ion, and:

$$\Delta\mu = Z_M F\xi_m + RT \ln \frac{[M^+]_i}{[M^+]_o} \quad \ldots \quad \ldots \quad \ldots \quad (1)$$

where Z_M is the valency of the cation M^+, F is the Farady, ξ_m is the transmembrane potential, R is the universal gas constant and T is temperature in degrees absolute. When $\Delta\mu$ is zero, the ion is in equilibrium and:

$$\xi_M = \frac{RT}{FZ_M} \ln \frac{[M^+]_o}{[M^+]_i} \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (2)$$

Equation (2) is the Nernst equation and ξ_M is known as the equilibrium potential for that ion. This equation can also be derived from the Gibbs-Donnan equilibrium conditions as described by Boyle and Conway (1941) for the more permeable ions K and Cl:

$$\xi_m = \frac{RT}{F} \ln \frac{[K]_o}{[K]_i} = \frac{RT}{F} \ln \frac{[Cl]_i}{[Cl]_o} \quad \ldots \quad \ldots \quad \ldots \quad (3)$$

and

$$\frac{[K]_o}{[K]_i} = \frac{[Cl]_i}{[Cl]_o} \quad \ldots \quad \ldots \quad \ldots \quad (4)$$

In nerve and skeletal muscle and also smooth muscle, the membrane potential varies linearly with the log of the external potassium

concentration, as predicted by this equation, provided that $[K]_o$ is greater than 25 mEq/l. At low $[K]_o$ values, increments of $[K]_o$ cause less than the predicted 61mv change in membrane potential per 10-fold change in $[K]_o$, at 37°C (Casteels and Kuriyama, 1965). This presumably reflects the permeability of the membrane to other ions. Thus, when considering diffusion potentials in cell membranes, several univalent ionic species are present and the membrane has a finite permeability to them all. The equation derived by Goldman (1943) and later applied by Hodgkin and Katz (1949) to the potentials of squid axon, considers only potassium, sodium and chloride:

$$\xi_m = \frac{RT}{F} \ln \frac{P_K[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_o}{P_K[K]_o + P_{Na}[Na]_o + P_{Cl}[Cl]_i} \quad \ldots \quad \ldots \quad (5)$$

where E is the outside minus inside potential, and P_K, P_{Na}, and P_{Cl} are the permeability coefficients for potassium, sodium and chloride respectively. These coefficients are proportional to the product of the ionic mobility (u) and the ionic partition coefficient (β) in the membrane and solution, and inversely proportional to the membrane thickness (a). P has Units of $cm.sec^{-1}$.

$$\text{Thus:} \qquad P_K = u_K.\beta_K.\frac{RT}{aF} \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (6)$$

The basic assumptions of this equation are that 1) the electric field in the membrane is uniform and constant; 2) the activity coefficient of an ion is the same as that in open solution, such that ions in the membrane move under diffusion and electrical forces as in free solution; 3) the concentrations of ions at the membrane interface are proportional to those in aqueous solution; 4) the membrane is homogenous.

The ionic current flowing across the cell membrane is zero, and the net movement of sodium, potassium and chloride ions cancel out. The equation empirically fits the experimental data quite well for nerve and skeletal muscle, the membrane potential being at a level between the equilibrium potential for sodium, potassium and chloride ions (Hodgkin, 1958; Harris, 1960).

The ratio of $P_{Na} : P_K : P_{Cl}$ has been empirically determined by Hodgkin and Katz (1949) for the squid axon; values of 0.04:2:0.45 were found to fit the experimental data for axons exposed to different ionic environments. Bennett (1966) calculated that P_{Na}/P_K for smooth muscle is 0.05, from the data of Kuriyama (1966) using chloride-free solutions. Under these conditions, $[Cl]_i$ should fall rapidly and

$$\xi_m = \frac{RT}{F} \ln \frac{[K]_o + \alpha[Na]_o}{[K]_i + \alpha[Na]_i} \quad \ldots \quad \ldots \quad \ldots \quad (7)$$

where $\alpha = P_{Na}/P_K$

Both Kao and Nishiyama (1964) and Casteels and Kuriyama (1965) estimated the ratio to be about 0.1 for rat myometrium, while Goodford (1968) reported a value of 0.06 for intestinal smooth muscle. Casteels and Kuriyama (1966) found that taenia coli did not obey the Boyle and Conway theory and did not agree with Bennett's conclusions.

Equations (4) and (7) become invalid if chloride is not distributed passively; there is some evidence to indicate that chloride is actively accumulated in smooth muscle cells (Kao and Nishiyama, 1964; Casteels, 1971). Downhill movement of ions occurs in excitable tissues and the ionic distribution at rest is maintained by an active transport of sodium outwards. Such a pump may be electrically neutral (1:1 ratio of sodium moved outwards to potassium moved inwards) or electrogenic. Although evidence exists that an electrogenic pump exists in myometrial smooth muscle (Taylor, 1969; Taylor, Paton and Daniel, 1969) it is not known whether it contributes significantly to the resting membrane potential under normal conditions. If such a pump does contribute to the resting potential then there will be a net ionic current flowing at rest and the above assumptions do not apply.

Estimation of the membrane potential of smooth muscle cells is subject to many difficulties *(vide infra)*. Values of resting potential of smooth muscle cells measured with intracellular electrodes range from 40 to 80 mV, inside negative (Bülbring, 1962; Burnstock, Holman and Prosser, 1963; Casteels, 1970). The values

are clearly lower than for nerve and skeletal muscle; one possible cause of deviation from the potential from that predicted by the Goldman equation has been mentioned, and a further possible cause of deviation - internal ion binding - is discussed in Part IE. In smooth muscle either the sodium permeability is higher or the potassium permeability lower than for skeletal muscles, or ion binding is more significant.

IB Electrical activity of uterine smooth muscle cells

The first attempts to measure membrane potentials of uterine muscle using microelectrodes were made by Woodbury and McIntyre (1954, 1956). Resting membrane potentials of the myometrium have been found to be between 35 and 65 mV. (mouse : Kuriyama, 1961a, 1964; rat : Marshall, 1959; Marshall, 1962; Csapo and Kuriyama, 1963; Casteels and Kuriyama, 1965; guinea-pig : Bülbring, Casteels and Kuriyama, 1968; cat : Bülbring, Casteels and Kuriyama, 1968; rabbit : Goto and Csapo, 1959; Kuriyama and Csapo, 1961; Kao and Nishiyama, 1964). Potassium equilibrium potentials have been estimated to be 70-80 mV. (rat : Cole, 1950; Casteels and Kuriyama, 1965; rabbit : Kao and Nishiyama, 1964; cat : Daniel and Singh, 1958). The membrane potential, like many other parameters of the uterus, changes with different hormonal conditions. In rat myometrium, Casteels and Kuriyama (1965) reported that the membrane potential increases from 42 mV up to 60.5 mV at the fifteenth day of pregnancy, and falls in the last few days of gestation to 54 mV at

parturition. Potentials measured from the region of the placenta are reported to have a higher membrane potential than the nonplacental regions (Goto and Csapo, 1959; Casteels and Kuriyama, 1965; Csapo, 1969). These changes due to pregnancy were not due to changes in ionic distribution or content (Kao and Siegman, 1963; Casteels and Kuriyama, 1965; Bülbring, Casteels and Kuriyama, 1968) in the rat, rabbit or guinea-pig, and changes in ionic permeability have been considered as the basis for the changes in resting membrane potential. (In the cat, Bülbring, Casteels and Kuriyama (1968) showed an increase in the equilibrium potential for chloride). Such permeability changes, in turn, have been ascribed to an effect due to progesterone (Marshall, 1959; Goto and Csapo, 1959; Csapo, 1961). Progesterone suppresses the activity and pharmacological responsiveness of the myometrium and these regulatory effects of progesterone were subsequently explained by a "blocking" action (Csapo, 1969). According to Csapo (1969), increased progesterone levels during pregnancy is a compensatory effort to offset the activity-inducing effect of increase in uterine volume. Progesterone withdrawal leads to increased uterine activity and increased sensitivity to oxytocin.

Cable-like properties of myometrial muscle have recently been demonstrated (Abe, 1968, 1970, 1971). They appear to resemble those reported for the taenia coli (Tomita, 1966ab; Abe and Tomita, 1968; Tomita, 1970) and vas deferens (Tomita, 1967). The space constant and time constant can be fitted by the core-conductor

equations of Hodgkin and Rushton (1946). The membrane resistance has been found to decrease significantly during pregnancy (Abe, 1971).

Bozler (1948) showed that mechanical changes in smooth muscle were associated with the initiation of electrical impulses and that the cause of the discharge was probably depolarisation of the cell surface. He classified vertebrate smooth muscle into two categories: a) "Unitary" muscles which include intestine, ureter and uterus and which behave as single units; they contract spontaneously: b) "Multiunit" muscles, which includes the urinary bladder and vascular smooth muscle and which is activated by nerves; they are not spontaneously active.

Most uterine muscles, when placed in a suitable external environment, will contract spontaneously during all stages of hormonal condition. Contractions are accompanied by action potentials, the amplitude of which depends upon the hormonal condition but which may exceed 70 mV in pregnant uterine muscle. The origin of such spontaneous activity is likely to be myogenic. Conduction velocity appears to vary widely, from 0.26 $cm.sec^{-1}$ (Melton, 1956) to 10 $cm.sec^{-1}$ (Goto, Kuriyama and Abe, 1961) and it seems likely that this parameter is influenced by hormonal status. Daniel (1960) found conduction of electrical activity to be most regular in pregnant cat uteri (8-12.5 $cm.sec^{-1}$) and highly variable in rat and rabbit uteri. Progesterone treated uteri had slower conduction velocities and in some cases action potentials were not conducted more than 1cm in progesterone treated rabbit uteri.

The origin of automaticity in uterine muscle is not known, but it seems that labile pacemaker regions are formed within the tissue (Kuriyama, 1961b; Marshall, 1962). Propagation of action potentials occurs as if the tissue were a syncytium; rather than by protoplasmic continuity, however, adjacent cells appear to mediate transmission by regions known as nexuses (see Cobb and Bennett, 1969; McNutt and Weinstein, 1970).

The active properties of nerve and skeletal muscle have been discussed in terms of the ionic hypothesis (Noble, 1966). This hypothesis considers that depolarisation initiates a self-regenerative activation process which increases g_{Na}, the sodium conductance. g_{Na} is returned to its resting value by an inactivation process which, like the activation process, is dependent upon time and potential. Repolarisation is also effected by an increase in g_K. Although the ionic hypothesis provides the basis of the interpretation of the action potential in smooth muscle, the ionic mechanisms have not been clarified. Specifically it is not known to what extent sodium or calcium carry the total current during the action potential. Most studies have used the guinea-pig taenia coli. The interpretation of data obtained from tissues exposed to solutions of different ionic strengths (e.g. substitution of sucrose for NaCl) is fraught with difficulties. Effects occuring during deficiency of one ion on the action potential may reflect direct changes due to the replacing substance; furthermore indirect effects due to changes in permeability to other ions may be manifest, especially if calcium and sodium compete

at the membrane as proposed by Niedergerke (1963) for cardiac muscle and Goodford (1967) for taenia coli. Yet another problem is that data obtained during abnormal conditions (such as Na-deficiency in the external solution) does not necessarily reflect the normal mechanisms.

Tomita (1970) has summarised the data available for taenia coli, and concluded that sodium contribution to the spike is small, because the rate of rise and overshoot of the action potential were actually increased in low sodium solution, and spikes can be evoked in a solution deficient of sodium, but containing calcium (Bülbring, Kuriyama and Twarog, 1962; Bülbring and Kuriyama, 1963; Brading and Tomita, 1968; Brading, Bülbring and Tomita, 1969a; Bülbring and Tomita, 1970). In the rat uterus, reduction of external sodium (substitution with sucrose) has been reported to decrease the size of the action potential (Goto and Woodbury, 1958; Marshall, 1963; Kao, 1967). In contrast, Daniel and Singh (1958) and Daniel (1960) found no reduction of spike amplitude or conduction of cat uteri when NaCl was partially (to one ninth) replaced by sucrose or choline chloride; in rat uteri, replacement of sodium (by 50%) reduced the amplitude and conduction velocity of spikes in most cases. Kuriyama (1961, 1964) reported that an overshoot potential could be recorded when sodium concentrations were reduced to 20mM (replacement with choline or tris) in mouse and rat uterus. Abe (1971) found that spontaneous spikes of rat uteri deteriorated in low sodium solution (16.7mM) after 30 minutes; however, for periods up to 4 hours,

spikes of larger amplitude and faster rate of rise could be evoked with depolarising current.

Reduction of calcium concentration in the external medium causes depolarisation (Kuriyama, 1961; Csapo and Kuriyama, 1963; Marshall, 1965) and the rate of rise and amplitude of the action potential is decreased. Complete removal causes spikes to cease. Increasing calcium concentration hyperpolarises the membrane and increases these parameters of the action potential. Interpretation of the effect of altering $[Ca]_o$ is especially difficult since it seems likely that calcium performs more than one function directly related to membrane potential and contractility in smooth muscle, including stabilisation of the membrane potential.

The voltage clamp technique has been used to follow the ionic currents during the action potential. Anderson (1969) found that in Na-free solution, the peak transient current decreased and its equilibrium potential shifted towards a more negative potential inside, and concluded that a sodium-dependent regenerative process was involved in spike generation. However, Kumamoto and Horn (1970) criticized the work of Anderson on the basis that smooth muscle goes through a period of inexcitability when transferred from a higher to a lower sodium concentration, which may last for a period of up to 10 minutes. Anderson measured current voltage relations at 1.5 minutes and 11.5 minutes after exposure to Na-free medium. There are also both theoretical and practical difficulties in application of this technique to smooth muscle.

Tetrodotoxin, which is thought to specifically inhibit the voltage-dependent early transient conductances in membranes which utilize a sodium mechanism of excitation (Moore and Narahashi, 1967), fails to affect action potentials in smooth muscles (Kuriyama, Osa and Toida, 1966), including uterus (Abe, 1971). Kao questioned the value of the use of tetrodotoxin since *tetrodon* fish and *taricha* newts are resistant to its action, despite using a sodium mechanism for excitation (Kao, 1966; Moore and Narahashi, 1967). This could, however, be accounted for as a teleological self-protective mechanism peculiar to these animals. Experiments in which manganese ions, which are able to block calcium action potentials in crustacean muscle (Hagiwara and Nakajima, 1966) have been used to prevent spike activity in smooth muscle (Nonomura, Hotta and Ohashi, 1966; Hashimoto and Holman, 1967; Brading, et al, 1969b; Bülbring and Tomita, 1969b; Abe, 1971) are not conclusive since Mn at low concentrations (10^{-5}M), will inhibit spikes of taenia coli in Ca-free solution which, under the conditions used, were also sensitive to tetrodotoxin (Golenhofen and Petrányi, 1969). Thus the evidence is conflicting as to whether calcium is the current carrying ion during the upstroke of the action potential. It seems likely, in the final analysis, that calcium ions contribute to the depolarising current and carry the depolarising current completely in an Na-free environment. Certainly, calcium must play a considerable regulatory role in the control of permeability of the membrane to ions, including calcium itself, and the potential across it.

IC Excitation-contraction coupling in smooth muscle

The coupling process links the stimulus at the membrane with the rise or fall of the free intracellular calcium initiating contraction or relaxation of the contractile proteins. In skeletal muscle the structural site of coupling is thought to reside in the sarcoplasmic reticulum, and calcium is included in the functional components of the system (Sandow, 1965). In smooth muscle the sarcoplasmic reticulum is poorly developed and Peachey and Porter (1959) suggested that the short diffusion distances in smooth muscle would make a highly refined sarcotubular system superfluous. Some studies of smooth muscle have claimed to have demonstrated a sarcotubular system (Lane, 1965). The surface sarcoplasmic reticulum or micropinocytotic vesicles, (Burnstock and Merrillees, 1964; Burnstock, 1970) could be involved in the excitation-contraction mechanism (Yamauchi, 1964). Carsten (1968) demonstrated calcium-binding in the presence of ATP and magnesium, by a fraction of the bovine uterus considered to be isolated sarcoplasmic reticulum.

Batra and Daniel (1970) have demonstrated an ATP-dependent calcium uptake by the microsomal and mitochondrial fractions of rat myometrium; the speed of removal of calcium and capacity for calcium would be compatible with a causal relationship between uptake by the microsomal fraction and relaxation of the muscle.

The fine structure of smooth muscle has recently been reviewed (Burnstock, 1970) and the proteins of the contractile mechanism in uterus by Needham and Shoenberg (1967). Myosin (probably

in dispersed form), actin filaments, troponin and tropomyosin may be extracted from smooth muscle (Burnstock, 1970). However, the mechanism of contraction of smooth muscle is still obscure. Electron microscopic studies (Lane, 1965; Kelly and Rice, 1969) on relaxed and contracted smooth muscle suggest that contraction is due to some sliding filament mechanism. Needham and Shoenberg (1964) and Shoenberg (1969) have suggested that myosin aggregates into filament form with increase in intracellular concentration of ionized calcium, so that a sliding filament mechanism becomes a possibility. More recently, Rice, McManus, Devine and Somlyo (1971) have demonstrated the presence of thick myosin filaments in a 'quasi-regular' packing arrangement in electron micrographs of relaxed anterior mesenteric vein. The qualitative similarity between stimulus-response, force-velocity and length-tension relationships of smooth and striated muscle suggests that the same basic design of contractility is operating both types of muscle (Csapo, 1962; Stephens and Chiu, 1970).

Whatever the mechanism of the final step of the chain of events leading to contraction, the most likely penultimate step is an increase in the concentration of ionized calcium in the region of the myofilaments. Relaxation is probably brought about by the decrease of $[Ca]_i$ to a level of less than 10^{-6}M.

An increase in intracellular free calcium levels may result from a) release of intracellularly bound calcium; b) release of calcium ions from stores in or near the cell membrane; c) an influx of calcium ions from the extracellular solution; d) inhibition of

calcium efflux, active rebinding or pumping. However, the calcium exchange in smooth muscle is complex and the source of the calcium ions which activate contraction has not been elucidated (see Introduction Part E). If the action potentials of smooth muscle are due to the inward movement of calcium ions from the cell membrane then there may be a direct link between excitation and contraction in this tissue. It is of interest to calculate the influx of calcium necessary to cause contraction, assuming the threshold for contraction of the proteins to be in the range of 1×10^{-6}M (Weber, Herz and Reiss, 1964). If the extracellular space is taken to be 400 mls/Kg, then 1 Kg of tissue corresponds to $600cm^3$ of cell volume. Thus $1 \times 10^{-6} \cdot 6.0 \times 10^{-1}$ $1 = 6 \times 10^{-7}$ moles per Kg of tissue, or 0.6μM, is required for threshold response. If calcium is the current carrying ion, a calculation of the movement of calcium during impulse activity can be made in a similar manner to that for sodium movement in nerve (Keynes and Lewis, 1951). Thus, using the membrane capacity (C) determined by Tomita (1966b) as $3\mu F/cm^2$ and a surface to weight ratio of $4.1cm^2/mg$, the necessary quantity of electricity for a 60mV action potential, is $CV \times 4.1 = 3\mu F.cm^{-2} \cdot 4.1cm^2mg^{-1} = 738$ $\mu coulomb.mg^{-1}$, which may be transferred to the interior of the cell by:
$CV/FZ = 738 \times 10^{-9}/96500 \times 2 = 3.8 \times 10^{-12}$ moles of calcium per mg of tissue, or 3.8 μM/Kg of tissue It can be seen that calcium influx during an action potential could initiate contraction.

Depolarisation of the cell membrane, either transient (by action potentials) or sustained, leads to or is associated with, the

activation of the contractile mechanism. However, although electrical and mechanical processes are obviously linked in smooth muscles, they are probably only steps in a chain of events, since changes in membrane potential are not always associated with changes in tension and/or length of the muscle (see Introduction Part D).

ID The effects of drugs on electrical and mechanical activity and ionic fluxes of uterine smooth muscle

1. Stimulants

A variety of receptors are available for the initiation of contraction of smooth muscle, but it seems likely that some of the sequence of events occuring between combination of agonist with receptor and contraction, is common to all stimulants. Drug receptors are considered to reside on the surface of cells, principally because of the very short period of time between application of the drug and the response, and also by extrapolation from the striated muscle end plate where acetylcholine is ineffective when iontophoretically injected inside the membrane (Del Castillo and Katz, 1955; Goldsmith, 1963).

Electrical activity normally accompanies drug-induced contractions. Stimulants (potassium, acetylcholine, oxytocin, α-adrenergic agents, etc.) cause depolarisation, a train of spikes and contractions are initiated, or if spikes are present, the spike frequency is increased, when applied to uterine smooth muscle

preparations. High concentrations cause rapid firing of spikes, maintained depolarisation and contracture. If depolarisation is sufficient, spikes cease. Elevation of $[K]_o$ (hypertonically) has been shown to increase ^{45}Ca uptake and efflux in smooth muscles (Urakawa, Karaki and Ikeda, 1968; Hagiwara and Nagai, 1970). Isotonically added potassium did not alter ^{45}Ca uptake. When smooth muscle is completely depolarised by placing it in high potassium solutions, there is an initial contracture, from which the muscle will eventually relax partially or fully, (Evans, Schild, Thesleff, 1958; Durbin and Jenkinson, 1961b). Under these conditions, in the absence of electrical events, stimulants will still produce a reversible contractile response and are acting presumably by causing changes in permeability and/or release of ions. Daniel (1963) proposed a model in in which K_2SO_4 Ringer (i.e. depolarising) solution made calcium bound at the surface of the membrane more labile, and the membrane more permeable to calcium. On addition of acetylcholine the situation was considered to be qualitatively the same as in tissues depolarised by action potential discharge.

a) Acetylcholine

An increase in g_{Na} and g_K would account for many of the excitatory actions of acetylcholine in smooth muscle (Bülbring and Burnstock, 1960, Bülbring and Kuriyama, 1963b). Acetylcholine has been shown to increase potassium efflux (Durbin and Jenkinson, 1961; Weiss, Coalson and Hurwitz, 1961; Burgen and Spero, 1963; Von Hagen and Hurwitz, 1967). Durbin and Jenkinson (1961) used depolarised

taenia coli to prevent actions on membrane potential and showed that carbachol increased potassium efflux and influx, indicating an increase in potassium permeability. Although increase in K-efflux requires calcium, Durbin and Jenkinson (1961b) showed that in the presence of low concentrations of calcium, the increase in K-efflux could be elicited without any concomittant increase in tension. Ethanol inhibits acetylcholine-induced contractions but not the increased K-efflux (Hurwitz, 1964). Acetylcholine also increases ^{22}Na-efflux and uptake in ileal smooth muscle (Weiss, 1969). Depletion of calcium during acetylcholine contractions leads to relaxation (Daniel, 1963) and repeated stimulation with acetylcholine increases the rate of decline of responses in calcium-free media (Schild, 1964). This has been taken to indicate that acetylcholine mobilizes surface calcium, increasing calcium activity in the membrane, and leading to calcium penetration of the membrane (Daniel, 1963, 1964). Hurwitz and Joiner (1970) suggested that the movement of calcium into the cytoplasm of an excited fibre, either from a cellular pool or from the extracellular fluid, is regulated by a saturable transport mechanism. They further suggested that acetylcholine acts to increase the number of operational loci, increase the transport rate of calcium to the cytoplasm, or decrease extrusion of calcium. Goodman and Weiss (1971) claimed to have dissociated responses to potassium and acetylcholine by the use of lanthanum *(vide infra)*.

There are considerable differences in ability to demonstrate changes in calcium fluxes in response to acetylcholine between

different smooth muscle tissues and between different groups of workers on the same tissue. For intestinal muscle, the consensus appears to be that cholinomimetic drugs increase efflux of calcium (Durbin and Jenkinson, 1961b; Schatzman, 1964; cf. Joiner, 1970) and increase influx in depolarised muscle; in polarised muscle, effects on calcium influx are conflicting (see Lüllmann, 1970 for references). In the rat uterus, van Breemen and Daniel (1966) failed to demonstrate any changes in calcium fluxes in polarised muscle in the presence of acetylcholine, but found an increase in calcium influx in depolarised muscle. An enhanced efflux during the last phase of the washout was detected in the presence of high potassium. Krejci and Daniel (1970) later showed that contraction of the tissue altered fluxes of calcium by affecting diffusion through the extracellular space. Decreasing the electrical gradient across the membrane by increasing $[K]_o$ did not alter ^{45}Ca fluxes when tracer was allowed to equilibrate with the extracellular space before and after contractions.

b) Catecholamines

Excitatory responses to catecholamines are associated with α-receptors (Ahlquist, 1948, 1966). The uterus is innervated by the hypogastric nerves which release noradrenaline as neurohumoral transmitter. The uterus possesses both α and β receptors for catecholamines (Miller, 1967) and the response to adrenaline or noradrenaline depends upon the hormonal status. However, appropriate use of α or β blocking agents demonstrates the existence of both

types of receptor in all uteri studied (Marshall, 1970). The excitatory effects of catecholamines on membrane potentials of the myometrium are similar to those recorded after application of other smooth muscle stimulants. The ionic mechanisms of these contractile effects are not known and ionic fluxes have not been studied in this tissue. Hinke (1965) suggested that potassium-induced contractures in aortic muscle made use of a different source of calcium from that utilized by noradrenaline, and this has been supported by Hudgins and Weiss (1968). It has also been suggested that the phasic and tonic components of vascular smooth muscle contractions are activated by calcium from different sources (Godfraind and Kaba, 1969). Shimo and Holland (1966) considered that high potassium-induced contractions consisted of a tonic and a phasic component. The phasic response was thought to be due to calcium release due to depolarisation, while the tonic part to be due to a metabolically dependent calcium transport system. Such components have been disputed by Somlyo, Vinall and Somlyo (1969) who considered that the effects of depressants used by Godfraind and Kaba (1969) were compatible with a greater sensitivity of a delayed decay in Ca-conductance. Tonic and phasic responses to potassium have also been postulated for the taenia coli (Imai and Takeda, 1967ab).

The effects of noradrenaline on calcium fluxes accompanying contraction in vascular smooth muscle have been studied, but again are conflicting. Briggs and Melvin (1961) found an increased ^{45}Ca

uptake with adrenaline, while Hudgins (1969) found an increase in efflux with noradrenaline. Seidel and Bohr (1971) also found an increased uptake with noradrenaline and a decrease in efflux, while van Breemen and Lesser (1971) failed to find any increase in calcium uptake with this agent.

c) Oxytocin

The late pregnant, but not the mid-pregnant, rat uterus responds to oxytocin (Marshall and Csapo, 1961; Csapo, 1969). The suppression of the action of oxytocin has been suggested to be due to progesterone dominance at mid-pregnancy (Csapo, 1961). Marshall (1964) found that a prerequisite for the action of oxytocin was that the membrane potential be near threshold for the discharge of propagated action potentials. If the membrane is sufficiently hyperpolarised or depolarised, the action of oxytocin was said to be abolished.

Removal of calcium, sodium or potassium abolished the action of oxytocin and Marshall (1964, 1968) concluded that oxytocin causes a non-specific increase in membrane permeability to these ions. Kleinhaus and Kao (1969) and Kao (1967) considered that low doses of oxytocin do not change resting membrane potential but initiate spike activity by causing the appearance of pacemaker potentials. They postulated that oxytocin caused an increase in the number of sodium gates or carriers capable of responding to threshold depolarisation. This does not however explain the response to

oxytocin in muscles depolarised with potassium Ringer (Evans and Schild, 1957). Oxytocin does not affect ^{45}Ca uptake by uterus (Krejci and Daniel, 1970).

1. Spasmolytics

a) Catecholamines

Adrenaline relaxes the rat uterus irrespective of hormonal status (Paton, 1961) while to noradrenaline it may relax or contract depending upon the predominant endocrine influence (Marshall, 1970). As is the case for stimulant drugs, the spasmolytic action of catecholamines is, under normal conditions, accompanied by a change in membrane potential, viz. hyperpolarisation. The degree of hyperpolarisation produced by catecholamines depends upon the level of the resting membrane potential; the duration of the period of cessation of action potentials depends upon the concentration of adrenaline (Csapo and Kuriyama, 1963). Although the change in membrane potential is probably indicative of an associated alteration of ion movements, it must be emphasized that the spasmolytic action of catecholamines is still demonstrable in tissues depolarised by high potassium (Edman and Schild, 1961; Schild, 1964). The hyperpolarisation is therefore not a requisite part of the spasmolytic action of catecholamines.

Observation of changes in the membrane potential response to catecholamines when tissues are exposed to solutions of different ionic composition has been used to attempt to relate hyperpolarisation

to changes in the permeability to specific ions. In high potassium solution, there is no hyperpolarisation in response to adrenaline; in K-free solutions, adrenaline causes a large hyperpolarisation (Kuriyama, 1961). Excess sodium reduces the hyperpolarisation and depresses but does not block the inhibitory action of adrenaline. Decreasing $[Na]_o$ has no effect on the action of adrenaline, but in Na-free solutions, the action of adrenaline is reduced or abolished (Bülbring and Kuriyama, 1963b). In low calcium solutions, the effect of adrenaline is reduced, while in excess calcium it is enhanced. Burnstock (1958), suggested that inhibition of spontaneous activity by adrenaline is brought about by stimulation of an electrogenic sodium pump, from observations of the interaction of adrenaline and 2:4-dinitrophenol. Bülbring and Kuriyama (1963b) considered that adrenaline changes the sodium conductance. Adrenaline was said to increase the fixation of calcium at the membrane, resulting in stabilisation of the membrane, a decreased g_{Na}, and hyperpolarisation. However, since membrane potential, according to the ionic hypothesis, is dependent upon the ratio of g_K/g_{Na}, blockade of hyperpolarisation in Na-free solution could be accounted for if adrenaline increased g_K or decreased g_{Na}. Beuding and Bülbring (1964) considered that fixation of calcium at the membrane is brought about by an adrenaline-induced increase in the supply of metabolic energy. Bülbring and Tomita (1968a, 1969abc) showed that increased membrane conductance to potassium and calcium fixation in taenia coli were due to an α-adrenergic effect; β-adrenergic inhibition was considered to be due

to suppression of the generator potential.

Marshall (1968) suggested three possibilities for adrenaline-induced hyperpolarisation of rat myometrium:

a) a selective increase in potassium permeability

b) stimulation of an electrogenic sodium pump

and c) increased fixation of calcium at the membrane, diminishing sodium permeability

Adrenaline was found to bring the membrane potential closer to the calculated potassium equilibrium potential at all concentrations of $[K]_o$ and this suggested that adrenaline selectively increased potassium permeability of the membrane. However, this could equally well be explained on the basis of a decreased permeability to sodium. Marshall rejected the idea that adrenaline stimulated the sodium pump and considered that since the inhibitor action of adrenaline depends upon the presence of calcium ions in the external medium, calcium binding at the membrane could be involved. Daniel, Taylor, Paton and Hodgson (1970) showed that β-adrenergic agents did not affect the electrogenic pump in rat myometrium when the membrane potential was controlled by the pump. Isoprenaline can cause relaxation in the presence of ouabain in K-free solutions and the sodium pump is therefore not involved in the spasmolytic activity of catecholamines. Daniel *et al* (1970) concluded that although there is no direct evidence available, the spasmolytic action of catecholamines probably involves increased calcium binding.

Diamond and Marshall (1969ab) showed that while catecholamines

abolished spontaneous activity of rat myometrium, caused hyperpolarisation and reduced the resting tension by β-receptor stimulation, papaverine and tetracaine had no effect on resting membrane potential. They concluded that hyperpolarisation is not a prerequisite for inhibition of motility and that tissue resting membrane potential and resting tension are not necessarily related under physiological conditions. They further concluded that the most important effect of the drugs in the normally polarised preparation is to reduce the pacemaker discharge, resulting in a decreased action potential frequency, possibly reinforced by an alteration in cell-to-cell conduction. Schild (1966, 1967) postulated that stimulation of the adrenergic β-receptor activates a Ca-accumulating system, since adrenaline inhibits calcium contracture in depolarised muscle. Batra and Daniel (1970) have in fact demonstrated increased calcium uptake in their microsomal fraction of rat uterus in response to adrenaline but the time required for increased uptake was slower than would be expected.

Born and Bülbring (1956) have studied ^{42}K fluxes of the spontaneously active taenia coli and found that adrenaline had no effect on potassium loss but did increase potassium uptake. Jenkinson and Morton (1967abc) have clearly shown that noradrenaline causes an increase in potassium permeability in guinea-pig taenia coli, which is blocked by α-blocking agents, but not by pronethalol. Isoprenaline also causes relaxation of the preparation but has no effect on potassium permeability. They suggested that an increase

in g_K may be responsible for the inhibitory action of catecholamines which is mediated by α-receptors. Changes in sodium fluxes are difficult to measure in smooth muscles because of the fast rate of exchange (Goodford and Hermansen, 1961; Daniel and Robinson, 1970). Bülbring, Goodford and Setekeiv (1966) found an increase in sodium efflux induced by adrenaline, but the effect was small and the source of the sodium was uncertain. Jenkinson and Morton (1967b) did not find any increase in sodium uptake with noradrenaline. Calcium fluxes have not been observed to change in the presence of catecholamines in intestinal muscle (Banerjee and Lewis, 1963; Schatzman, 1964). Adrenaline did not affect calcium uptake in rat uterus (Krejci and Daniel, 1970a).

Sutherland and Robison (1966) and Sutherland, Robison and Butcher (1968), identified cyclic 3'5' AMP as the second messenger mediating a number of hormonally induced metabolic effects, and established criteria whereby the action of a hormone could be considered to be mediated via the adenyl cyclase system. The synthesis of the acylated derivatives of cyclic 3'5' AMP has helped to circumvent the problem of low permeability of cells to exogenous cAMP. Catecholamines active at myometrial β-adrenergic receptors stimulate adenyl cyclase activity and cause an increase in the level of intracellular cAMP simultaneously with inhibition of contractility (Diamond and Brody, 1967). These effects are enhanced by theophylline which inhibited phosphodiesterase and antagonized by β-adrenergic blocking agents. Levy and Wilkenfield (1969) dispute the mechanism

of action of theophylline, finding that theophylline potentiates the inhibitory action of noradrenaline on rat uterus by a non-specific mechanism. The relaxing effect of catecholamines can be mimicked by dibutyryl cyclic 3'5' AMP but Kim, Schulman and Levine (1968) considered that the relaxant effect of cAMP on the ileum was non-specific and that the adrenosine moiety was the active inhibitory agent. Polacek and Daniel (1970) showed that the isoprenaline-induced increase in cAMP levels remained elevated after reversal of inhibition of contractions with propranolol, indicating that cAMP is not the primary determinant of uterine contractility.

b) Papaverine

Papaverine is described as a non-specific relaxant, since it relaxes all smooth muscles and is not antagonized by any known blocking agent. Little work has been done on the mechanism of its relaxant effect; although it has been shown not to change resting membrane potential in the uterus (Diamond and Marshall, 1969), Imai and Takeda (1967a) and Takagi and Takayanagi (1969) found a hyper-polarisation to papaverine in the taenia coli. Substances such as papaverine and the nitrites are thought to interact at some step in the final common pathway to prevent calcium release (Daniel, 1964). Since papaverine abolished the tonic phase of the potassium response in taenia coli, papaverine was considered by Imai and Takeda (1967a) to act to decrease the size of the "bound" calcium, or increase the inactive form of this fraction. Ferrari and Carpenedo (1968) showed

that papaverine shifted the dose-response curve to calcium of taenia coli in K_2SO_4 Ringer to the right and considered that this indicated competitive antagonism between papaverine and calcium. Carpenedo, Ferrari and Furlanut (1968) showed that papaverine inhibits lipid-facilitated calcium transport from an aqueous to a chloroform phase and suggested that this could be related to spasmolytic activity at cell membranes. Kukovetz and Pöch (1970) demonstrated inhibition of phosphodiesterase by papaverine leading to accumulation of cAMP in arterial muscle. The effects of papaverine on ion movements have not been studied.

c) Lanthanum

Mines (1910) showed that lanthanum inhibits contractions of the heart. During the course of the research reported in this thesis, a number of papers have appeared in which lanthanum has been used to specifically prevent calcium fluxes through cell membranes. van Breemen (1969) and van Breemen and McNaughton (1970) have used lanthanum, which prevents calcium movements through artificial membranes, to inhibit contractions of the rabbit aorta to noradrenaline and potassium. Lanthanum changes the shape of the respective contractions; the phasic part of the noradrenaline contraction persists but the tonic phase is abolished, while the opposite is true for KCl contractions. van Breemen (1969) considered that this indicated that noradrenaline and potassium-induced contractions use calcium from different sources; thus noradrenaline was considered to release

intracellular calcium initially and the tonic phase was due to calcium influx from the intracellular fluid. Sanborn and Langer (1970) used lanthanum to inhibit contractility of the heart and showed that lanthanum caused release of the contractile-dependent calcium, which is thought to reside at superficial sites.

More recently van Breeman and Lesser (1971) have used lanthanum to remove extracellular non-specific calcium and prevent calcium fluxes from the smooth muscle cells. Uptake of ^{45}Ca and subsequent exposure of the tissues to lanthanum reveals a fraction of calcium which is said to represent intracellular calcium; this fraction can be increased by potassium but not by noradrenaline, which would confirm the previous observations concerning calcium influx during contraction in vascular smooth muscle.

IE Problems of smooth muscle research

Understanding of the intricate mechanisms and complex processes of smooth muscle cells makes it imperative to evaluate the effect of a single controlled variable, rather than attempting to analyse the numerous unknowns that influence such muscle *in vivo*. However, such evaluations are obfuscated by specific problems which arise in experiments which attempt to relate ionic composition and flux and membrane potential to function of, and the action of drugs on, smooth muscle preparations *in vitro*.

1. Extracellular space

In order to estimate intracellular concentrations of ions,

total ionic concentration must be used in conjunction with an estimate of the amount of water which is outside the cell. Estimates of the extracellular space made by determination of the volume of distribution of a marker substance which does not penetrate cells or does so at a very slow rate, depend upon a number of assumptions, which may not be valid. It is assumed: a) that the concentration of the marker in the extracellular space is the same as in the incubating medium; b) that the marker occupies the whole volume of the extracellular fluid and this implies that the extracellular space is homogenous; c) the marker is not bound in the extracellular space nor does it enter the intracellular volume; and d) the marker is not chemically modified by the extracellular fluid. Values for the extracellular space in smooth muscle depend upon the size of the molecular species used as marker and also upon the charges on the molecule.

Radioactive tracers including ^{14}C-inulin, ^{14}C-sorbitol, ^{35}S-ethanesulphonate, and ^{131}I-serum albumin have been employed (Burnstock, Dewhurst and Simon, 1963; Goodford, 1966; Goodford and Leach, 1966) and these give values which are in reasonable agreement (Goodford, 1968). Values determined by weighing portions of electron-micrographs which are not occupied by muscle (Yamauchi and Burnstock, 1969a) are consistently lower than those calculated from the uptake of markers. This may be due to swelling of cells during fixation, and/or selection of areas of the preparation which consist of only muscle fibres.

2. Ion Binding

Some electrolytes may be bound either extracellularly or intracellularly in an osmotically or electrochemically inactive state. The calculation of cellular ion concentrations assumes that the activity coefficients are the same (1.0) in the extracellular and intracellular compartments. Since the definition, and hence the numerical amount of "bound" ion depends upon the method employed for estimation, it is not possible to apply corrections for this phenomenon. The work of Hinke (1961) on squid axon and Lev (1964) on frog sartorious muscle indicate that for sodium, binding or a low activity coefficient is the case. Cope (1966, 1967) has used ^{23}Na nuclear magnetic resonance to investigate the state of sodium in frog muscle and found an "NMR-invisible" fraction of sodium which was considered to be bound. This finding was confirmed by Czeisler, Fritz and Swift (1970). In arterial smooth muscle, it has been demonstrated that a portion of the net sodium of the tissue is probably in a bound state rather than that of the free ion (Dodd and Daniel, 1960; Palaty, Gustafson and Friedman, 1969). Kao (1961) attempted to determine the bound fraction of the ions in rabbit myometrium and calculated that up to 29m Eq/kg potassium and 14m Eq/kg sodium could be considered bound. Radiotracer efflux curve analysis indicates that significant, but considerably smaller, amounts of sodium could be bound in rat myometrium (Daniel and Robinson, 1970). Batra and Daniel (1970b) found three types of binding sites for cations in homogenates of rat uterus; those from which sodium, potassium,

calcium and magnesium could be: a) removed by washing; b) removed by ion exchange and c) not removed by washing or ion exchange.

Since the threshold for contraction of the contractile proteins is in the region of 10^{-6}M, any intracellular calcium must be bound inactively. However, most, if not all, of the tissue calcium appears to be exchangeable as calculated from ^{45}Ca influx studies (Lüllmann, 1970).

3. Tissues in an *in vitro* environment

There is a rapid movement of cations down their concentration gradients when smooth muscle tissues are moved from an *in vivo* to an *in vitro* environment; (Daniel and Robinson, 1960; Dawkins and Bohr, 1960; Kao and Siegman, 1963; Rorive, 1969), this is probably due to manipulation and the small volume:surface ratio. The tissues do not fully recover from such changes, but an *in vitro* environment does not reduce all smooth muscles to an identical performance as demonstrated by the differences in electrolyte content (Daniel and Daniel, 1957) mechanical (Berger and Marshall, 1961) and electrical (Bülbring, 1960) performance of uterine muscle from animals under different endocrine influence.

4. Membrane potential recordings

The use of microelectrodes represents the most direct approach to the study of transmembrane potential changes. This method has been successfully applied to several smooth muscle preparations, but most work has been done on the guinea-pig taenia

coli and the uterus (Burnstock, Holman and Prosser, 1963). Technical difficulties arise due to the small diameter of the muscle cells (2-5μ) and their spontaneous activity which tends to dislodge the electrode. The latter has been overcome to some extent by the use of "floating" electrodes (Woodbury and Brady, 1956). In order to penetrate smooth muscle cells without causing injury, microelectrodes must have tips of less than 0.25μ (Kao and Nishiyama, 1964). Such electrodes tend to have a high resistance and high tip potentials (Shanes, 1958). Tip potentials may be reduced or eliminated by the use of thorium chloride, which is thought to prevent the formation of glass-electrolyte interface potentials (Agin and Hotzman, 1966). Kao and Nishiyama (1964) listed a number of criteria for the measurement of membrane potentials:

a) sharp deflection on penetration to new d-c level (inside negative)
b) "cleanliness" of shift on reaching new d-c level
c) persistence of new d-c level
d) return to original baseline

A further criterion, recording of large action potentials, was abandoned in studies where the criterion could not be met; i.e. when conditions were changed such that no action potentials occurred.

5. Tracer flux data analysis

The interpretation of observations concerning the kinetics of the distribution of an isotope within a biological system such as

smooth muscle, must consider the inhomogeneity of the tissue in terms of compartments. The term compartment refers to an homogenous region bounded by limiting barriers. Two physiologically recognisable compartments are obvious from the morphology of smooth muscle tissue viz. extracellular and intracellular regions. In practice more than two compartments must be considered in order to reproduce the observed kinetics of tracer distribution in terms of a simple model (Daniel and Robinson, 1970).

The following assumptions are made in the use of compartmental analysis of tracer kinetics:

a) each compartment is uniform in composition and hence, distribution of tracer is uniform

b) the behaviour of the tracer is chemically and physically identical to that of the unlabelled isotope, and the system cannot descriminate between them

c) flux across the membrane is proportional to the concentration gradient across the membrane

d) transfer across the compartmental boundary is rate limiting

The requirement that resistance to flow of tracer resides at the interface means that mixing within a compartment must be fast to ensure uniform distribution. The assumption of homogeneity of compartments is of dubious validity. Furthermore, diffusion from the

extracellular space may affect efflux since the diffusion coefficient has been found to have a low value (Daniel and Robinson, 1970). Homogeneity can be tested for by a number of techniques: parallelism of cpm and cpm^2 plots; analysis of log cpm vs log cpm^2 plot; and measurement of tracer equilibrium specific activities. Assumption 2) is probably valid for isotopes of atomic weight large enough such that the difference is negligible (i.e. greater than 30), but assumption 3) may be invalid if the electrical driving force on the tracer changes. The biological identification of compartments presents a considerable problem, and must depend upon independent evidence. (For further discussion of problems of flux data analysis, see Solomon, 1960; Daniel and Robinson, 1970).

Although compartment analysis may not provide a complete description of biological systems, it provides a valuable means of interpreting experimental results of tracer kinetics in a quantitative manner.

IF <u>The specific aims of the research</u>

There have been numerous studies on the drug-receptor interactions of smooth muscles and extensive kinetic theories have been evolved to account for the relationship between drug dose and the effect produced (see, for example, Ariëns, 1964). There are also many published reports on the effects of drugs upon the membrane potential and associated active properties of smooth muscles. However, in spite of advances in electrophysiology and the use of more refined

techniques for following the movement of ions into and out of cells (radiotracers) the immediate consequences of combination of drugs and their receptors are still uncertain. The following assumptions may be made concerning the responses of smooth muscle cells to drugs:

1. The initial event after the drug has diffused to the biophase is combination with discrete specialised sites - receptors.
2. Under normal physiological conditions, many drug responses are accompanied by effects on the membrane resting and active properties.
3. A polarized membrane is not necessary for the actions of drugs on smooth muscle.
4. Some substances (e.g. barium and potassium) cause contracture of nearly all smooth muscles and do not possess specialised receptors; these agents can presumably activate a step beyond the combination of drugs with receptors.
5. The penultimate step in the sequence of events in the elevation of the concentration of free calcium in the intracellular fluid, near the contractile proteins.
6. The ultimate step is the alteration of the interaction of myosin and actin in such a manner as to cause relaxation or contraction

of the muscle cell.

The actions of drugs on the smooth muscle cell to cause excitation or inhibition may be initiated by effects on tissue electrolytes. Such effects may be changes in passive permeability, ion binding or active transport. The interrelationships between effects on ion movements and electrical and mechanical events have not been determined. The present work therefore set out to answer the following questions in relation to the sequence of events which takes place between the combination of drug with its receptor, and the final response:

1. What changes in net ion movements and unidirectional fluxes do drugs and ions produce?
2. Can the demonstrated effects on ion movements be correlated with the electrical events which occur during drug action?
3. Do the changes in ion movements mediate the observed changes in contractility of the tissue?

More specifically, these questions were posed with reference to the movements of potassium and calcium.

II. METHODS

II METHODS

IIA Tissue Preparation

Pregnant wistar rats weighing 100-150 grammes were killed by a blow on the head, at least seventeen days after mating and as near to term as possible. The uterus was excised and cut along the line of mesenteric attachment, to form a sheet of muscle. The foetuses and placentae were removed. A segment of uterus approximately 2 cms square was placed on filter paper, moistened with Krebs solution, mucosal surface uppermost. It was stretched to cover an area approximately equal to that *in situ*. The line of demarcation between the longitudinal and circular muscle layers could be readily found using splintered forceps, and, holding one end of the segment, the endometrium and circular muscle layer was gently stripped away. Care had to be taken to ensure that the thin and delicate longitudinal muscle layer did not dry out. For ionic flux studies, as many as 36 pieces of tissue weighing 10-30 mg could be obtained from the two horns of uterus. All tissues were allowed to equilibrate with Krebs Ringer solution for one hour prior to experimental procedures. Only fresh tissues were used for flux studies. Tension experiments were occasionally carried out on tissues kept overnight at 4°C in Krebs Bicarbonate Solution. Tissues did not recover if kept in Krebs Tris Buffered Solution overnight.

IIB Materials

1. Solutions

The composition of the Normal Krebs solution used was as follows:

Krebs Bicarbonate Buffered Solution: NaCl, 115mM; KCl, 4.6mM; $CaCl_2$, 2.47mM; $MgSO_4$, 1.2mM; $NaHCO_3$, 21.9mM; Glucose, 50mM. The solution was bubbled with 95% O_2 and 5% CO_2; the pH was 7.4.

Krebs Tris Buffered Solutions: In some experiments, the bicarbonate was replaced by tris-(hydroxymethyl)aminomethane (tris), the pH of which had been adjusted to 7.4 using 10N HCl. In most experiments, the concentration of NaCl was increased to allow for a reduction in tris concentration: NaCl, 160mM; KCl, 4.6mM; $CaCl_2$, 2.47mM; $MgSO_4$, 1.2mM; tris, 4.2mM; Glucose, 15mM. The solution was bubbled with 100% O_2 and the pH of the tris was 7.4. The pH of the Krebs solutions did not change during experiments.

Lithium and Potassium Krebs were obtained by replacement of the sodium with lithium chloride or potassium chloride. For Ca-free solutions, calcium was omitted.

2. Drugs

Solutions of drugs were prepared fresh before each experiment, except for acetylcholine, which was prepared every few days. Solutions of ions were prepared from stock solutions kept at 4°C. The following drugs were used:

Acetylcholine bromide (Sigma Chemical Co.), carbachol (Aldrich Chemical Co.), chlorpromazine (a generous gift from Poulenc Limited, Montreal), l-adrenaline bitartrate (Sigma Chemical Co.), l-isoprenaline (Sigma Chemical Co.), lanthanum chloride (Fisher Chemical Co.), l-noradrenaline (Nutritional Biochemicals), oxytocin (Nutritional Biochemicals), papaverine (a generous gift from Ely Lilly Laboratories, Indianapolis), phenoxybenzamine (Smith, Kline and French), propranolol (Ayerst, McKenna and Harrison), SKF 525-A (2-diethylamino ethyl-2,2-diphenyl-valerate HCl, a generous gift from Smith, Kline and French, Montreal).

3. Isotopes

^{42}K was obtained from Atomic Energy of Canada as $^{42}KHCO_3$, and from International Chemical Nuclear Corporation as ^{42}KCl. The bicarbonate was dissolved in 0.1N HCl and made up to 10 mls with distilled water; the ^{42}KCl was in solution, and was made up to 10 mls with distilled water.

^{86}Rb was from New England Nuclear as chloride; ^{45}Ca from Amersham Searle as chloride; ^{89}Sr from Amersham Searle as chloride; inulin carboxyl-^{14}C from New England Nuclear; ^{14}C-mannitol from New England Nuclear; ^{140}La from International Chemical and Nuclear Corporation as $^{140}La(111)$ in 1N HCl. The pH of ^{140}La and ^{89}Sr solutions was adjusted to 6.8 with NaOH and the pH of Tris Buffered Krebs Solution containing ^{140}La or ^{89}Sr was 7.4. Radiometric purity of these compounds was stated to be greater than 99% for all except

lanthanum, which was stated to be greater than 98% pure. Decay of ^{42}K and ^{86}Rb was examined, and the half-lives found to be 12.38 ± 0.11 hours (λ = 0.000938 min^{-1}) and 17.9 ± 0.3 days (λ = 0.00158 $hours^{-1}$) respectively. These are in good agreement with quoted values (Goodier, 1969; Heath, 1964).

Samples of approximately 1 μC of ^{42}K and ^{86}Rb were introduced into a gamma-ray spectrum multichannel analyzer which had been calibrated using ^{60}Co and ^{137}Cs. For ^{86}Rb, the gamma peak at 1.085 Mev was the only one visible indicating purity of the radiotracer. ^{42}K was found to be contaminated with ^{24}Na, the spectrum having ^{24}Na peaks at 495 Kev (β^+); 1.03 Mev (β^+); 2.75 Mev (γ) and 4.2 Mev (γ). Contamination however, was found to be less than 0.5%.

Fluor solution for counting β-emission was that due to Bray (1960) and contained: 60 g naphthaline, 100 mls methanol, 20 mls ethylene glycol, 4 g PPO (2,5-diphenyloxazole) and 0.2 g POPOP [1,4-bis-2-(5-phenyloxazolyl)-benzene], made up to 1 litre with 2,4, dioxane.

IIC Recording of electrical activity

A portion of fresh tissue about 8 mm x 3 mm was mounted on a neoprene block and secured by three pins at each end. One set of pins was joined and was connected by a silk thread to a Grass force displacement transducer for recording of mechanical activity (Figure 1). A resting tension of 0.2 - 0.5 g was applied to each

tissue. Flow rate of Krebs through the bath was 2-4 mls/min. In approximately one half of the experiments where membrane potential was recorded, stripped tissues were used, either completely stripped or, since such tissues tended to tear from the mounting pins, were stripped in a central region (2-3 mm) from which potential recordings were taken.

Membrane potentials were recorded using glass capillary ultra-microelectrodes filled with 3M potassium chloride and 2mM potassium citrate. The electrodes were generally pulled by hand but in some experiments had been pulled using a commercial micro-electrode puller (Industrial Science Associates). Electrodes were filled with a solution of equal parts methanol and distilled water by boiling under reduced pressure, and then transferred to a solution of 3M KCl containing 2mM potassium citrate and were left to stand overnight.

The glass microelectrodes were suspended from a flexible helix of 0.002" platinum wire, connected to a high input impedance probe of a negative capacitance electrometer (Medistor Instruments, Model A-35), which provided a calibration circuit for measurement of microelectrode resistance. Input resistance of the cell was measured as the increase in resistance in this circuit after penetration. The output of the electrometer was displayed on a Tektronix oscilloscope (Model 584) and recorded on a Beckman Dynograph (Type R, response time less than 5 msec). The output was differentiated for obtaining maximum rate of rise of action potentials using an RC circuit

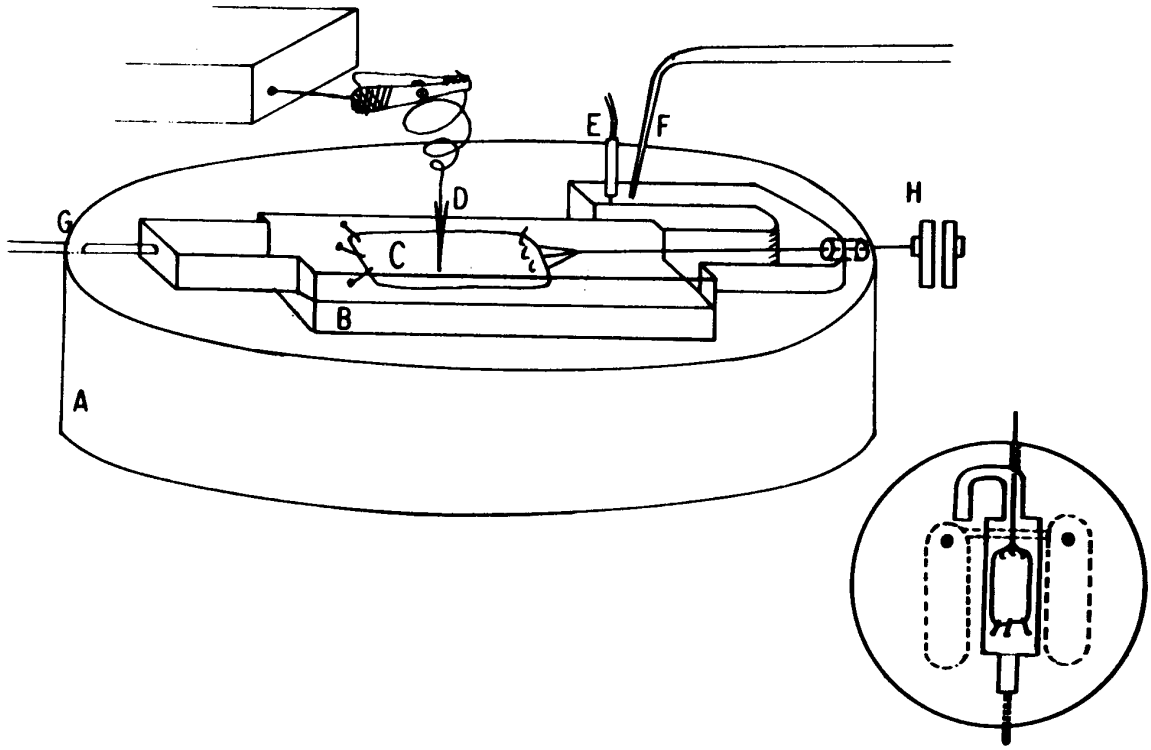

Figure 1. Isolated organ bath for recording mechanical and electrical activity of smooth muscle. A, perspex block in which bath was machined; B, neoprene block; C, tissue; D, microelectrode suspended from platinum helix attached to medistor probe; E, Ag:AgCl reference electrode; F, vacuum tube for removing oxygenated Krebs solution flowing into the bath continuously at G; H, Grass force displacement transducer. Inset shows view from above illustrating constant temperature bath adjacent to organ bath.

using a capacitance of 1000 pf in series with the 100 kΩ input resistance of the oscilloscope. The differentiating circuit was calibrated using saw tooth pulses from a Tektronix wave form generator (Type 162). Thorium chloride (10 μM) was usually added to the perfusing solution to abolish tip potentials. Membrane potential records were required to meet the criteria of Kao and Nishiyama (1964, see Introduction Ie). In addition, electrodes had to demonstrate that action potentials of reasonable amplitude and rise time (30 mV and 0.7 V/sec) could be recorded from them. In all records, the calibration of 50 mV shows zero potential at the top of the scale.

IID Total ion content determination

10-30 mg samples of longitudinal muscle were suspended from stainless steel hooks for incubation. After removal from the Krebs medium, they were blotted and weighed. In some cases they were then dried in an oven at 95°C for 24-36 hours and reweighed. Samples were wet ashed using 0.2 ml of concentrated nitric acid and 0.05 ml hydrogen peroxide (Fisher Reagents, ACS), at 200-250°C in 'hysil' test tubes in a sand bath. The ash was dissolved in 10 or 25 mls of distilled water and sodium and potassium contents determined using an Evans Electroselenium flame photometer. A standard curve, obtained using solutions of known sodium and potassium concentrations, was constructed at each experiment. For chloride estimations, tissues were extracted with 0.1N HNO_3 for 48 hours and chloride content determined using the titration method of Cotlove (1963) using an automatic

titrator (Buchler Instruments).

For calcium content, tissues were blotted, weighed, placed in crucibles and dry ashed at 550-600°C for eight hours in a muffle furnace. The ash was dissolved in 2.0 or 5.0 mls of 0.1N HCl and calcium concentration was measured using an SP90A Unicam flame Spectrophotometer at a wave length of 422 mμ, and by comparison to a standard curve constructed for each experiment. Standard solutions contained Na and K in approximately the same amounts as unknowns. Solutions and standards contained 0.5% $LaCl_3$. Ion contents were expressed as mmoles/kg wet weight of tissue.

IIE Determination of extracellular space

After incubation in Krebs solution for equilibration, tissues, suspended on stainless steel hooks, were placed in Krebs media at 37°C containing inulin-carboxyl-^{14}C or ^{14}C-mannitol (0.05 μC/ml and 0.01 μC/ml respectively). Samples were incubated in tracer-containing medium for 20, 60 or 120 minutes and then rinsed rapidly in four successive solutions of inactive Krebs solution, blotted, weighed and digested in scintillation vials (7 mls capacity) using 0.3 ml NCS solubilizer, (Amersham Searle) for 10 hours in a water bath at 40°C. 5 ml of Bray's solution was added, and ^{14}C was counted using a Picker-Nuclear 'Liquimat' Scintillation spectrometer (Model 650-513). Two samples of duplicate aliquots of media were also counted. Samples were counted to a relative standard error of less than 2% (10 minutes for ^{14}C mannitol and four minutes for ^{14}C inulin). A quench curve

was constructed using the channels ratio method, and acetone as quenching agent, and tissue and media counts were corrected to dpm. ^{14}C-inulin and ^{14}C-mannitol spaces were expressed as mls per 100 g tissue by dividing dpm/100 g tissue by dpm/ml incubation medium.

IIF Measurement of tracer uptake (^{42}K, ^{45}Ca, ^{140}La and ^{89}Sr)

Samples of longitudinal muscle 15-30 mg wet weight were mounted on stainless steel hooks. After equilibration in Krebs solution, they were transferred to Normal Krebs or KCl Krebs containing ^{42}K (0.03 μC/ml), ^{45}Ca (0.01 μC/ml), ^{89}Sr (0.01 μC/ml) or ^{140}La (0.02 μC/ml) at 25°C (^{42}K) or 37°C (^{45}Ca, ^{89}Sr and ^{140}La). For ^{89}Sr uptake, tissues were equilibrated for 1 hour with normal Krebs solution, thirty minutes in Ca-free Krebs solution and then 40 minutes in Ca-free Krebs containing 2.47mM strontium chloride. Tissues were removed from the radioactive solution at 10, 20 or 60 minutes, rinsed rapidly in four successive solutions of isotonic choline chloride (^{42}K) or Ca-free Krebs (^{45}Ca, ^{89}Sr and ^{140}La), ashed at 550-600°C (^{45}Ca) or digested with NCS (^{140}La and ^{89}Sr) as described above. If the tissues were exposed to drugs during uptake of radiotracer, the drug was added 2 minutes after transfer of tissues to the radioactive solution.

The ashed tissues containing ^{42}K were dissolved in 10 mls distilled water and counted for 5 minutes using a Picker Well-Type Scintillation Detector (Model 2956-4), automatic well counter (Model 2956) and spectroscaler III with a window setting to give a

range of 1.35 to 1.65 Mev. Ashed tissues containing ^{45}Ca were dissolved in 2 mls 0.1N HCl and 1 ml and 0.5 ml of this were transferred to either aluminium planchets and dried under infra red, or scintillation vials for counting using the Picker-Nuclear Scintillation Spectrometer. Planchets were counted using a Nuclear Chicago Gas Flow Proportional Counter (Model D47).

At least two aliquots of the uptake media were taken before and after incubation of tissues, and in the case of ^{140}La, after each series of tissues was removed (i.e. at 10 and 20 minutes). Media aliquots were diluted 1:100 either with deionized water or with 0.1N HCl and were counted in duplicate. For ^{42}K, tissues and media were assayed for potassium after the radioactivity had decayed. Media for ^{45}Ca uptake were prepared Ca-free and $CaCl_2$ added from concentrated stock solution so that all solutions contained precise concentrations of calcium. In all experiments involving ^{42}K or ^{140}La, a correction was made for decay of each sample from time zero (the time of counting the first sample)using a decay factor of 0.000932 min^{-1} for ^{42}K and 0.000287 min^{-1} for ^{140}La.

IIG Tracer effluxes from myometrium (^{42}K, ^{86}Rb, ^{45}Ca, ^{140}La and ^{89}Sr)

Tissues weighing 25-30 mg were incubated in 8 mls of Krebs solution containing tracer, for a period of two to three hours at 37°C. For ^{42}K loading, a special K-free Krebs was prepared which allowed for the addition of ^{42}K.

After incubation, tissues were suspended on stainless steel [†] hooks and rinsed for one or two seconds in isotonic choline chloride or Ca-free Krebs solution. They were then transferred to successive tubes containing 10 mls (^{42}K, ^{86}Rb), 4 mls (^{45}Ca and ^{89}Sr) or 2 mls (^{140}La) of inactive Krebs solution for specific time intervals, for a total of up to six hours. Tubes were bubbled with 95% O_2/ 5% CO_2 (^{42}K) or 100% O_2 (^{45}Ca, ^{89}Sr and ^{140}La) through stainless steel tubes, to ensure mixing and oxygenation of tissues. Solutions containing tracer were counted as described previously for respective isotopes. Tissues, at the end of the efflux period, were blotted, weighed and digested or ashed and counted. Spectrum analysis of the radioactivity remaining in tissues after ^{42}K efflux showed that such radioactivity was due only to ^{42}K. Specific activity of the tissue was obtained by analysing for total potassium.

IIH Analysis of efflux curves

Efflux data was initially calculated manually but subsequently a computer programme for the APL/360 system which gave satisfactory analyses was used (Cook and Taylor, 1971). Data was analysed according to the method of Soloman (1960) as subsequently

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[†] A number of different designs of hooks for efflux was used. Initially tissues were suspended from hooks under no tension. Hooks were then designed in which tissues were suspended as a sheet, under tension (0.5-1 g) exerted by a small spring. Considerable time was involved in setting up tissues in this manner. Finally, tissues were attached to an alligator clip by a silk thread, the clip hanging outside tube and exerting a constant tension of 1.1 g, as used by Creese (1968). None of these hooks was considered ideal.

applied by Daniel and Robinson (1970, 1971a,b,c).

In many experiments, the effects of drugs or ions were determined during efflux and curves were constructed of amount of radioactivity lost relative to the amount remaining in the tissue. This is defined as the rate coefficient and is $\Delta c/\Delta t.C_m$ (min^{-1}) where Δc is the amount of radioactivity lost during time Δt and C_m is the logarithmic mean of the content of tracer at times t and $t + \Delta t$.

In experiments where there was no change in the composition of washout solution and no drug added during the whole of efflux, the washout curves of log cpm vs time and log cpm^2 were constructed (Keynes and Swan, 1959; Persoff, 1960). The washout curves of radioactivity were found in most cases, to be described, with the least standard error, as the sum of three exponents:

$$Y_t = Ae^{-k_1 t} + Be^{-k_2 t} + Ce^{-k_3 t} + D$$

where Y_t is the amount of tissue radioactivity; A, B and C are the sizes of three compartments obtained by extrapolation to zero time; D is the bound fraction of an ion as determined by parallelism of cpm and cpm^2 curves and extrapolation of cpm to zero cpm^2 (Dick and Lee, 1964). k_1, k_2, and k_3 are rate constants of efflux from the respective compartments in min^{-1}.

III Tension experiments

Stripped tissues were attached at one end to a stainless steel hook and at the other to a force transducer, by silk thread. They were placed in Magnus isolated organ baths of 20 mls volume, at a constant temperature of 37°C and tension of 0.5-1 g. Four tissues could be suspended at any one time. Dose-response curves to ACh and KCl were constructed by washing out each dose. A three minute time cycle with one minute drug contact time, was used. A random dosage schedule was used in a restricted latin square design, and responses to each dose meaned. When the effects of a spasmolytic drug were investigated, tissues, after the initial dose-response relationship had been established, were continuously exposed to the spasmolytic. Measurements were made when responses to stimulant had stabilized. When effects on constant doses of ACh and KCl were studied, tissues were stimulated at regular intervals (usually every 3 minutes) either by only one of these stimulants or by alternating doses. Doses were used which gave rise to an 80% of maximal to just-maximal response. After application of the spasmolytic, the relationship to the maximal response was determined by occasional application of two times the constant dose. For calcium dose response curves in Ca-free Potassium chloride Krebs, cumulative curves of the type used by Van Rossum and Ariëns (1959) were used. One hour elapsed between successive curves and tissues were washed every 15 minutes during this time. The tone in tissues decreased gradually throughout exposure to

Ca-free KCl Krebs solution and tension was returned to approximately 0.2 g at each wash. Calcium dose-response relationships were found to be quantitatively repeatable for some uteri, in which case three control curves to cumulative concentrations of calcium (contact time three minutes for each dose) were constructed prior to drug administration; the effect of the drug was compared to the mean of the control curves. For some uteri, the Ca dose-response relationship showed a progressive deterioration, in which case dose-response curves of treated tissues were corrected for change in sensitivity of a concurrent control tissue.

When tissues were exposed to Lithium Krebs Solution, 30 minutes elapsed between successive exposures, and sensitivity to ACh and KCl was monitored during the elapsed time.

IIJ Measurement of ^{140}La uptake of isolated subcellular fractions of uterine muscle.

For each experiment, stripped uteri from 8 oestrogenised, non-pregnant rats were equilibrated with Krebs solution, and then exposed to Krebs solution containing 5 μM ^{140}La (2 μC/ml), for 1 hour at 37°C. Tissues were then divided into two equal portions and one portion placed in 0.25M sucrose; the other was placed in 0.25M sucrose containing 10 μM non-radioactive lanthanum. Tissues were then homogenised and the plasma membrane and mitochondrial fractions prepared as described by Kidwai, Radcliffe and Daniel (1971). After collection of fractions each of 1 ml volume, 0.5 ml of each was transferred to

counting vials and counted as described previously for ^{140}La. The remainder was used for estimation of protein, as described by Kidwai *et al* (1971). Uptake of ^{140}La was expressed in terms of μM La/g protein.

III. RESULTS

III RESULTS

IIIA Parameters of the longitudinal muscle of the pregnant rat myometrium

1. Morphology

Microscopic examination of sections of the stripped preparation of the pregnant rat uterus revealed that the longitudinal muscle layer could be separated from the circular muscle and endometrial layers, and that this could be done without gross morphological damage. Figure 2 shows sections of whole and routinely stripped pregnant rat uteri; it can be seen that the stripped preparation consisted entirely of longitudinal muscle. When a large number of portions of uterus had to be stripped in a short period of time, it was possible for small amounts of circular muscle to be present in the preparation; but in any case, no endometrium was ever present. The thickness of the longitudinal muscle layer was measured from 25 micrographs of whole and stripped tissues. Whole uterine segments were in both the relaxed (Figure 2c) and 'stretched' (Figure 2a) states, while the stripped tissues were apparently stretched during fixation (Figure 2b and d). The mean thickness of the stripped preparation was 34.5 μ (range 25-50 μ) while the thickness of the longitudinal layer was 52 μ (range 35-70 μ) in sections such as in Figure 2a and 73.5 μ (range 55-100 μ) in uteri which were clearly not stretched. A reasonable maximum thickness of the preparation from this data would be 75 μ. The preparation was from 9 to 12 cells thick which

Figure 2. Longitudinal sections of pregnant rat myometrium. A, longitudinal muscle layer of whole uterus. B and D, longitudinal muscle stripped away from circular muscle and endometrium. C, low power view of whole uterus in relaxed state. Calibration refers to smallest division of that scale. Right hand for A, B and D. Left hand for C. (H & E: A, B and D x 160; C x 40)

implies a cell width of about 6-7 μ. The stripped preparation was found to comprise 42.9% (±1.1%, n=10) of the whole uterus.

2. Mechanical and Electrical Activity

After removal from the rat and stripping, the longitudinal muscle, when placed in Krebs solution at 37°C, gradually increased in tone and spontaneous contractions commenced usually within 10 minutes. The tension generated by spontaneous contractions gradually increased for a period of about 15 minutes, after which spontaneous contractions (of 0.5 to 4 g isometric tension for tissues approximately 15 mm x 10 mm x 0.1 mm) stabilized at a frequency ranging from 1/minute to one every 10 minutes. Sometimes, and more frequently in stripped tissues, spontaneous contractions were uncoordinated (Figure 3B), but usually they consisted of a smooth and coordinated increase in tension.

The mean resting membrane potential of all penetrations which satisfied the criteria for successful impalement from untreated whole and stripped uteri at 17-21 days after conception was 46.4 ± 0.3 mV negative inside (n=323). When potentials of less than 39 mV are eliminated, the total mean is 47.6 ± 0.3 mV (n=291). A number of variables can be discerned. The mean membrane potential for stripped tissues was 48.3 ± 1.2 mV (n=18) for 17-18 day pregnant uteri and 43.8 ± 1.0 mV (n=24) for 20-21 day pregnant uteri. For whole tissues, membrane potential was 50.5 ± 1.0 mV (n=37) for 17-18 day pregnant uteri and 48.7 ± 1.2 mV (n=24) for 20-21 day pregnant uteri. The difference between all membrane potential measurements for stripped

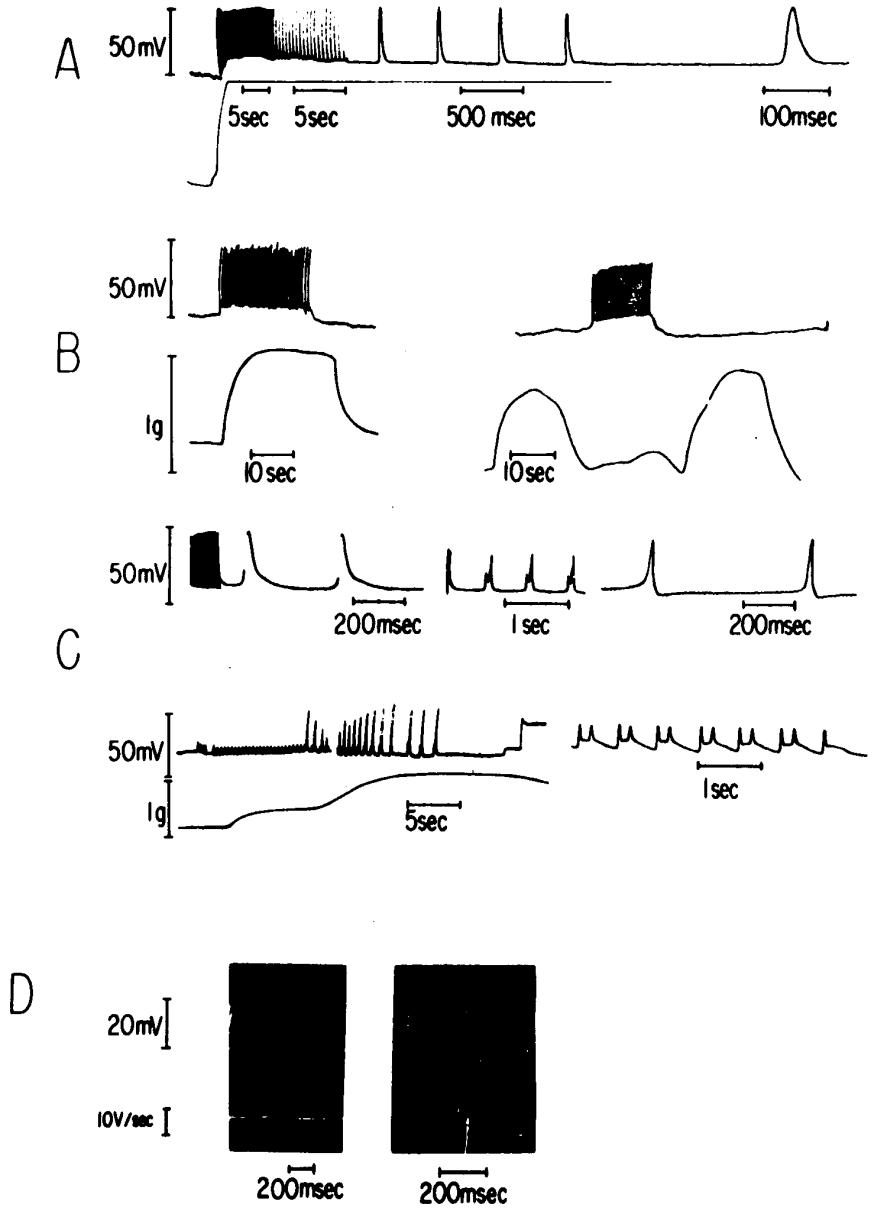

Figure 3. Spontaneous activity of pregnant rat myometrium. Isometric tension is shown as lower recording in A, B and part of C. A; whole uterus, action potentials recorded at different sweep speeds; B, C and D from stripped uteri. Note uncoordinated contractile activity in B, and action potentials recorded when only small amount of tension was generated. C and D show different forms of spikes, including double spikes and electrotonic potentials.

and whole tissues is significant ($p<0.001$) and also between 17-18 day and 20-21 day stripped tissues ($p<0.01$). Variation between stripped, whole uteri and differing lengths of pregnancy for other parameters were not significant, being less than the variation within any one group. Whole tissues were used in approximately one half of the membrane potential experiments because of greater ease of mounting the tissue and of penetrating cells with microelectrodes.

When tris was substituted for the bicarbonate in the Krebs solution, great difficulty was encountered in successfully penetrating cells. Furthermore, although the membrane potential did not change significantly when measured for 15 minutes after commencement of superfusion with Tris Krebs solution, after a period of greater than about 3 hours, the membrane potential was reduced to 37.8 ± 1.3 mV ($n=14$), and spontaneous and drug-induced contractions were reduced in amplitude. For this reason, all experiments in 26mM tris were completed in less than 3 hours, and later, the concentration of tris was reduced to 4.2mM, when irreversible changes did not occur.

Contractions of myometrium were accompanied by action potentials which could be reproduced and recorded on the Beckmann pen recorder (Figure 3D, 4B). The first action potential of trains of spikes had a mean amplitude of 49.1 ± 0.9 mV ($n=58$) compared to a mean membrane potential of 45.9 ± 0.7 mV observed at the same time; this is significant when compared using a paired t-test ($p<0.01$). For 17-18 day pregnant uteri, the mean difference of first action potential of a train to membrane potential (3.7 ± 1.2 mV, $n=27$) is

significant at the 5% level ($p=0.02$); for 20-21 day pregnant uteri, the mean overshoot of 6.3 ± 0.3 mV ($n=25$) is also significant ($p<0.001$). Overshoot potential did not differ significantly between stripped and whole tissues. The mean amplitude of action potentials, other than the first, was 44.7 ± 1.16 ($n=20$) which is not significantly different from the membrane potential. The mean maximum spike frequency for spontaneous trains of action potentials was 152 ± 5.7 per minute. Trains of action potentials were sometimes superimposed upon a depolarization of a few mV. Rarely, the potential hyperpolarized after a train of spikes (Figure 3). The action potential lasted usually 70-100 msec. The shape of action potentials recorded from the myometrium was often highly variable (see Figure 3C and D, Figure 4 and Figure 5). In general, three types of spikes could be differentiated from the 'normal' action potential: a) pacemaker-type, with a slow depolarization between action potentials, and a fast repolarization and, usually, an undershoot; type a is seen coincident with the central portion of a contraction; b) spikes with a slowed repolarization; these consisted of spikes with a fast initial repolarization but delayed positive potential of 3 to 15 mV; c) spikes with two distinct rising phases (see Figure 3C). Types b) and c) were only observed at slow rates of action potential discharge, usually towards the latter phase of a spike train. Thus to some extent, spike shape can be considered to have a temporal relationship within the train of spikes. Spikes with more than two rising phases were rarely observed, as were spikes with delayed depolarization.

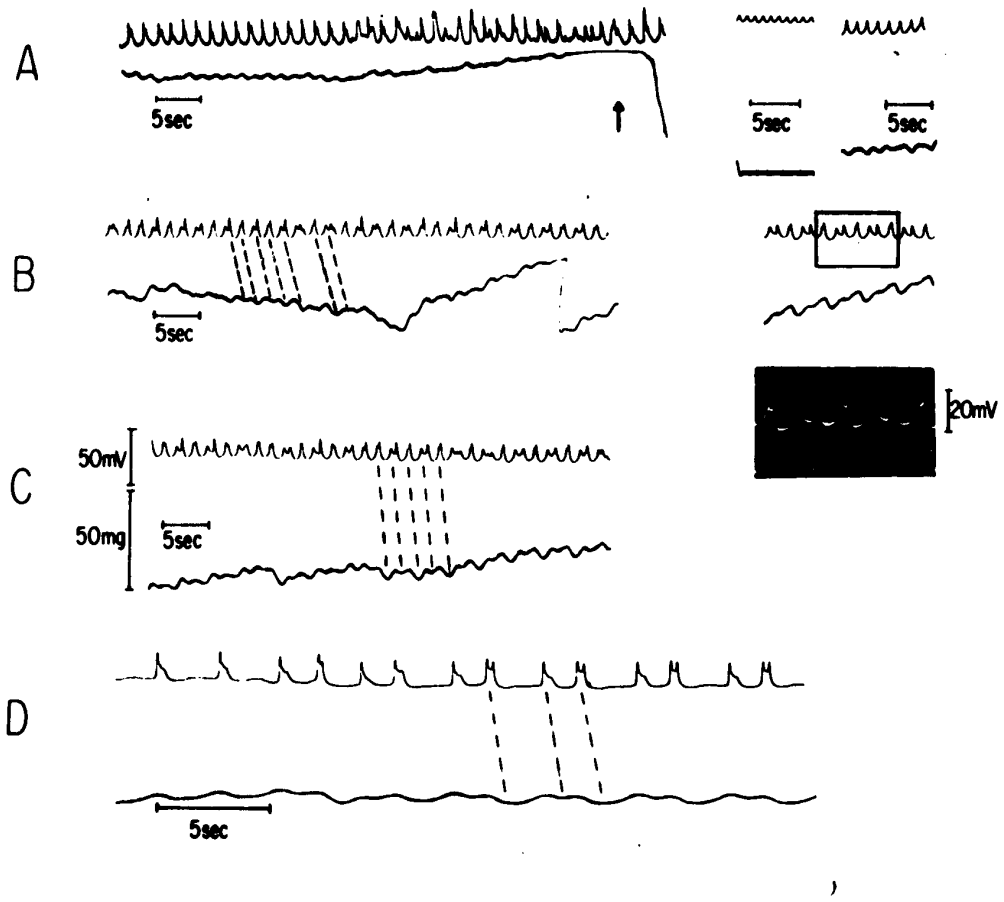

Figure 4. The relation of action potentials to tension. Bizzarre action potentials from stripped tissues. Upper records are membrane potential and lower records tension, contraction downwards (this figure only). A; addition of 1.4×10^{-6}M acetylcholine at arrow, and 1 and 3 minutes later (tension record has been rezeroed). B; 6 and 6.5 minutes after acetylcholine. C and D; 7 and 9 minutes later.

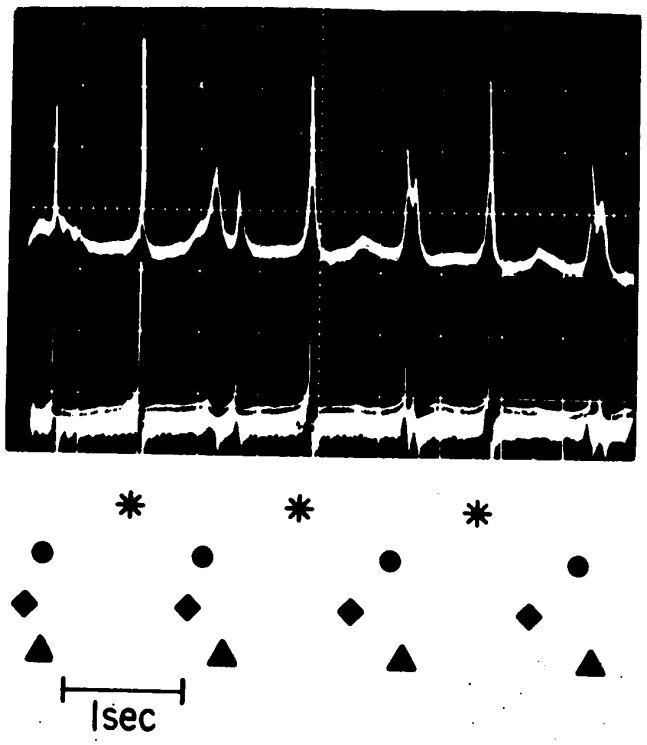

Figure 5. Analysis of complex spikes as asynchronous discharge. Oscilloscope recording of spikes of different form. Analysis reveals that the recording represents the sum of 4 distinct sources of discharge, as indicated by symbols. Interspike intervals are 1.37, 1.32, 1.54 and 1.37 seconds for respective spike sources represented by upper to lower symbols. Vertical division is 10 mV for (upper) membrane potential record and 1 v/sec for (lower) differentiated potential record.

Electrotonic potentials of a few mV could be recorded during contractions (Figure 3C; see also Figure 17). Under normal recording conditions, it was not possible to demonstrate a phasic tension response to each action potential as is frequently illustrated for taenia coli. However, when a highly sensitive mechano-electronic transducer (5734 RCA) was used it was possible, as shown in Figure 4, to show that each spike elicits a small but finite increase in tension. In the experiment shown in Figure 4, particularly bizzarre action potentials were observed. Not only did the cell penetrated show a relationship of spikes to tension, but also a clear relationship between spike shape and amplitude of the tension response can be seen. Action potentials having two rising phases gave rise to a larger tension response than other types of action potential.

3. Ion Content of Longitudinal Muscle

Table I shows the ion content of the longitudinal muscle of rat myometrium, both fresh and after incubation in Bicarbonate Krebs and Tris Krebs. Fresh tissues which had not been moistened with Krebs solution had a higher potassium and lower sodium content than moistened tissues. Although these tissues had visibly dried during dissection, the values were also significantly different when expressed as mmoles/kg dry weight (369 ± 18.5 and 364 ± 20 mmoles/kg, n=12, for sodium and potassium respectively for unrinsed tissues, and 443 ± 15 and 305 ± 10.5 mmoles/kg, n=30, for sodium and potassium respectively for rinsed tissues). Chloride content of fresh tissues was

TABLE I

Ion Content of Longitudinal Muscle of Pregnant Rat Myometrium (mmoles/kg wet wt)

Treatment	$[Na]_t$	$[K]_t$	$[Ca]_t$	$[Na]_i^{\dagger}$	$[K]_i^{\dagger}$	H_2O mls/kg
Fresh*	84.5	74.4	--	47.6	123.0	749
	3.6 (15)**	4.2				5.1
Fresh***	88.2	64.2	4.15	53.9	105.4	788
	2.8 (30)	2.3	0.37 (16)			5.3
2 hrs Bicarbonate Krebs	97.7	50.6	--	70.0	82.5	798
	3.4 (6)	2.1				8.6
4 hrs Bicarbonate Krebs	91.3	50.8	--	59.5	82.8	--
	6.3 (6)	5.3				
6 hrs Bicarbonate Krebs	100.4	38.9	--	74.6	62.7	816.0
	4.8 (18)	1.5				6.2
2 hrs Tris Krebs 26mM	139.2	36.4	--	152	58.5	834
	5.0 (8)	2.0				6.2
2 hrs Tris Krebs 4.2mM	114.5	40.4	4.3	98.5	65.3	829
	4.3 (8)	2.4	0.35			5.8

†calculated from inulin space of 410 mls/kg
*without contact with Krebs solution
**standard error of mean; number of observations in parentheses
***kept moist in Krebs solution

102.6 ± 3.7 mmoles/kg (n=12). Over a period of hours, the tissues gain sodium and lose potassium, and this occurs much more rapidly in solutions buffered with tris. Values given for calcium were obtained from wet ashed tissues; contamination of crucibles as indicated by comparison of radioactive uptake of calcium and total calcium, made very high values often obtained after dry ashing of dubious validity. Calcium content obtained for fresh non-pregnant uteri was 3.22 ± 0.2 mmoles/kg wet weight (n=8). Potassium equilibrium potential was 87 mV for the highest value of $[K]_i$ and 67 mV for the lowest value. Even the latter value is higher than the membrane potential recorded after 6 hours in Krebs solution (41.3 ± 1.2 mV n=15).

The uptake of inulin and mannitol by longitudinal muscle of the myometrium are shown in Figure 6. Values for the inulin space were not significantly different at 60 and 120 minutes and the intercept of the values at these times with the ordinate was 410 mls/kg. Mannitol was taken up rapidly at first but the mannitol space did not become constant and reached a value of 660 ± 32.5 mls/kg at 120 minutes.

4. Uptake of ^{42}K

The uptake of ^{42}K by longitudinal muscle at 25°C followed an exponential process which is shown in Figure 7. Mean values of the fraction of potassium exchanged (f) were 0.234, 0.462 and 0.869 at times 10, 30 and 60 minutes, after correction for concommittant efflux according to the method of Keynes (1954). Curves could be

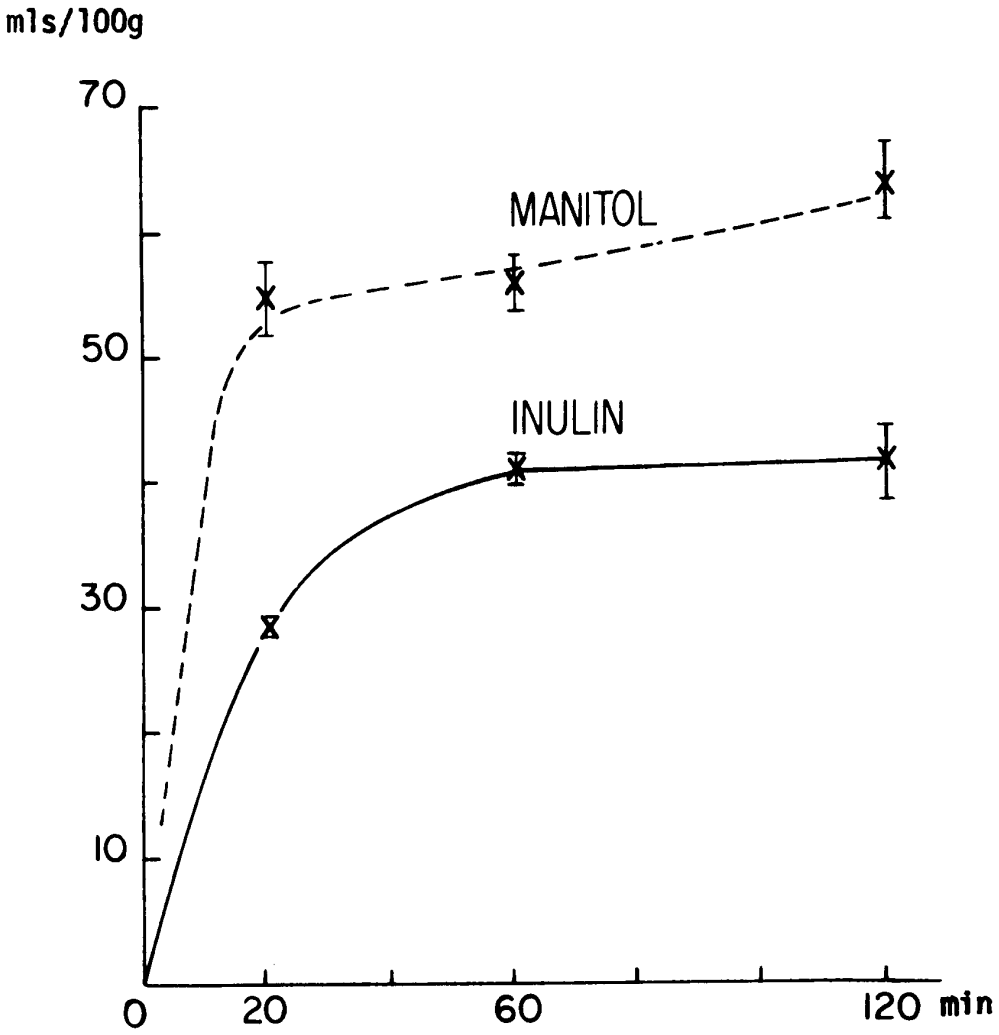

Figure 6. The uptake of ^{14}C-inulin and ^{14}C-mannitol by the longitudinal muscle of the pregnant rat myometrium. Ordinate: uptake expressed as mls/100 g tissue. Abscissa: time of exposure to radiotracer in minutes. Each point is the mean of 5 tissues and vertical bars are twice the standard error.

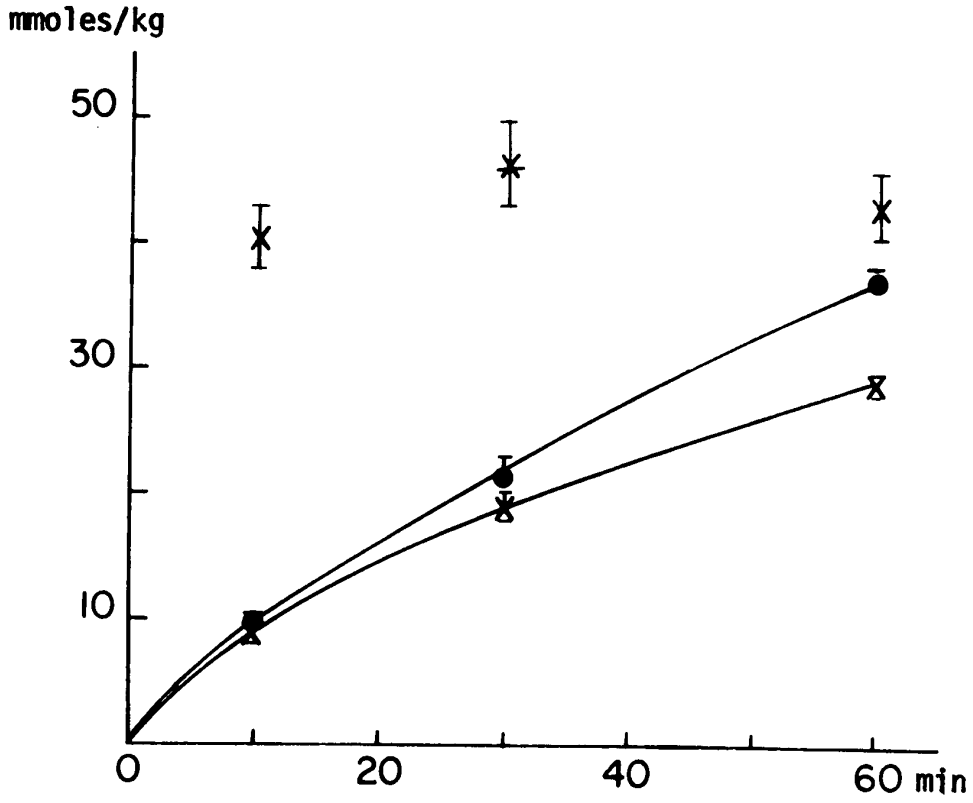

Figure 7. Uptake of ^{42}K by longitudinal muscle of pregnant rat myometrium (lower curve). Upper curve, uptake corrected for concommitant efflux using value of k_3 for efflux measured independently (see Table II). Curves have not been corrected for extracellular uptake. Total potassium is shown for each time. Ordinate: mmoles/kg ^{42}K uptake obtained by dividing counts $min^{-1}kg^{-1}$ by counts min^{-1} mM^{-1} of the medium. Abscissa: time in minutes in radioactive solution.

fitted by the equation $Yt = A(1 - e^{-k_1 t}) + B(1 - e^{-k_2 t})$, to yield values of k_1 and k_2 by trial and error after finding a value for A from the intercept at time zero and B by subtraction from total potassium. (Since the first time of observations was 10 minutes, A in this case represents a fraction with t 1/2 of 4-10 minutes and B a fraction with t 1/2 of 90-155 minutes. A and B correspond to fractions B and C in efflux analysis, *vide infra).* However, in view of the small number of observation times, the number of variables involved and the fact that the computer could generate a large number of curves with different values of k_2, which fitted the observed influx closely, this seemed a somewhat pointless exercise. Values of k_2 from a plot of ln (1-*f*) against time were from 0.01 to 0.042 for the slow fraction.

5. Efflux of ^{42}K and ^{86}Rb

Figures 8 and 9 show efflux curves obtained for the loss of ^{42}K and ^{86}Rb from the longitudinal muscle of the pregnant rat myometrium in Bicarbonate Krebs solution. In nearly all cases it was necessary to subtract a small but significant bound fraction of potassium from the counts per minute remaining in the tissue, to obtain complete parallelism with the rate of loss of counts per minute per minute curve. Analysis of the curves by the peeling technique is shown in Figure 8. This operation was routinely performed objectively using an APL program on an IBM 360 computer (see Methods IIII). Mean values obtained are shown in Table II. ^{86}Rb was added to Krebs solution which contained 4.6mM potassium chloride, on the

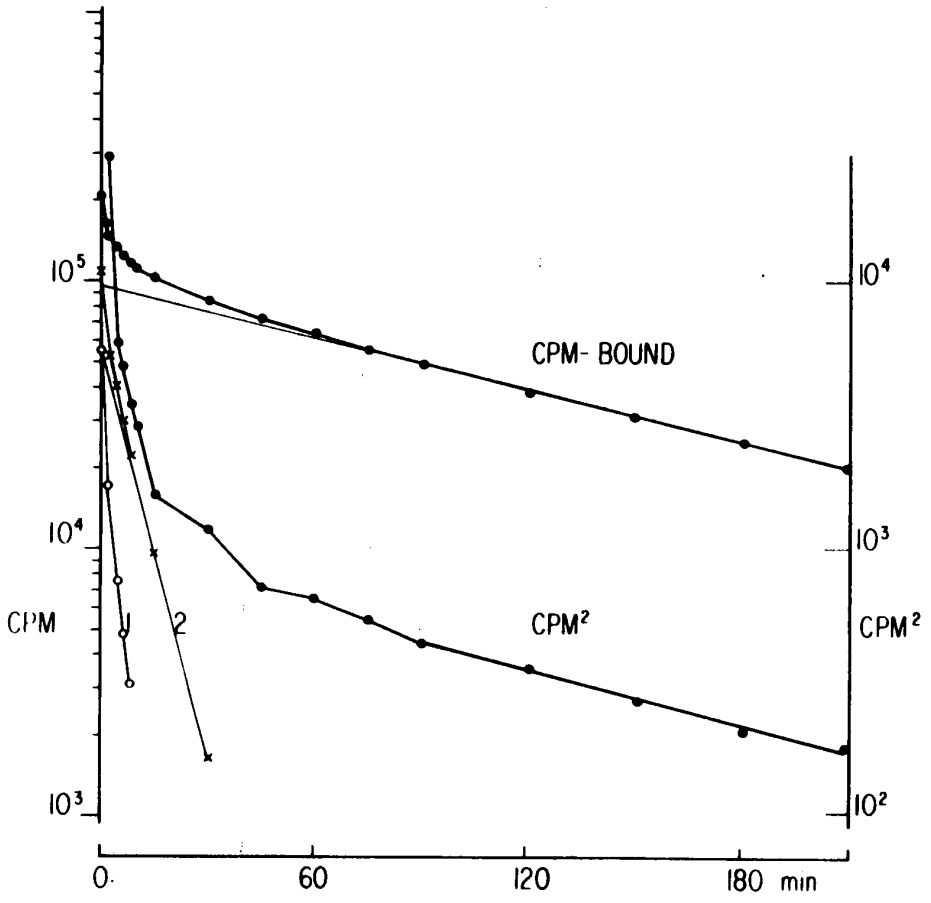

Figure 8. Efflux of ^{42}K from longitudinal muscle of pregnant rat myometrium. Original curve (not shown) after subtraction of a small amount of bound ^{42}K, is parallel to rate curve (cpm^2). Curve-peeling by extrapolation backwards of final phase of efflux, subtraction from cpm-bound curve to yield curve 2 and the same procedure to yield curve 1 is illustrated. Ordinate: counts min^{-1} remaining in tissue at left and rate of efflux (counts min^{-2}) at right. Abscissa: time after removal from radioactive solution.

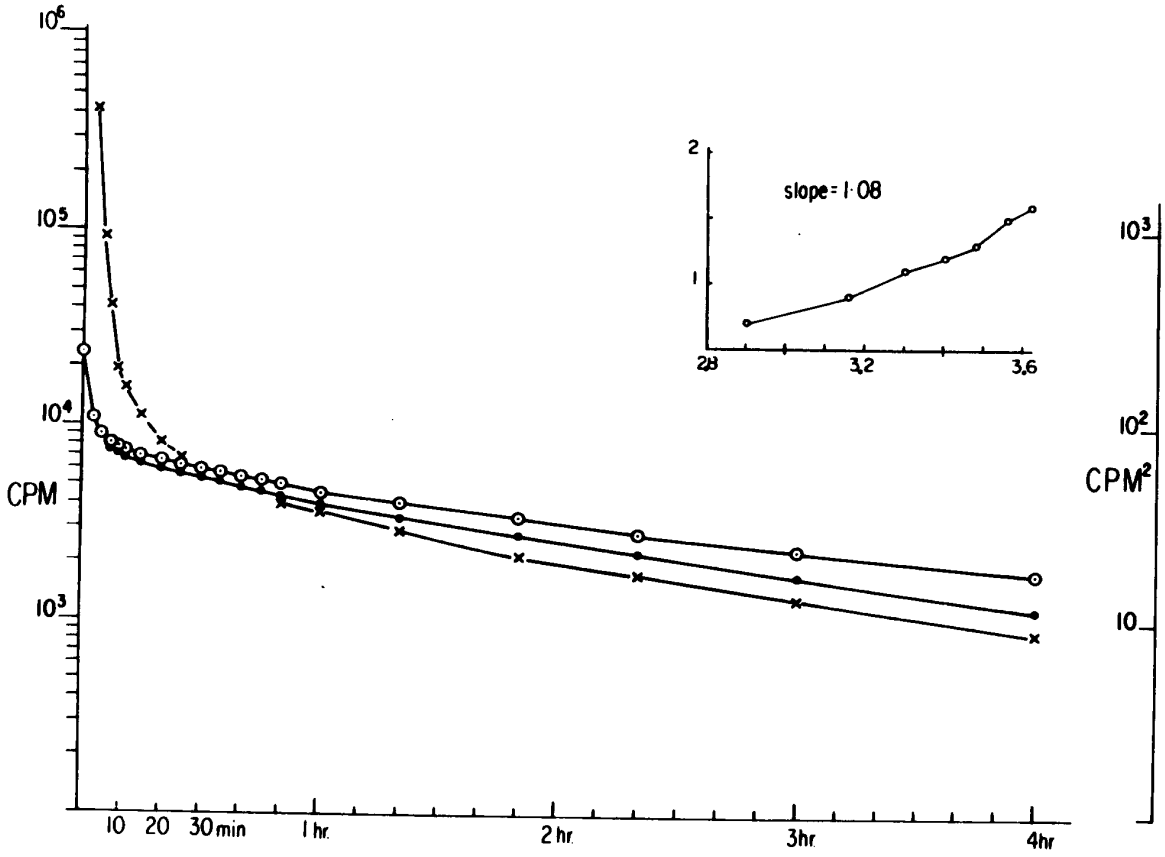

Figure 9. Efflux of ^{86}Rb from longitudinal muscle of pregnant rat myometrium. Original efflux curve o-o, original curve after subtraction of the bound fraction ·-·, and rate curve (counts min^{-2}) x-x. Inset shows curve of log counts min^{-2} (ordinate) against log counts min^{-1} (abscissa). Ordinate: counts min^{-1} remaining in the tissue at left, and counts min^{-2} at right. Abscissa: time after removal from radioactive solution.

TABLE II

Analysis of ^{42}K and ^{86}Rb from the Longitudinal Muscle of the Pregnant Rat Myometrium

		A			B			C			D	Y_t
Isotope	n	%	k_1	t 1/2	%	k_2	t 1/2	%	k_3	t 1/2	mm/kg	
^{42}K	(6)	32.8	0.49	1.7	21.3	0.084	9.0	45.7	0.0087	81.4	1.68	36.0
		5.2	0.10	0.20**	7.0	0.02	1.3	5.1	0 0009	4.6	0.22	
^{86}Rb	(8)	30.4	0.57	1.4	20.3	0.062	12.1	49.3	0.0057	124.6	1.31	--
		7.0	0.06	0.14	3.4	0.005	1.06	5.2	0.0004	9.3	0.6	

*percent of exchangeable potassium
**standard error of mean (as in the case of all subsequent tables)

premise that the system would not be able to distinguish between potassium and rubidium. Since the half-time for loss of rubidium from the myometrium is increased compared to that of ^{42}K, the meaning of the sizes of the fractions for ^{86}Rb and their comparability to values from analysis of ^{42}K efflux curves is questionable. The slope of the log counts min^{-1} against log counts min^{-2} of ^{42}K or ^{86}Rb was 1.0 for the final compartment (range 0.98-1.12) indicating efflux from a single homogenous compartment.

6. ^{45}Ca and ^{89}Sr

In spite of a gain in total calcium in stripped tissues, values for total calcium were reproducible within uteri as determined by the use of radiotracer and values from tissues which had been wet ashed. The uptake of ^{45}Ca and ^{89}Sr (in Strontium Krebs solution) by the pregnant rat uterine longitudinal muscle is shown in Table III. Strontium is taken up at the same rate as is calcium. Since facilities for measurement of total strontium were not available, and values for the specific activity of calcium indicated that total calcium values obtained from dry ashed tissues were sometimes unreliable, specific activities are not included.

The loss of ^{45}Ca and ^{89}Sr from longitudinal muscle incubated for 2 1/2 hours in respective isotope is shown in Figure 10. Tissues for ^{45}Ca efflux were rinsed and put into successive solutions of either Ca-free or normal (2.47mM calcium) Tris Krebs. For ^{89}Sr efflux, tissues were exposed to Ca-free Krebs for one hour, and then loaded in Ca-free

TABLE III

Uptake of ^{45}Ca and ^{89}Sr by Longitudinal Muscle of Pregnant Rat Myometrium (mmoles/kg wet wt)

Isotope	n	Time (mins) 10	20	60
^{45}Ca	8	2.42	2.91	3.56
		0.17*	0.32	0.28
^{89}Sr	4	2.50	3.23	3.52
		0.19	0.09	0.18

*standard error

Krebs containing 2.47mM strontium and ^{89}Sr. Washout of ^{89}Sr was followed into either Ca-free Krebs or into Ca-free Krebs containing 2.47mM strontium. Values in Figure 10 have been plotted as percentage of original amount of radioactivity in the tissue, in order to compare the curves. Bound fraction has not been subtracted from the curves. Difficulty was encountered when it was attempted to analyse the efflux curves compartmentally. The standard error for representation as the sum of three exponentials was less than that for one or two exponential terms. However, in some cases the second compartment had an inconsistent half-time, and/or was of negligible size; the bound fraction was often not consistent when estimated by the three available methods. Analysis indicates that efflux of ^{45}Ca and ^{89}Sr was not from a single homogenous compartment. Thus the slope of the log count min^{-1} against log count min^{-2} curve at the end of efflux

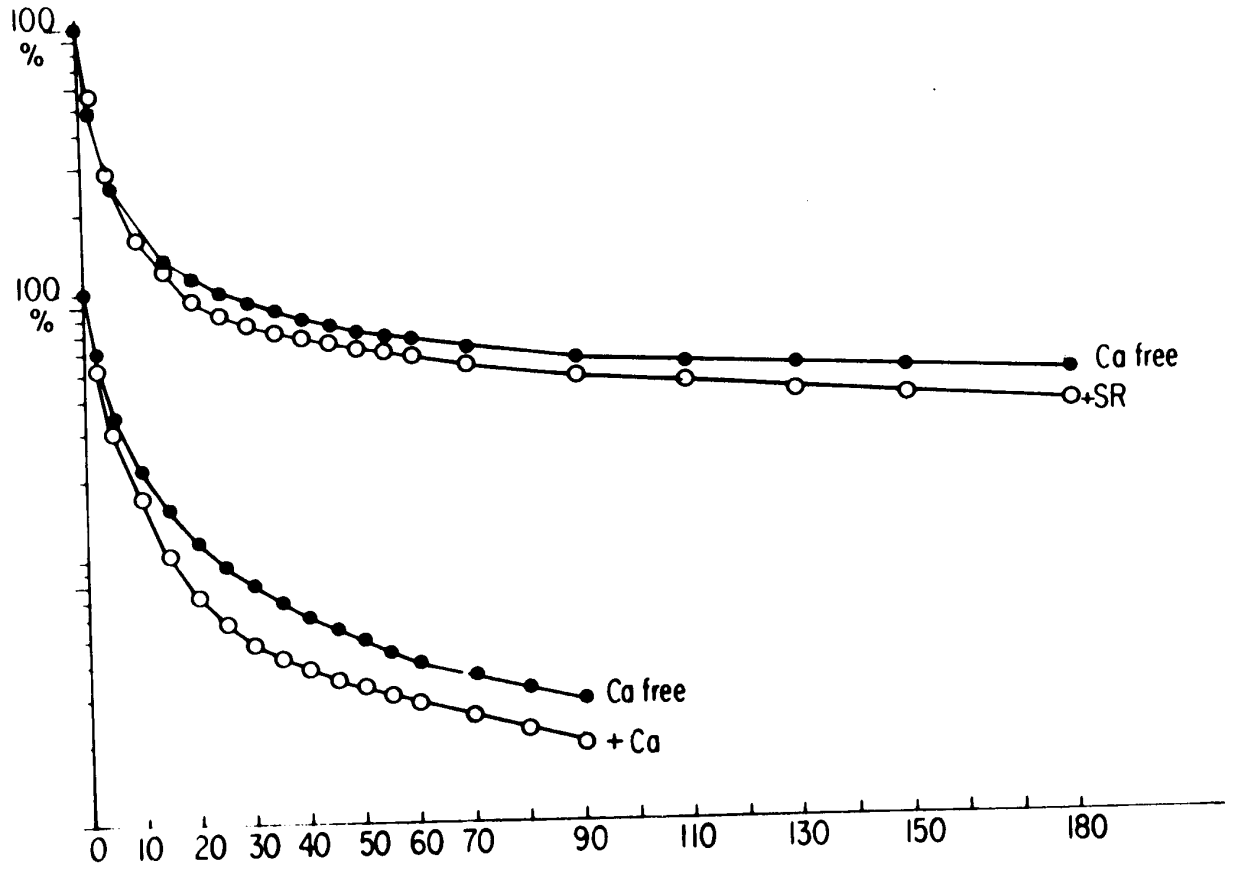

Figure 10. Efflux of ^{45}Ca and ^{89}Sr from the longitudinal muscle of the pregnant rat myometrium. Each curve represents the mean of two tissues. Bound fraction has not been subtracted from curves. Upper 2 curves are ^{89}Sr efflux and lower 2 curves are ^{45}Ca efflux. Filled circles are efflux into Ca-free Solution. Open circles are efflux into Krebs solution containing either 2.47mM calcium (^{45}Ca) or 2.47 mM strontium (^{89}Sr). Ordinate: counts min^{-1} remaining in the tissue as percentage of counts at zero time. Abscissa: time in minutes after commencement of efflux. Note that ordinate has been moved downward for ^{45}Ca efflux.

was not one, even after subtraction of a bound fraction (slopes before subtraction were least for ^{45}Ca effluxing into calcium-containing Krebs solution, being 1.81-2.02; for ^{45}Ca effluxing into Ca-free solution and ^{89}Sr effluxes, the slope was between 4 and 6. After subtraction of the bound fraction, the slope for ^{45}Ca and ^{89}Sr was 1.18-1.83). Values for rate constants of ^{45}Ca and ^{89}Sr efflux are given in Table IV. Owing to the uncertainty as to the applicability of curve peeling to these curves, these values must be considered only as a means of quantitatively comparing the curves. From these values, it can be seen that the presence of calcium (for ^{45}Ca) or strontium (for ^{89}Sr) increased the rate of loss of respective tracer. This can be readily seen from Figure 10 for calcium efflux, but is not obvious from the curves of ^{89}Sr efflux.

In any case, there is little difference between the curves for ^{45}Ca and ^{89}Sr for early efflux (0-15 minutes). Late efflux is not strictly comparable, since efflux was continued for different times for the two radioisotopes. However, the cut-off point for the slower compartment was 30 minutes for both ^{45}Ca and ^{89}Sr (i.e. a least squares plot was constructed on all points after 30 minutes). Furthermore, the continued faster rate of efflux of calcium is confirmed by data from two tissues for which ^{45}Ca efflux was continued to 4 hours into Ca-free solution (not shown in Figure 10). (Rate constants of 0.0073 and 0.0081 min^{-1} for the slower fraction). Thus, late efflux does appear to be faster for calcium than for strontium. (Rate constants correspond to half-times of 131 minutes and 51 minutes

TABLE IV

Rate constant for ^{45}Ca and ^{89}Sr efflux from the Longitudinal Muscle of Pregnant Rat Myometrium* (min^{-1})

Isotope	Efflux into	A	B
^{45}Ca	-Ca	0.134 (0.130-0.138)	0.0053 (0.00460-0.00601)
	+Ca	0.179 (0.163-0.195)	0.0134 (0.0122-0.0146)
^{89}Sr	-Sr	0.061 (0.078-0.045)	0.00310 (0.00302-0.00319)
	+Sr	0.161 (0.146-0.176)	0.00250 (0.00210-0.0029)

*Bound fraction has not been subtracted before analysis. Each value is the mean of two tissues, values in parentheses.

for ^{45}Ca efflux into Ca-containing and Ca-free Krebs respectively; 223 and 280 minutes for ^{89}Sr efflux into strontium-containing and Ca-free Krebs respectively). Bound fraction was highly variable but was between 100 and 15 μmoles/kg. Sizes of the two fractions A and B were 1.6-3.0 mmoles/kg and 0.23-0.62 mmoles/kg respectively.

IIIB Responses to Excitatory Drugs and Ions

1. Mechanical and electrical activity

The effects of drugs and ions used in this study which cause contraction of the rat uterus are shown in Figures 11 and 12. Acetylcholine at low doses (2.8×10^{-7}M) did not alter the membrane potential when it was measured between spike trains (42.1 ± 0.9 mV

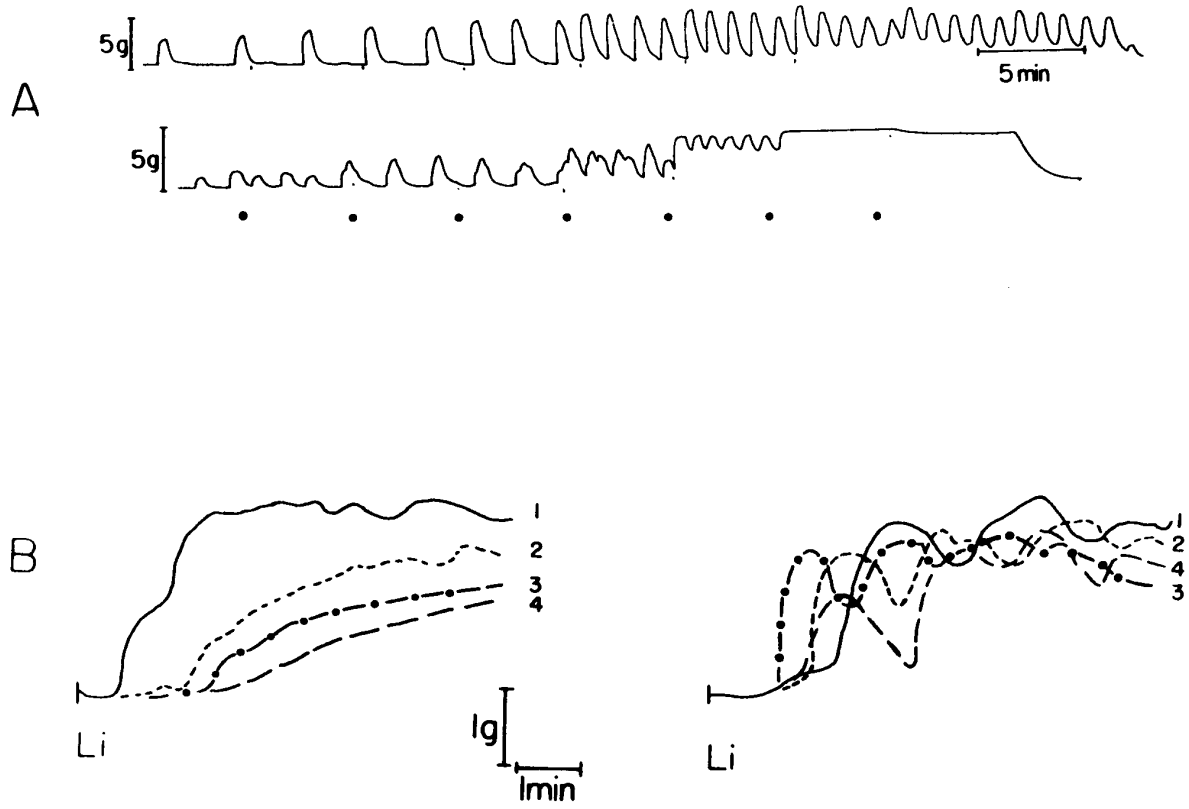

Figure 11. Effects of acetylcholine, potassium chloride and Lithium Krebs on the contractile activity of the longitudinal muscle of the pregnant rat myometrium. A: increasing doses of acetylcholine (upper record) and increasing doses of potassium chloride (lower record) at each filled circle, at 5 minute intervals. Final concentrations are: acetylcholine (M): 7×10^{-8}, 1.4×10^{-7}, 2.8×10^{-7}, 1×10^{-6}, 2.4×10^{-6}, 5.7×10^{-6}, 1.3×10^{-5} and 2.8×10^{-5}. (Last dose coincides with wash for potassium chloride-dosed tissue). Potassium chloride (M): 1×10^{-3}, 2×10^{-3}, 4.5×10^{-3}, 9.5×10^{-3}, 1.95×10^{-2}, 3.95×10^{-2} and 8×10^{-2}. B: Responses to Lithium Krebs Solution. Responses 2, 3 and 4 were obtained 1, 2 and 3 hours after initial responses (1). At left, tissue exposed every 10 minutes and at right, tissue exposed every 30 minutes to Lithium Krebs.

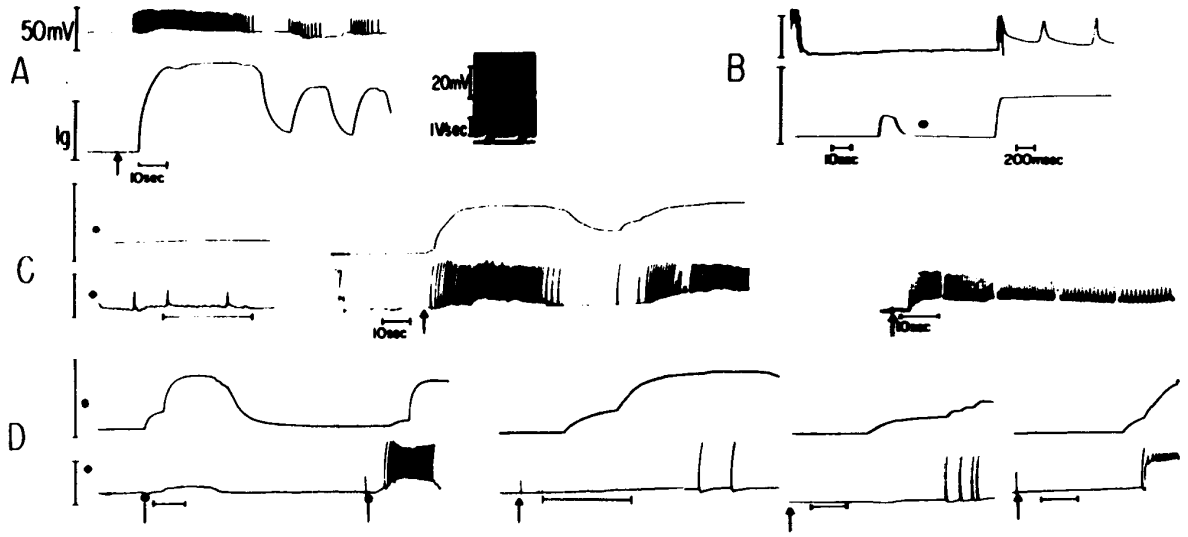

Figure 12. Electrical responses of the longitudinal muscle of the pregnant rat myometrium to excitatory drugs and ions. A: 4.4 x 10^{-6}M acetylcholine at arrow; oscilloscope recording of evoked pacemaker type action potentials shown at right. B: Propranolol (3.9 x 10^{-5}M) Krebs, noradrenaline, 5.9 x 10^{-6}M at filled circle. C: (1 to r) response to 10 mM potassium chloride; response to 20 mM potassium chloride; response to 25 mM potassium chloride, at application and 1, 2, 4 and 5 minutes later. (tension record, not shown, consisted of contracture) D: (1 to r) application of 0.5 mU oxytocin, 10 mM potassium chloride; 0.5 mU oxytocin; 0.5 mU oxytocin and 1 mU oxytocin. Tension is lower record in A and B and upper record in C and D.

n=20 before and 42.3 ± 1.3 mV, n=12 during exposure). Even at high doses, when a depolarisation of 6-7 mV could be observed, acetylcholine never caused a prolonged contraction of the rat uterus at 37°C. As shown in Figure 11, increasing doses of acetylcholine (cumulatively) leads to increased frequency amplitude of spontaneous contractions to a maximum after which there is a slight increase in tone and decreased frequency of contractions. Acetylcholine caused contraction within seconds of application and spike frequency rapidly increased to a maximum. The mean maximum spike frequency in the presence of acetylcholine was 239 ± 10 per minute. Acetylcholine was able to shorten spontaneous action potentials which had a plateau, and often converted them to pacemaker type. Spike frequency gradually declined in the presence of acetylcholine and relaxation usually commenced when spike frequency fell below about 80/min. After the initial fast contraction the period of sustained contraction depended upon dose; for submaximal doses, tissues relaxed almost immediately, while for supramaximal doses, the initial contraction rarely lasted longer than one minute. In the continued presence of acetylcholine, the tissue relaxed either partially or completely and subsequently contracted at a frequency of about one per minute. Cumulative doses of carbachol did not cause contracture, and records for carbachol were not different from Figure 11A.

The effect of elevation of the concentration of potassium chloride in the external medium was similar in many respects to that of acetylcholine. However, depolarisation could be observed (see Figure 12) and the shape of the contraction differed from that due to acetylcholine. At submaximal doses (usually maximum response

was elicited with an elevation of the potassium chloride by 20mM) the tissue relaxed after an initial contraction, but a gradual increase in tone with superimposed 'spontaneous' contractions usually occurred. At doses which were just maximal, the tissue usually exhibited a fast contraction followed by a slight relaxation and then a continued increase in tone (Figure 11; see also Figure 23).

Phenylephrine failed to cause contractions of this preparations (3 different tissues) when applied at doses of 4×10^{-6}M to 2×10^{-3}M, in the presence of propranolol. Noradrenaline (6×10^{-6}M to 6×10^{-4}M) after propranolol, sometimes caused contraction but this was preceded by a delay, or in some cases, relaxation (5 different tissues, at least two of which were at term). Although α-receptors could be demonstrated, dose-response curves could not be established. A constant dose of noradrenaline which caused a contraction at one time did not necessarily do so subsequently; increasing the dose of noradrenaline did not necessarily increase the response, although the response was clearly not maximal.

Oxytocin caused contractions of the rat uterus but in many tissues, relatively high doses (1 mU/ml) were necessary to consistently cause rapid action potential discharge (Figure 12D). The onset of contraction was delayed compared to that due to acetylcholine (by about 30-60 seconds) and time for washout was longer (2-3 minutes).

When lithium was substituted for all sodium except that in the bicarbonate buffer, spikes of normal amplitude and rate of rise could be recorded for over 35 minutes (Figure 13). When tris was

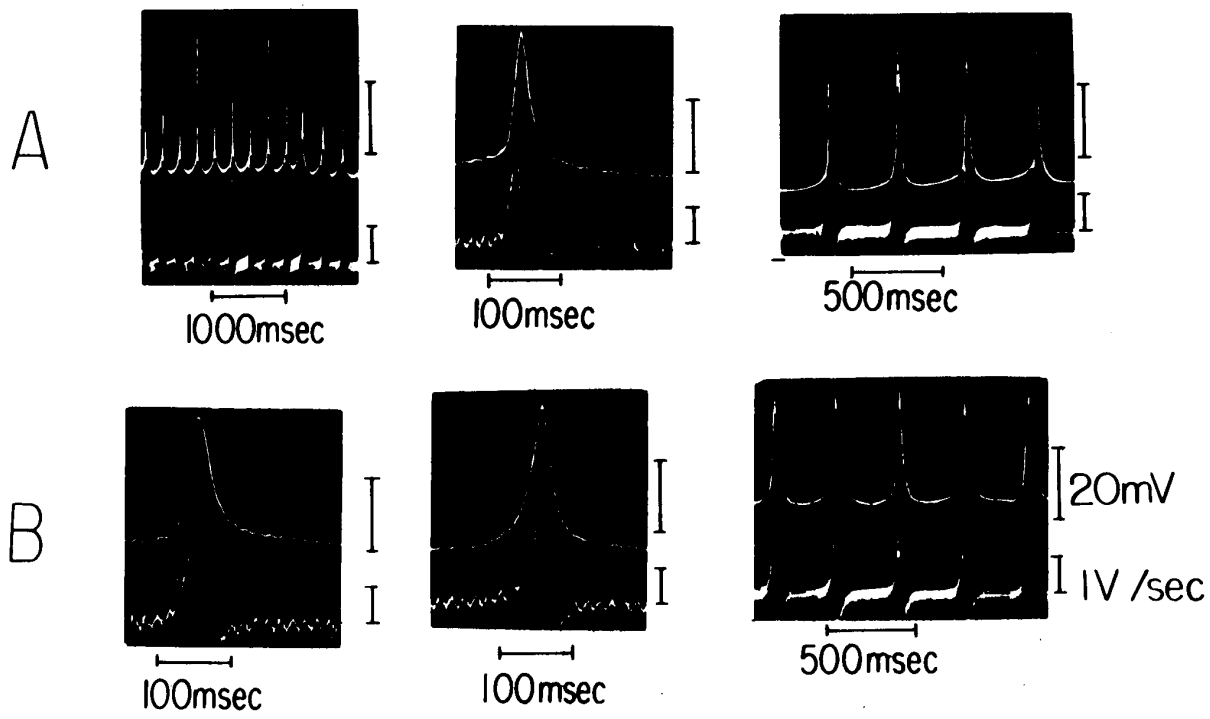

Figure 13. Action potentials recorded in Low Sodium Krebs Solution. A: (1 to r) action potentials recorded after application of 1.4×10^{-6} M acetylcholine, in Normal Bicarbonate Krebs Solution; action potential recorded after 4 minutes in low sodium (18%); after 15 minutes in low sodium. B: (1 to r) action potentials recorded after 20 minutes in low sodium; after 24 minutes; after 30 minutes. Substitution by lithium.

as buffer in Lithium Krebs so that no sodium was present, spikes persisted for longer than 20 minutes (Figures 14 and 15) in a stripped preparation. When the sodium in the medium was removed and lithium or sucrose used as substitute, tissues contracted after a period of up to 2 minutes (Figures 11 and 14). Sucrose, within 10 seconds of the onset of contraction, elicited spikes having a plateau such that action potentials lasted up to 200 msec, especially during the central portion of a train of spikes. Spikes could be recorded in a stripped preparation in sucrose for periods in excess of 20 minutes (Figure 16). Acetylcholine (1.4×10^{-5}M), applied during prolonged action potentials has little effect on the rate of repolarisation. The changes in spike shape in sucrose appear to be irreversible, prolonged action potentials persisting for over 90 minutes after return to normal Krebs solution (Figure 16).

Tissues could be repeatedly stimulated by application of Lithium Krebs, provided that sufficient period was allowed for recovery between contractions. Figure 11 shows the responses of two tissues, one stimulated every 10 minutes and the other every 30 minutes, by Lithium Krebs solution. Contractions gradually declined over a period of 3 hours when lithium was applied more frequently and responses to acetylcholine and potassium chloride declined in a similar fashion between contractions to Lithium Krebs. When lithium (10-40mM) was added hypertonically, tissues contracted but the maximum contraction was about 20% of the maximum to acetylcholine or potassium chloride. Tris applied in a similar manner

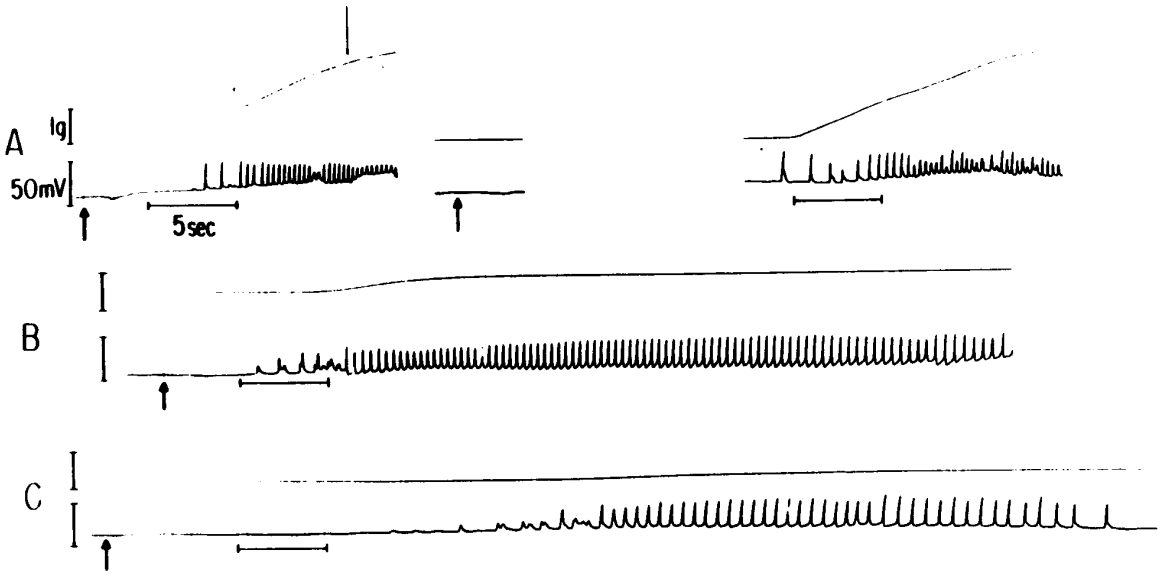

Figure 14. Effect of Lithium Krebs on electrical activity.
A: Response to 5.7 x 10^{-6}M acetylcholine (first arrow); start superfusion with Sodium-free, Lithium Krebs at second arrow. Contraction commenced 2 minutes later.
B: Same tissue as in A, 25 minutes after A and still in Lithium Krebs; addition of 2.8 x 10^{-6}M acetylcholine at arrow. Note that tone is increased in absence of spikes before addition of acetylcholine.
C: superfusion with Normal-Sodium Krebs at arrow, 30 minutes after A.

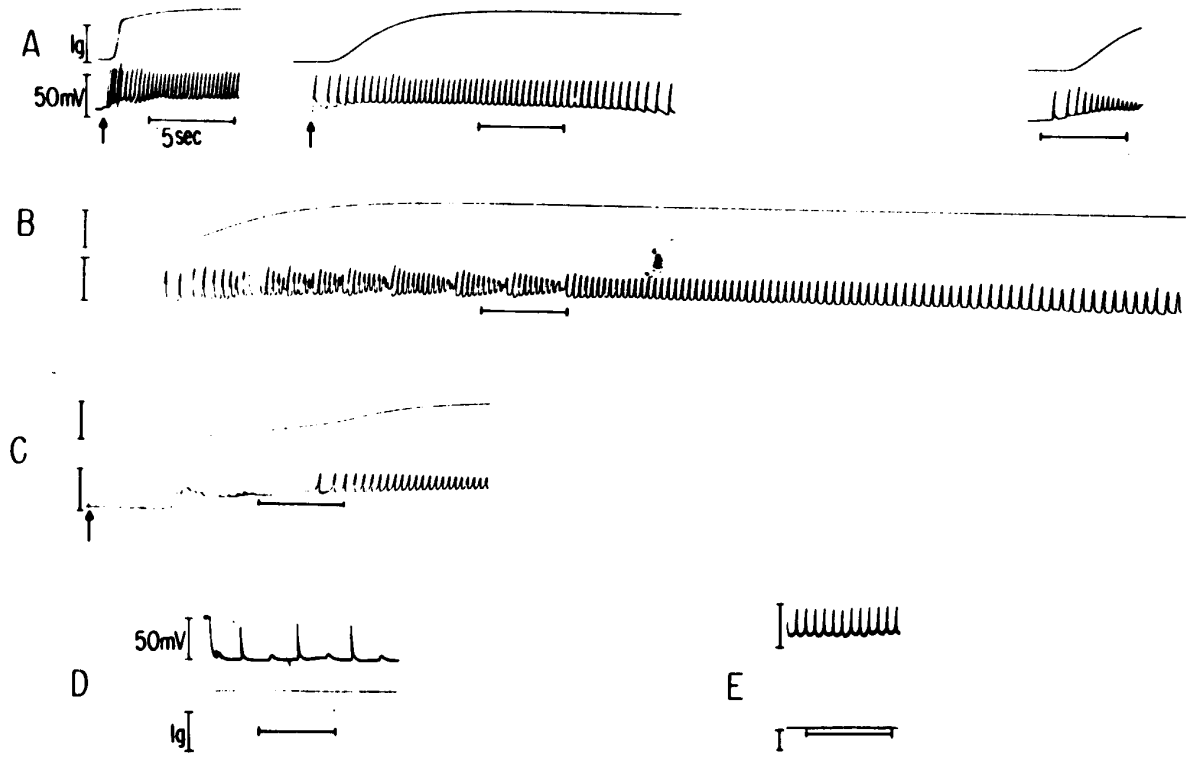

Figure 15. Action potentials recorded from the stripped longitudinal muscle in Lithium Krebs. A: (l to r) responses to acetylcholine (2.8×10^{-5}M), potassium chloride (15 mM) and potassium chloride (20 mM) after 7 minutes in Lithium Krebs. B: Response to barium chloride (5 mM) after 15 minutes in Lithium Krebs. C: responses to acetylcholine (2.8×10^{-5}M) after 20 minutes in Lithium Krebs. D and E from different tissues; spikes recorded 18 and 14 minutes after Lithium respectively. All time base calibrations are 5 seconds.

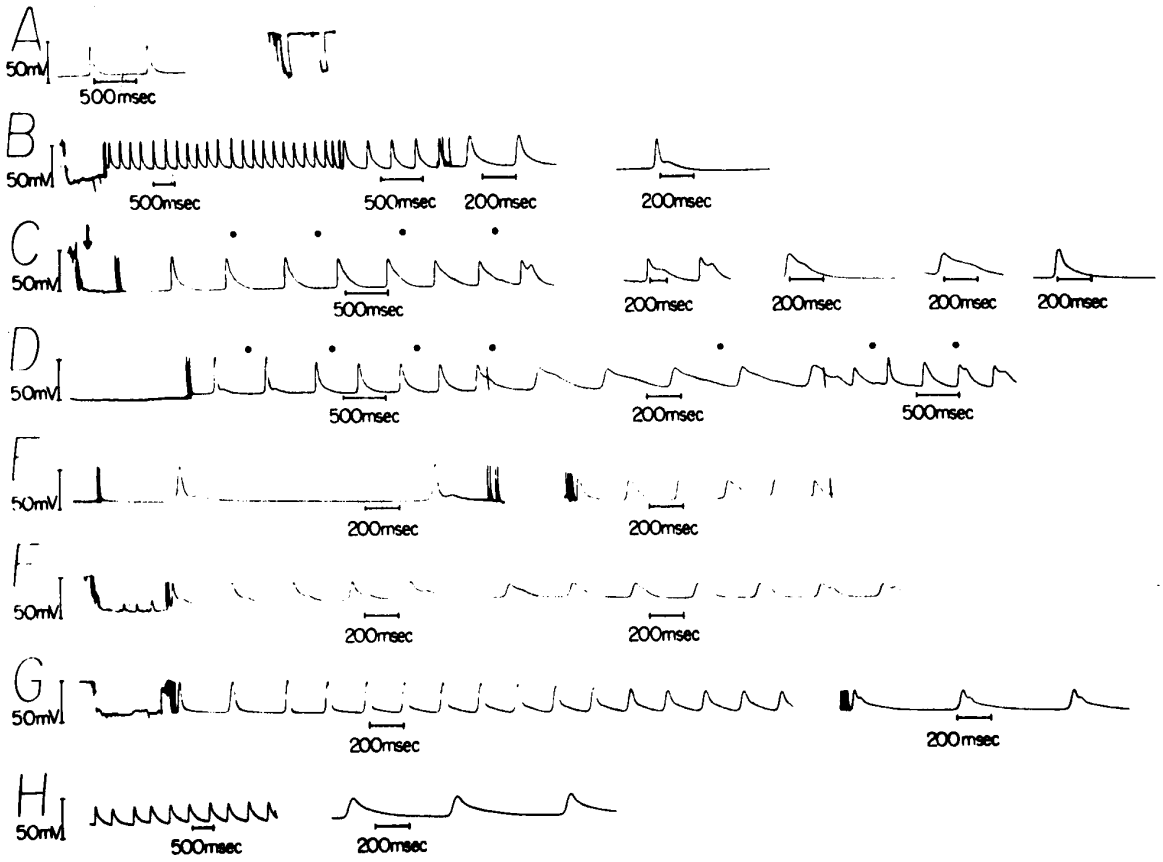

Figure 16. Effect of Sucrose Krebs on action potentials. (1 to r) A: spikes during spontaneous contraction and two penetrations of 4 seconds each. B: spikes evoked by 1.4×10^{-5}M acetylcholine at three sweep speeds, and spike towards the end of the acetylcholine-induced contraction. C: commencement of superfusion with Sucrose Krebs at arrows (time=zero). Contraction and spikes commenced at 6/sec. Time markers at 63 to 66 sec; 2 spikes at 74 sec; spike at 76 and 80 sec, and at 102 sec, the end of first train. D: second train of spikes in Sucrose Krebs, time markers at t=121 to 127 sec. E: 3 minutes and 14 minutes. F: In presence of 1.4×10^{-5}M acetylcholine, at 16 and 19 minutes. G: t=30 min, after wash at t=23. H in presence of 20mM potassium chloride, at 140 minutes.

caused a brief contraction. Glucose (40mM) added hypertonically, did not cause a contraction.

Application of barium ions to the uterus at a dose of 1-5mM caused a prolonged contraction and persistent depolarisation. Action potentials of bizzarre shape (see Figure 17, A and B) were readily observed in the presence of barium. Invariably, the last action potentials of a train induced by barium demonstrated delayed repolarisation and, often, formation of double spikes. Delayed repolarisation was not quantitatively consistent and in some cases only amounted to 2 or 3 mV, as shown in Figure 17C. In the experiment illustrated (Figure 17C), delayed repolarisation of action potentials during a barium-induced contraction appeared just prior to the onset of relaxation. During a second barium-induced contraction, acetylcholine was added when delayed repolarisation could be expected (i.e. was added at commencement of relaxation). Under these conditions, acetylcholine caused a contraction of the previously relaxing tissue; uncoordinated action potentials were coincident with the onset of contraction, superimposed upon a depolarization of a few mV. Spikes reverted to fast repolarization after maximum contraction was achieved. Spikes with delayed repolarization were seen at the end of the acetylcholine-induced train of spikes.

Figure 18 shows the effect of replacement of calcium in the external medium with strontium on action potentials. After exposure to Ca-free Krebs solution for 4 minutes, the tissue was superfused with Ca-free solution containing 2.47mM strontium. After 15 minutes

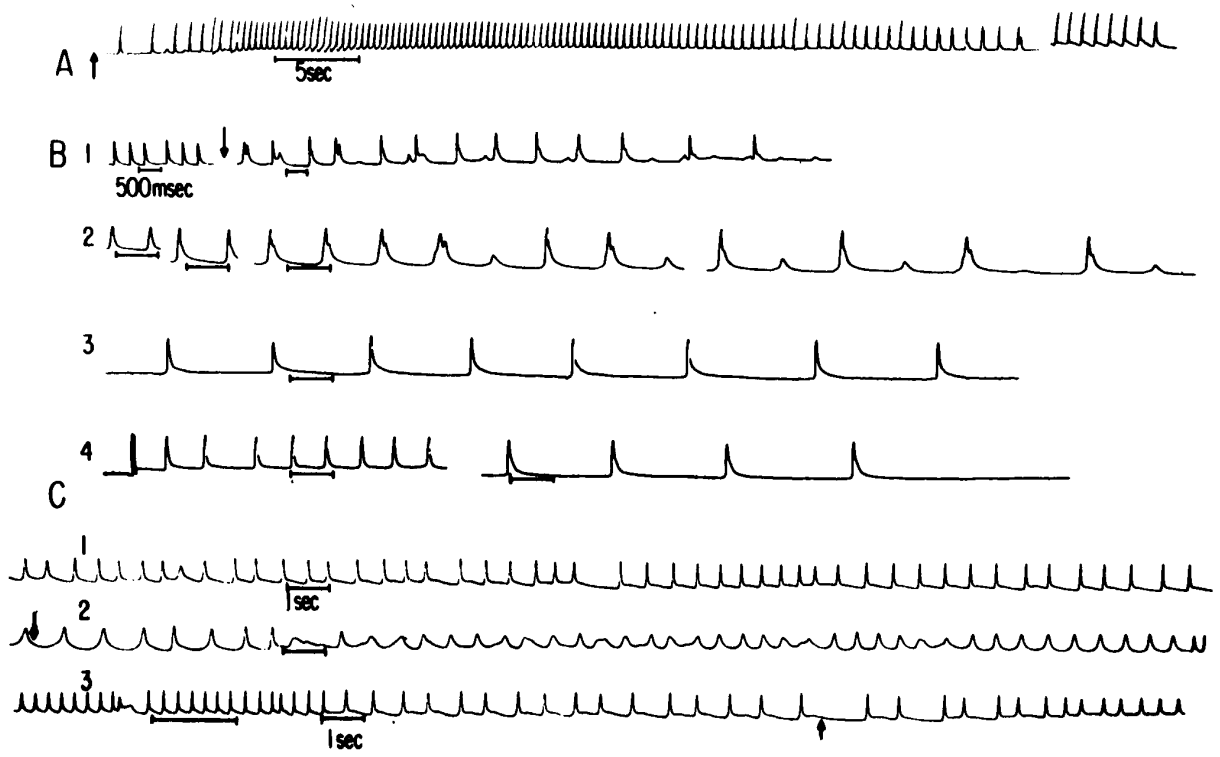

Figure 17. Effect of barium on action potential shapes. A. 5mM barium at arrow. Whole spike train, in Lithium Krebs for 10 minutes; end of barium spike train in lithium for 20 minutes. B. 1. spontaneous spikes. End of first spike train at 36 secs in 2mM barium. 2. (1 to r) beginning 2nd spike train 85 secs in barium; 102 secs after barium; 110 secs in barium; last spikes of 2nd train. 200 μM lanthanum on between 2 and 3. 3. last spikes of 3rd spike train 210 secs in barium. 4. beginning and end of 4th spike train (263 secs in barium). All time scales 500 msec. C. 1. end of spike train 5mM barium added during relaxtion 2. end of 2nd contraction, acetylcholine 4.4 x 10^{-6}M at arrow. Time scale 500 msec. Note uncoordinated spikes (contraction occurred at this point). 3. End of contraction induced by acetylcholine, and acetylcholine at arrow. Time scale 1 sec. Tension records not shown for simplicity. A, B and C are different times.

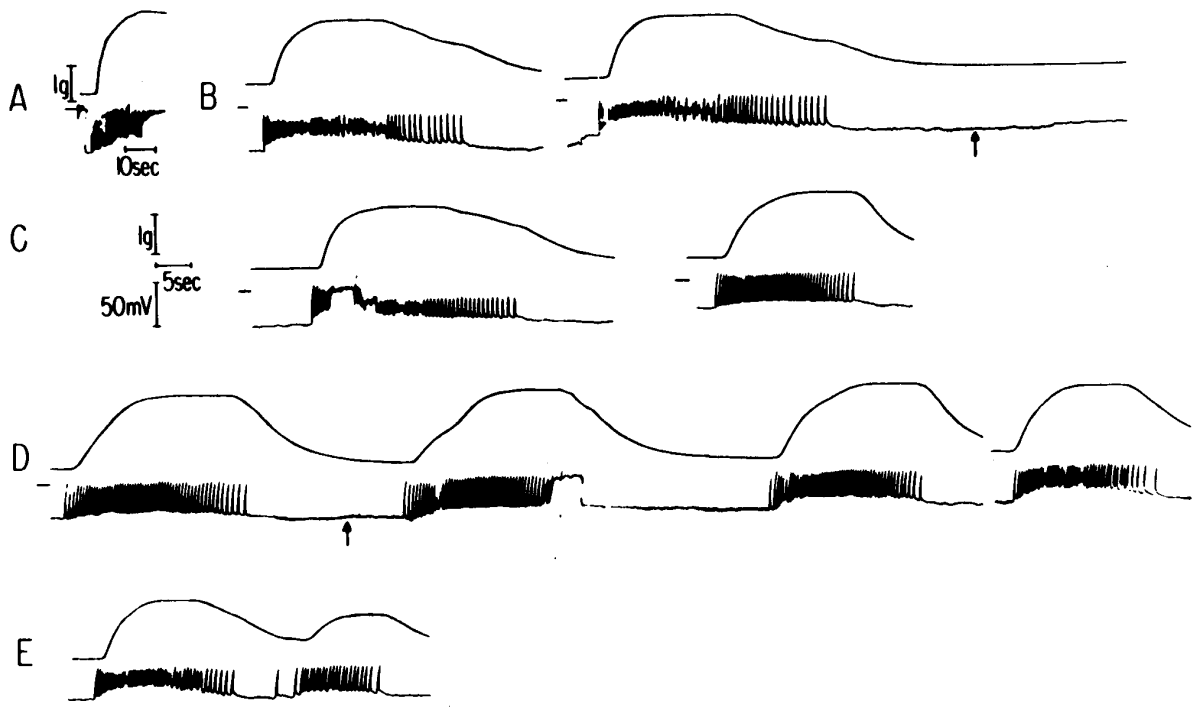

Figure 18. Effect of Strontium Krebs on action potentials. A: Contraction and action potentials in Normal Krebs Solution. B: 14 minutes after exposure to Ca-free Krebs for 4 minutes and then Ca-free Krebs containing 2.47mM strontium. Normal Krebs on at arrow. C: continuous with B, contractions at 1 and 2 minutes after Normal Krebs on. D: 5 minutes after C, shows return to Strontium Krebs at arrow and at right 3 minutes later. E: 6 mins after Strontium Krebs on.

in Strontium Krebs, rate of onset and shape of spontaneous contractions did not differ from those in calcium solution. Relaxation proceeded in two phases, however, the initial phase being accompanied by action potentials, as shown in Figure 18. Action potentials accompanying contractions were clearly impaired (Figure 18B), and this appears to be due to a slowing and in some cases, prevention of repolarisation. This effect is rapidly reversed upon return to calcium-containing medium. Furthermore, when the tissue is now returned to Strontium-containing Krebs, some impairment of action potentials occurs within seconds (see Figure 18D). After further exposure to Strontium-containing Krebs Solution (20 minutes), the tissue became depolarized to 36.8 $\pm$ 1.7 mV (n=10); spontaneous contractions were frequent but were reduced in amplitude, and accompanying spike frequency was reduced to 110/minute, from rates of 190-210/minute during the first 5 minutes of exposure. Acetylcholine applied after prolonged exposure is able to cause contraction, and is able to increase spike frequency to 160/minute but this rapidly declines to 70/minute. During the acetylcholine-induced contraction, the spikes were irregular but after about 1 minute became regular in shape although the tissue was relaxing (Figure 18E).

The effects of strontium on contractile activity were studied further using tissues suspended in 20 ml isolated organ baths. Tissues were exposed to Ca-free Krebs solution until there was no response to agonists (see Section IIIC) and then immersed in Ca-free Krebs containing 2.47mM strontium. Under these conditions, maximal

acetylcholine- and potassium chloride-induced contractions were 30-40% of the respective maximum in calcium solution. Contractions to these agonists could be elicited for periods in excess of 2 hours. Since it was found that the amplitude of the contraction to either acetylcholine or potassium chloride was dependent upon the frequency of exposure to agonist (being greatest for a 2 minute cycle but declining to 30% of this when a 10 minute cycle was used), rigid time cycles were used, usually 3 minutes.

When tissues were depolarised in Potassium chloride Krebs drugs failed to alter the membrane potential, which had a mean value of 7.3 mV (n=23). However, both acetylcholine and oxytocin were able to cause contractions of the depolarised tissue. Although full-dose-response curves were not obtained for these agonists acting on depolarised tissues, the doses required for maximum responses were greater (x 3-5) than those necessary to cause maximum response in Normal Krebs Solution. The shape of contraction to these agonists consisted of a fast increase in tension, followed immediately by a decline over a period of 30-60 seconds, to the pre-drug tension.

2. Ion contents

Tissues exposed to Potassium chloride Krebs rapidly take up potassium (see IIIB). The sodium content of the longitudinal muscle in Potassium chloride Bicarbonate Buffered Krebs was 14.5 ± 1.7 mmoles/kg wet weight (n=12) and the potassium content 104.3 ± 4.1 mmoles/kg wet weight. Total ion content in the presence

of acetylcholine or oxytocin did not differ significantly from controls. (In one experiment, of three, acetylcholine and oxytocin appeared to increase sodium content on a wet weight basis, but this was of marginal significance - p=0.05 - and not significant on a dry weight basis). The extracellular space of tissues after one hour in KCl Krebs was found to be reduced to 36.2 ± 0.70 mls/100 g, indicating swelling of cells, or mechanical extrusion of extracellular fluid. Sodium and potassium levels were not studied in tissues exposed to Lithium Krebs. Total calcium was not changed after exposure to Lithium Krebs (1 experiment, n=5 for control and exposed tissues).

3. ^{42}K and ^{86}Rb fluxes

Elevation of $[K]_o$ increases the rate of exchange of ^{42}K in pregnant rat myometrium. For uptake, uncorrected f value approaches 0.6 after only 10 minutes of incubation with ^{42}K. The half-time for the slowest fraction being decreased from 81.4 minutes in Normal Krebs, to 38.6 ± 2.1 (n=9) minutes; k_3 increased to 0.016 min^{-1}. For the fraction B (*vide supra*) t 1/2 decreased from 9.30 to 5.39 ± 0.5 minutes. Half-time for loss from the fastest fraction was not significantly different from that in Normal Krebs solution. Values for sizes of fractions were 5.3, 15.0, 26.2 and 53.5% for bound fraction and compartment C, B and A respectively (A fastest fraction).

The effects of excitatory drugs on ^{42}K fluxes have been summarised in Table V, and are illustrated in Figures 19, 20 and 21.

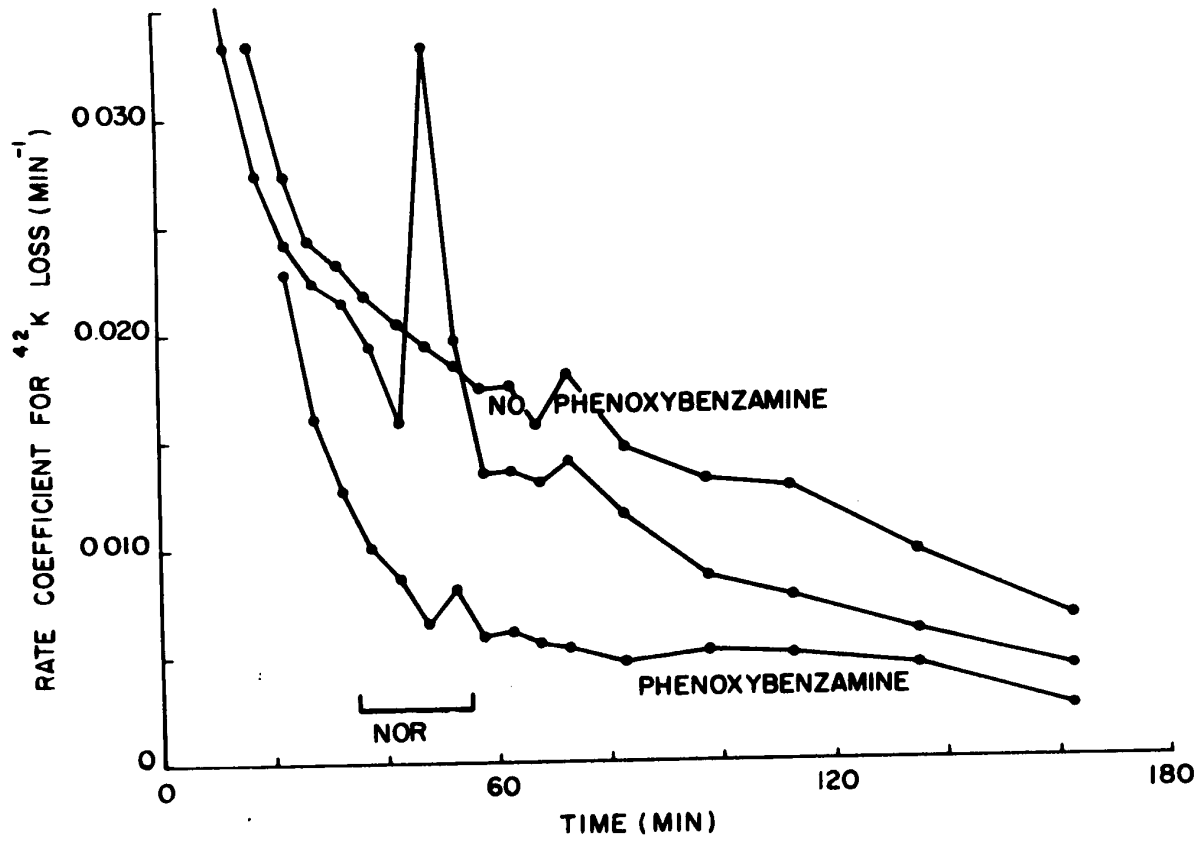

Figure 19. The effect of noradrenaline on the loss of ^{42}K, into Potassium chloride Krebs, from the longitudinal muscle of the pregnant rat myometrium. Upper curve, tissue exposed to 5.9 x 10^{-6}M noradrenaline; middle curve, exposure to 5.9 x 10^{-5}M noradrenaline; lower curve, exposure to 5.9 x 10^{-5}M noradrenaline after exposure to 1.6 x 10^{-7}M phenoxybenzamine during the first 15 minutes of efflux. Ordinate, rate coefficient (min^{-1}). Abscissa: time after commencement of efflux.

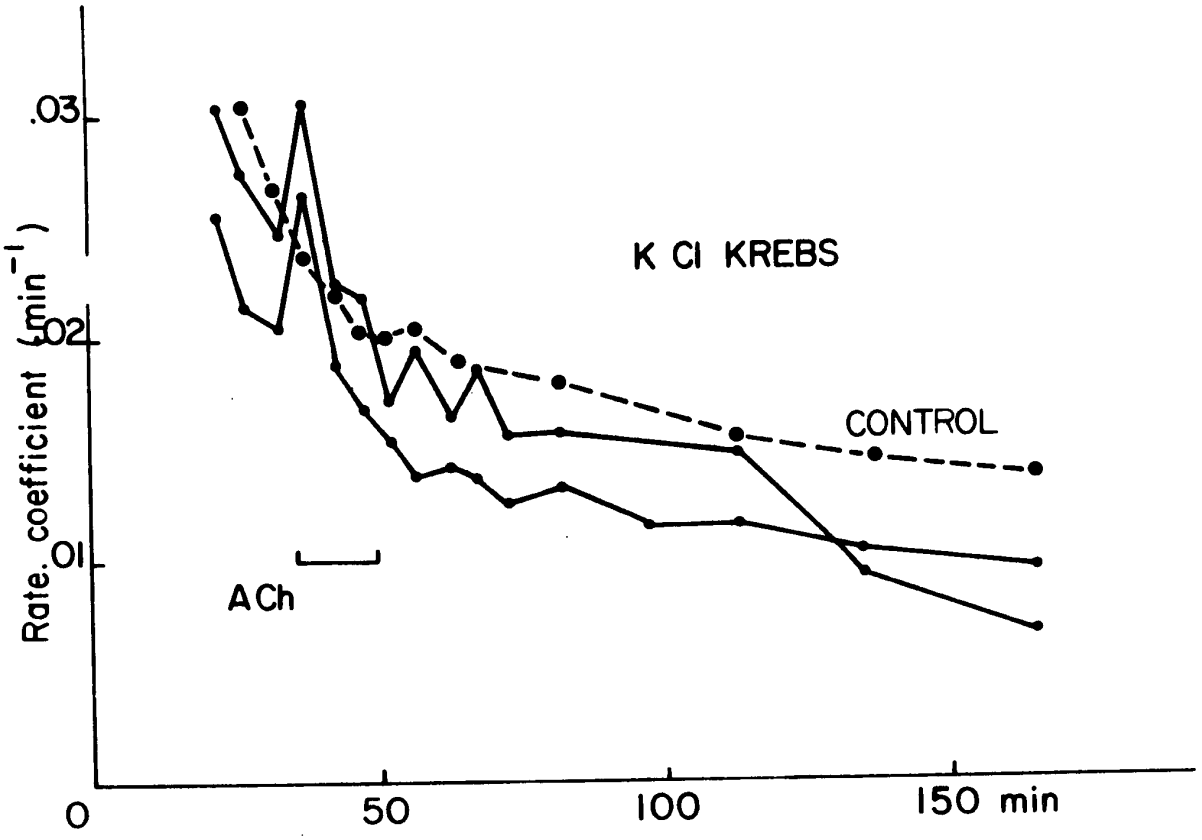

Figure 20. Effect of acetylcholine on the loss of ^{42}K into Potassium chloride Krebs, from the longitudinal muscle of the pregnant rat myometrium. Continuous curves: efflux rate coefficient (min^{-1}) for 2 tissues, both exposed to $2.8 \times 10^{-6}M$ acetylcholine at period shown. Dashed curve: efflux rate coefficient (min^{-1}) for concurrent control of one (upper) drug-treated tissue . Ordinate: rate coefficient (min^{-1}). Abscissa: time in minutes after commencement of efflux.

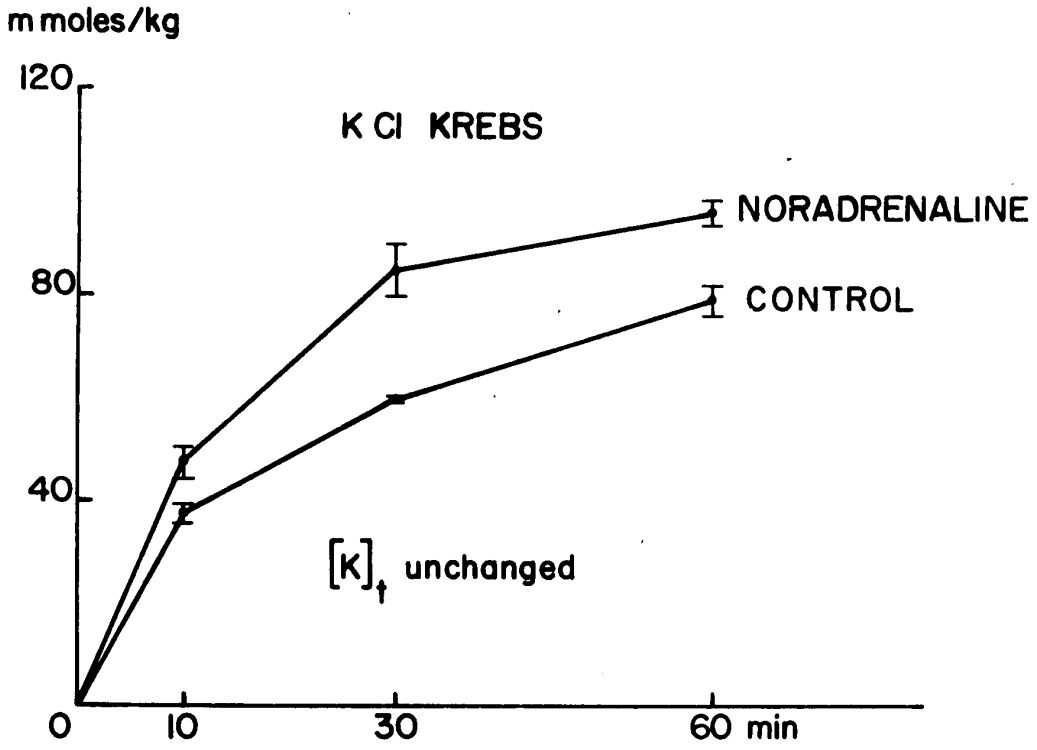

Figure 21. Effect of noradrenaline on uptake of ^{42}K from Potassium chloride Krebs Solution, by the longitudinal muscle of the pregnant rat myometrium. Each point is the mean of 4 tissues. Vertical bars are equal to twice the standard error. Upper curve: tissues exposed to $5.9 \times 10^{-5}M$ noradrenaline in the presence of $3.9 \times 10^{-5}M$ propranolol. Lower curve: control tissues. Values for total potassium $[K]_t$ were not significantly different from the control (60 mins) value of 103.8 ± 3.4. Ordinate: mmoles/kg potassium exchanged. Abscissa: time in minutes in radioactive solution.

TABLE V

Effect of Drugs on ^{42}K Fluxes

	POLARIZED		DEPOLARIZED	
	EFFLUX	INFLUX	EFFLUX	INFLUX
ACh	↑ (3)	↑↓	↑ (8)	↑↓
NORADR.	↑↓ (10)	↑	↑ (6)	↑
OXYTOCIN	↔ (4)	↔	↔ (5)	↔

↑ indicates increase
↓ indicates decrease
↔ indicates no effect
combinations indicate variable or marginal effect
numbers of efflux experiments in which exposure to drug was included in parentheses.

Acetylcholine, at a dose of 2.8×10^{-6}M, increased the rate coefficient (min^{-1}) by a mean of 49% (41-64%, n=4) in Potassium chloride Krebs. In Normal Krebs acetylcholine increased the coefficient by 230, 60 and 93%. (Obtained by comparison of the efflux coefficient estimated from values before and after drug). At a dose of 2.8×10^{-7}M, rate coefficient increased by 13.4% (n=2, 12-14.8%) and had no effect at doses below this. (It should be noted that in Bicarbonate Krebs, the stripped preparation would respond to doses as low as 4×10^{-9}M of acetylcholine). The increase in rate coefficient induced by acetylcholine was prevented by atropine

Carbachol, at 5 x 10^{-6}M, increased efflux coefficient of ^{86}Rb by 41-47% (n=2).

Noradrenaline which had inconsistent effects on contractile events, increased efflux coefficient, in the presence of propranolol (3.9 x 10^{-5}M) in KCl Krebs. At a dose of 5.9 x 10^{-7}M, it had no effect on rate coefficient; of 3 tissues exposed to 5.9 x 10^{-6}M noradrenaline, one showed no effect, the other two showed increases of 7% and 23% of the rate coefficient. At a dose of 5.9 x 10^{-5}M, the rate coefficient was increased by 48, 52, 61 and 130% in four tissues. This increase could be prevented by 1.6 x 10^{-7}M phenoxybenzamine or dibenamine. In Normal Krebs solution, noradrenaline did not increase efflux; the graph of efflux coefficient, previously stable, became erratic in the presence of the drug.

Oxytocin 1-5 mU/ml, which causes contractions of the uterus, failed to affect fluxes under any circumstances. Thus in 4 tissues in Normal Krebs and 5 in KCL Krebs, ^{42}K efflux was not changed in comparison to the concurrent control. Efflux of ^{86}Rb was likewise unchanged by oxytocin, although in the same experiment carbachol caused an increase of about 40%. Influx of ^{42}K was also not significantly changed by oxytocin in two experiments, one of which is shown in Table VI.

A number of efflux experiments were carried out in the presence of drugs throughout, and some in which tissues were exposed repeatedly to different drugs. The former are not included owing to insufficient data in the presence of variability in control

TABLE VI

Effect of Oxytocin on ^{42}K Uptake (mmoles/kg)

n=4 for all values

Treatment		Time (mins) 10	30	60	$K_t(60)$
KCl Krebs	Control	61.2	87.6	92.4	104.0
		1.1	3.6	2.1	2.3
	Oxytocin* 1 mU/ml	66.4	79.1	82.4	105.8
		2.9	5.0	4.6	3.2
Bicarbonate Krebs	Control	4.9	9.3	15.1	33.1
		0.56	0.8	2.1	4.0
	Oxytocin* 1 mU/ml	4.92	8.01	13.9	30.7
		0.37	1.4	2.1	3.2

*when controls are compared to tissues exposed to oxytocin, $p>0.05$ in all cases.

parameters. The latter type of experiments are not included because of delayed effects of drugs on ^{42}K efflux which confused interpretation. However, in a number of these experiments, tissues were exposed first to phenylephrine; this drug consistently (n=9) failed to increase potassium efflux.

4. ^{45}Ca and ^{89}Sr fluxes

When the uptake of ^{45}Ca was measured after exposure to drugs, no increase between experimental and control uptake was observed for uptake at 10, 20 and 60 minutes in isotonic Potassium chloride Krebs,

hypertonic KCl (40mM), acetylcholine, and oxytocin. Lithium Krebs was found to increase the uptake of ^{45}Ca but not ^{89}Sr. At times 10 and 20 minutes, uptake in isotonic potassium chloride was decreased compared to control values. Table VII shows the uptake of ^{45}Ca and ^{89}Sr in the presence of drugs and different media. However, Lithium Krebs increased the efflux of ^{45}Ca and ^{89}Sr (see Figure 34 and Section IIID 1). In an attempt to determine the site of increased calcium uptake in Lithium Krebs, tissues were loaded with ^{45}Ca in this medium and efflux compared to that of tissues loaded in Normal Krebs. Since the counts min^{-1} mg^{-1} curve was elevated throughout efflux after loading in Lithium Krebs, calcium uptake appeared to be increased at all sites.

IIIC Inhibitory Responses to Drugs

1. Mechanical and Electrical activity

Isoprenaline inhibits the contractions of the longitudinal muscle, and causes hyperpolarization (Figure 22). Membrane potential increased by a mean of 10.3 ± 0.98 mV (n=50) in three experiments. The mean membrane potential in each experiment before and during exposure to isoprenaline was compared using a paired t-test, and was highly significant ($p<0.001$). Values for input resistance not attributable to the microelectrode were variable and ranged from 2 mΩ to more than 20 mΩ. Due to this variability and the small number of observations where the same microelectrode was used before and during exposure to drug, it was not possible to determine effects

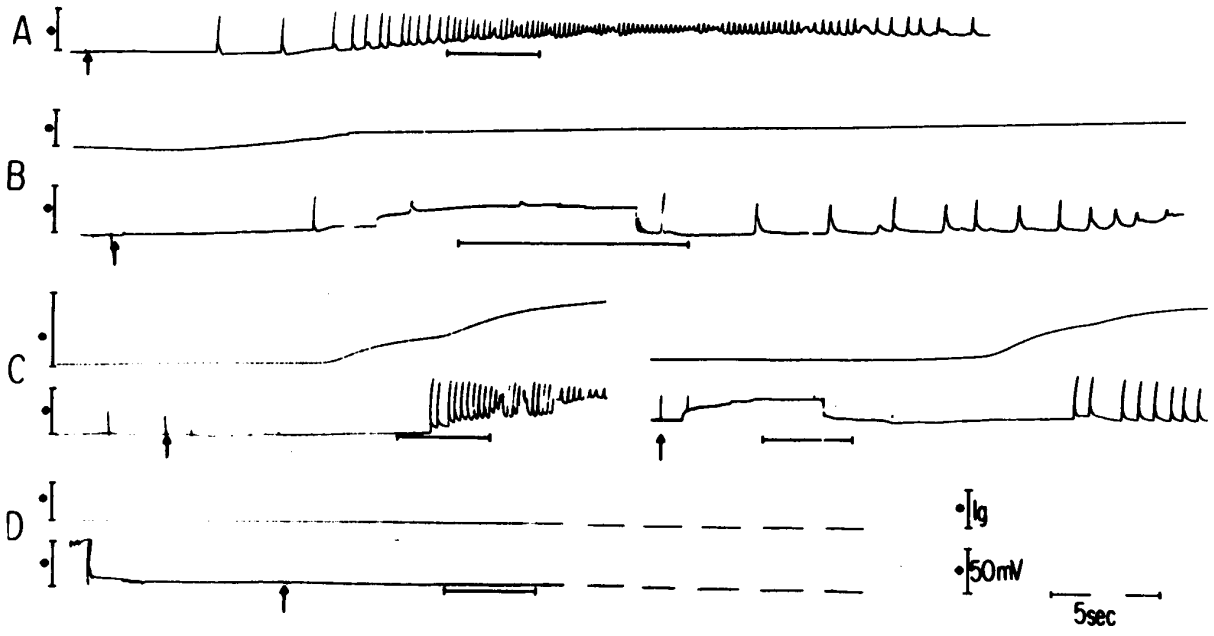

Figure 22. Effects of isoprenaline on electrical activity.
A. Control response to 4.4 x 10^{-5}M acetylcholine. (contraction not shown).
B. Same tissue as A, 10 minutes in 5 x 10^{-7}M isoprenaline. 4.4 x 10^{-5}M acetylcholine at arrow (although electrode was coming out of cell, note spike frequency).
C. Effect of barium (2.5mM) on electrical activity, 3 minutes in 5 x 10^{-7}M isoprenaline. Same tissue and same dose barium, 12 minutes in isoprenaline.
D. Effect of isoprenaline on membrane potential, added at arrow at left, 35, 45, 55 and 65 seconds in isoprenaline 1 x 10^{-6}M.

TABLE VII

Effect of Drugs on the Uptake of ^{45}Ca and ^{89}Sr (mmoles/kg)†

Treatment	^{45}Ca	^{89}Sr
Control Tris Krebs	3.46 0.35	3.97 0.26
Acetylcholine 2.2 x 10^{-5}M	2.91 0.08	3.30 0.12
Oxytocin 1 mU/ml	2.83 0.23	3.52 0.26
Lithium Krebs	3.81* 0.41	3.23 0.19
KCl Krebs	3.70 0.35	--
KCl 40mM (hypertonic)	3.69** 0.09	3.48 0.16
Control KCl Krebs	3.52 0.18	--
Acetylcholine 2.2 x 10^{-5}M in KCl Krebs	3.43 0.07	--
Oxytocin 1 mU/ml in KCl Krebs	3.82 0.32	--

†uptake was for 60 minutes. Each value represents the mean ± standard error of at least 4 tissues.
*different experiment; control 2.89 ± 0.20, $p<0.05$
**different experiment; control 3.37 ± 0.29

of drugs on input resistance with any precision. In three experiments isoprenaline appeared to cause an increase in input resistance. Thus the ranges of input resistance values were 4-16, 2-29 and 7-12 before exposure to isoprenaline, and were 18-42, 24-53 and 8-17 respectively in the presence of 5×10^{-6}M isoprenaline. Isoprenaline, at a dose which caused complete inhibition of acetylcholine contractions (0.5×10^{-6}M, was only partially able to inhibit contractions induced by isotonic Potassium chloride Krebs or Lithium Krebs. Mean maximum inhibition of lithium-induced contractions was 28% (44-17) for 6 tissues. Adenosine, when added at concentrations up to 4×10^{-4}M, failed to cause any inhibition of acetylcholine-induced contraction.

Papaverine at 2.5×10^{-5}M, was able to abolish contractions to constant doses of acetylcholine and potassium chloride; at lower doses, (1×10^{-5}M) papaverine changed the shape of contractions such that acetylcholine caused a slow contraction while the fast contraction persisted for potassium chloride induced contractions. However, when the dose of agonist was doubled, it was possible to show that inhibition consisted of a shift of the dose-response curve to the right. Thus acetylcholine contractions returned to the original (fast) shape of contraction on doubling the dose, the original, previously-maximal dose now being submaximal (and, in fact, near threshold). Changes in shape of contractions were dependent upon dose of agonist used.

Similar findings were encountered with chlorpromazine, which at 2×10^{-5}M changes the shape of the acetylcholine-induced

contraction and produced a delay of up to 30 seconds between dose and effect of acetylcholine (Figure 23). Electrophysiological studies indicated that such changes in shape are probably due to a delay in the appearance of action potentials, and the slowed rate of increase in tension due to a slowed rate of action potential discharge. Note that in Figure 24A, a high dose of acetylcholine causes a contraction in the absence of action potentials in the cell penetrated, a high dose of potassium chloride depolarised the cell and a fast contraction is elicited. Significant inhibitory effects on acetylcholine and potassium chloride induced contractions are seen with 1×10^{-6}M chlorpromazine, when 25% inhibition of maximum contraction to potassium chloride and 50% inhibition for acetylcholine contractions occurs. Lithium Krebs-induced contraction was inhibited by 50-60% in the presence of 10^{-6}M chlorpromazine. Dose-response curves to acetylcholine and potassium chloride were moved both to the right and downwards by doses of 10^{-6} and 10^{-5}M chlorpromazine, the acetylcholine dose-response curve being moved downwards to a greater extent than that for potassium chloride (Figure 25). Chlorpromazine at these doses had little effect on the dose-response curves obtained to calcium in Ca-free potassium chloride solution.

Acetylcholine-induced contractions were also more sensitive to SKF 525-A, which inhibited potassium chloride-induced contractions by 17% and acetylcholine by 49% after 1 minute exposure to 2.6×10^{-5}M. n=8 Spike frequency was 90/minute under similar circumstances when membrane potential was recorded after addition of potassium chloride (compared

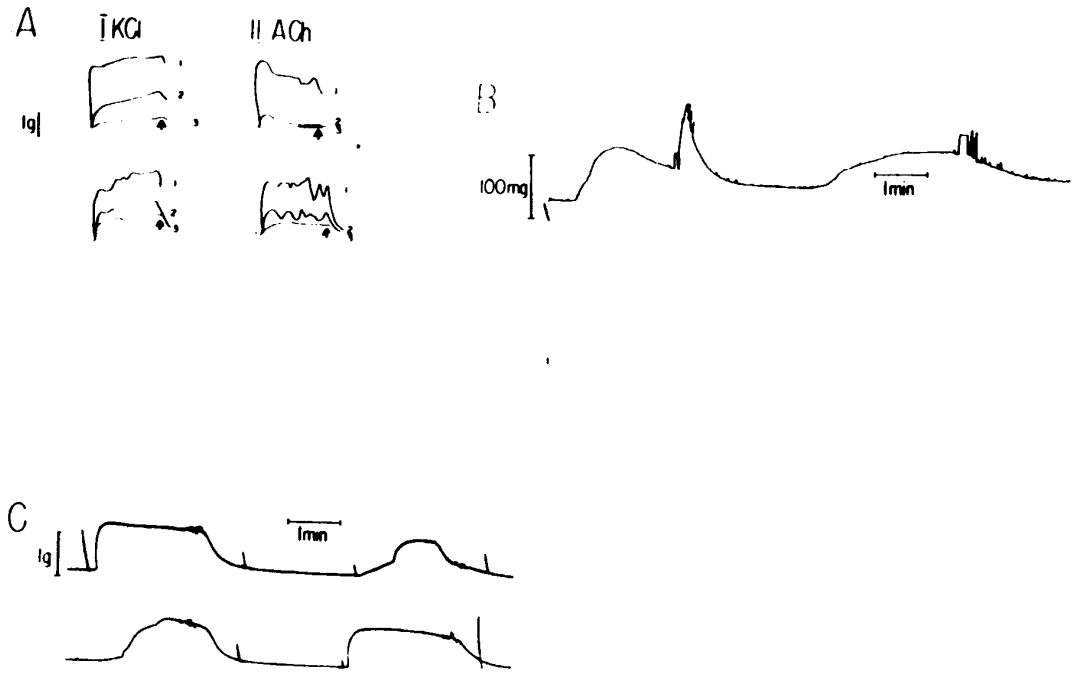

Figure 23. Effect of inhibitory drugs on shape of acetylcholine and potassium chloride induced contractions. A: 2 tissues each exposed to 40mM potassium chloride (I) and 5.7 x 10^{-6}M acetylcholine (II) for 3 minutes. Curves: 1-Control; 2-after 20 μM lanthanum; 3-after 200 μM lanthanum. B: exposure to acetylcholine 5.7 x 10^{-6}M and then 40mM potassium chloride, 15 minutes after exposure to 500 μM lanthanum for 3 minutes; contractions represent approximately 7 and 6% respectively of original maximum. C: responses of two tissues to upper record potassium chloride 20mM, and acetylcholine, 2.8 x 10^{-6}M and lower record in reverse order, after exposure to 1 x 10^{-6}M chlorpromazine for 5 minutes. Contractions are 80% (potassium chloride) and 60% (acetylcholine) of respective maximal responses.

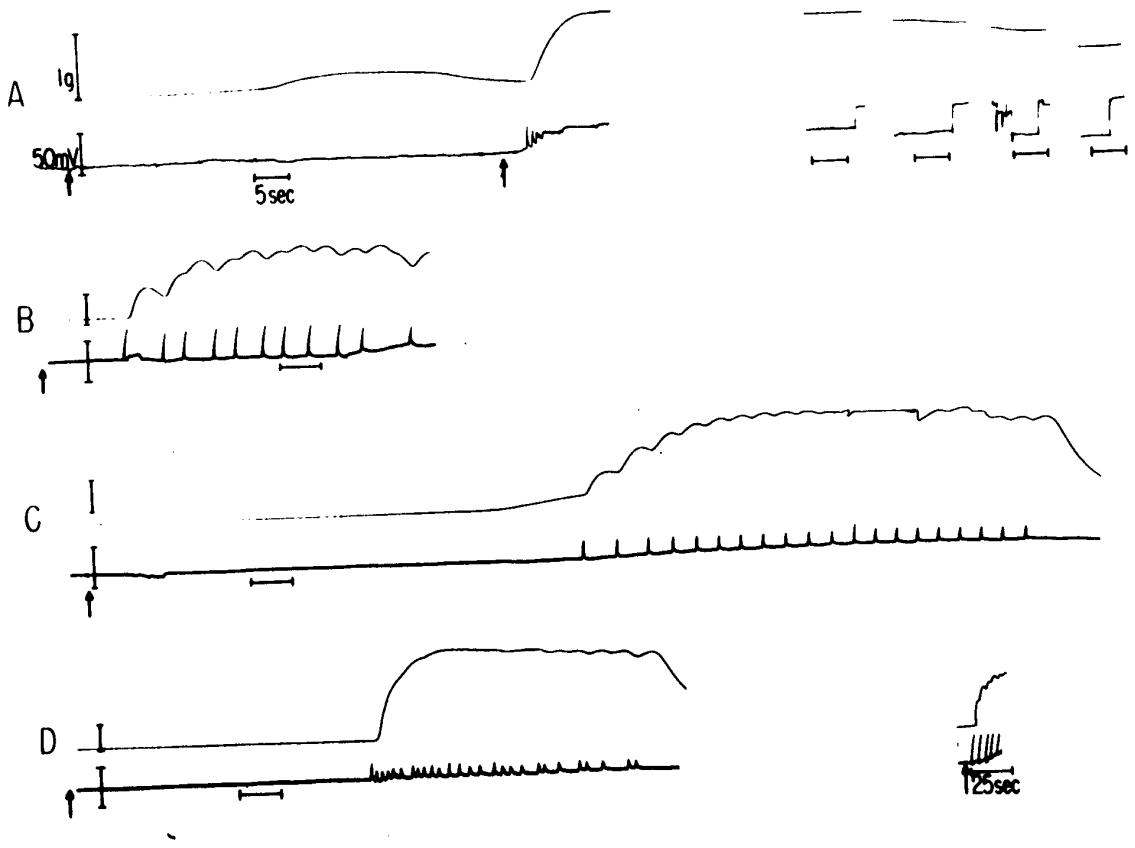

Figure 24. Effects of chlorpromazine and SKF 525-A on electrical events in response to agonists. A: (l to r) after 10 minutes exposure to 1 x 10^{-5}M chlorpromazine; application of 4.4 x 10^{-5}M acetylcholine at first arrow, and 60mM potassium chloride at second arrow; subsequent repolarisation after potassium chloride. B: response to 40mM potassium chloride after 7 minutes of superfusion with SKF 525-A, 2.6 x 10^{-6}M. Note very slow spike discharge, each spike causing a contraction. C: acetylcholine, 4.4 x 10^{-3}M acetylcholine 2 minutes after B. D: application of 10 mU oxytocin 2 minutes after C; at right, response to acetylcholine, 2 minutes after superfusion with Krebs solution not containing SKF 525-A. Note: electrodes probably acting as surface electrodes in C and D; note spike frequency. All time scales 5 sec.

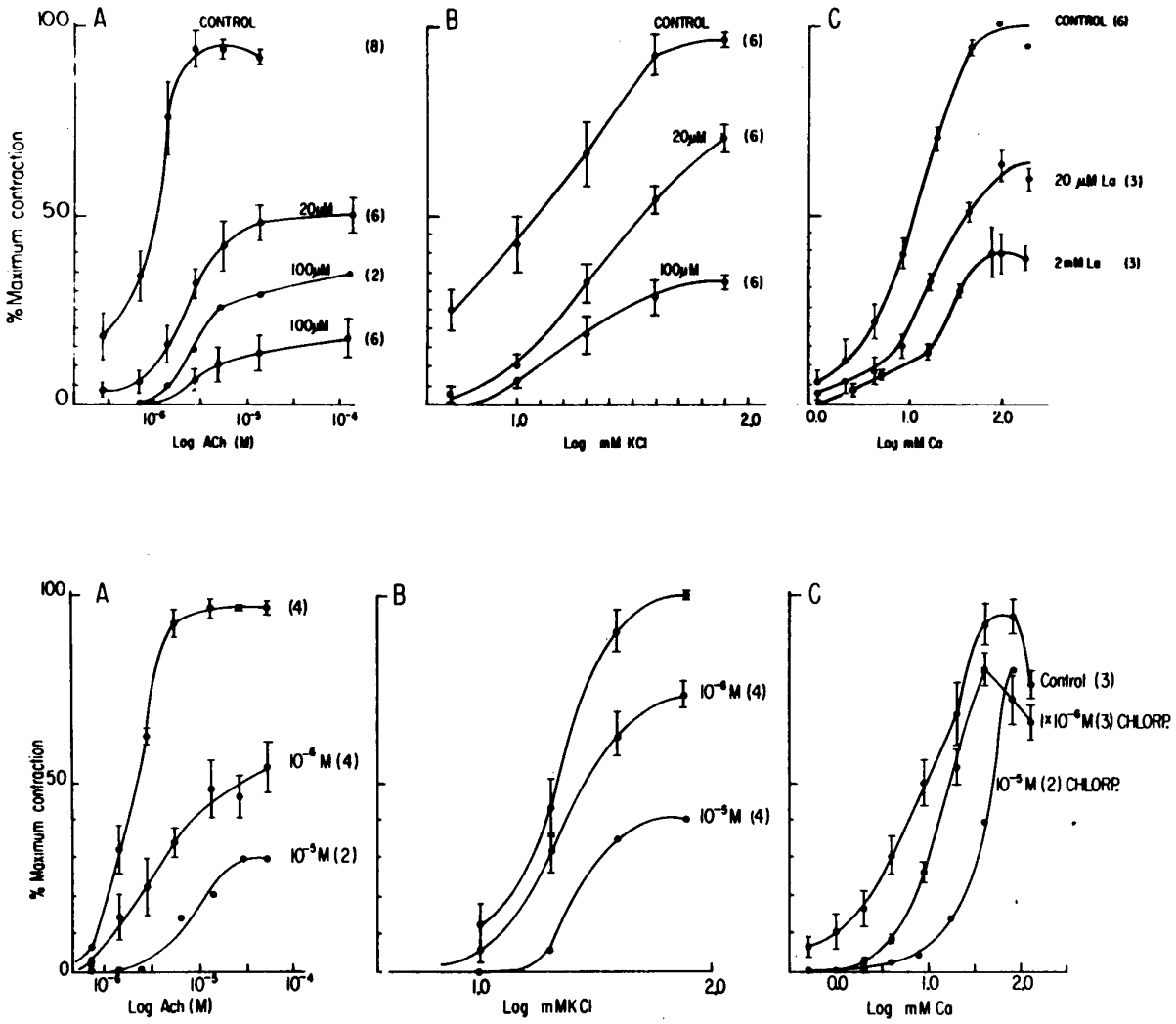

Figure 25. Dose-response curves in the presence of chlorpromazine and lanthanum. (1 to r): dose-response relationships established for acetylcholine, potassium chloride (by washout after each dose, in the continued presence of a constant dose of lanthanum or chlorpromazine) and calcium (by cumulatively increasing doses of calcium in Ca-free Potassium chloride Krebs solution). Numbers of tissues used in parentheses and vertical bars are twice the standard error. For acetylcholine curves in the presence of lanthanum, two curves are shown for 100 μM lanthanum because of effect of tris on sensitivity to lanthanum. Upper curve is in 26mM Tris Buffered Krebs Solution. Ordinate: response as percentage maximum; Abscissa: log dose of agonist.

to a previous rate of 210/minute prior to SKF 525-A, in response to a similar dose of potassium chloride). Dose-response curves were especially difficult to construct in the presence of SKF 525-A as inhibition increased progressively with time of exposure, but the effects were reversible on removal. SKF 525-A did not alter membrane potential significantly, but slowed spike discharge to a very slow rate (24/minute); each spike caused a large contraction (Figure 24). Cinnarizine (1-benzhydryl-4-cinnamylpiperazine,dihydrochloride) was used in two experiments. A dose of 7×10^{-6}M inhibited acetylcholine and potassium chloride-induced contractions by 50-60% but abolished Lithium Krebs-induced contractions.

Lanthanum was able to inhibit contractions to all agonists. In 26mM tris solutions, potassium chloride- and acetylcholine-induced contractions were equally sensitive to lanthanum, as determined by dose-response relationships and when tissues were contracted by constant doses (Figures 25 and 26). In 4.2mM tris solutions, acetylcholine-induced contractions were more sensitive to lanthanum. However, 20 μM lanthanum inhibited acetylcholine and potassium chloride-induced contractions approximately equally regardless of tris concentration. When drug-induced contractions were elicited in Strontium Krebs, tissues showed approximately the same sensitivity to lanthanum as did those in calcium solution (Figure 26). The inhibitory effect of lanthanum appeared to be due only to a decrease in maximum spike frequency, which for acetylcholine was reduced to 160-200 per minute. In the presence of 200 μM lanthanum, maximum spike frequency was 120-190/

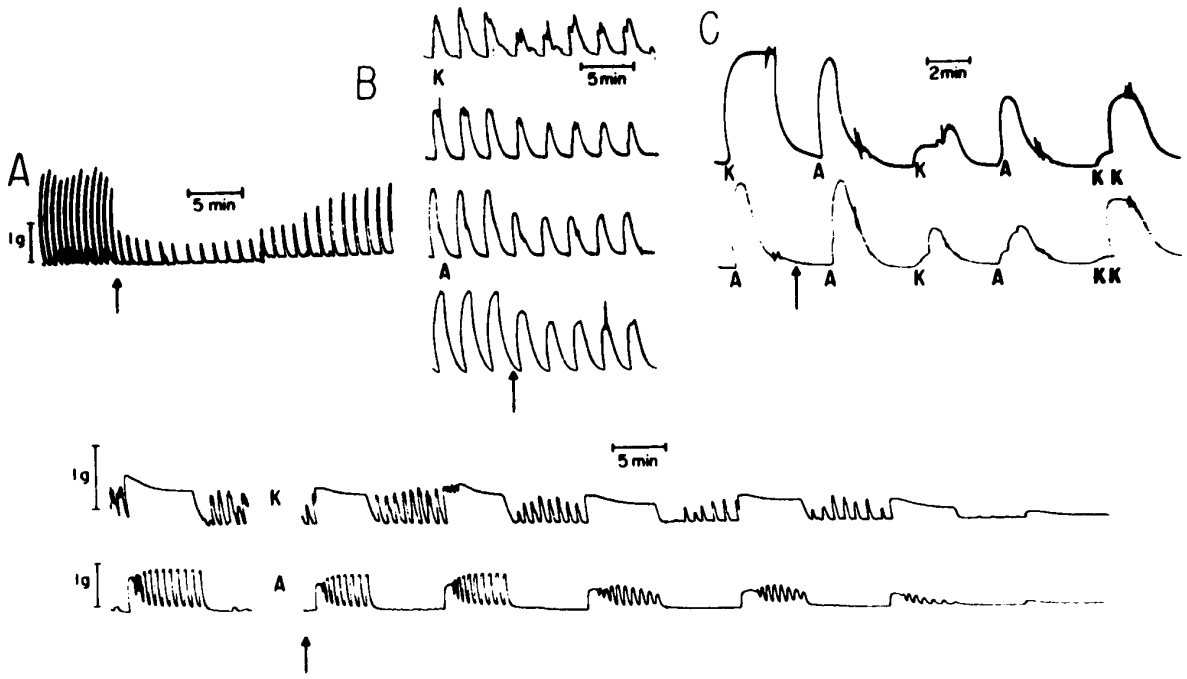

Figure 26. Effects of lanthanum on contractile activity of the longitudinal muscle of the pregnant rat myometrium. A: effect of 10 μM lanthanum (arrow) on spontaneous contractions. B: upper two tissues contracting to potassium chloride and lower two tissues to acetylcholine. All doses 80-90% of maximum. 100 μM lanthanum added at arrow. Inhibition is 60, 44, 57 and 50% from the top respectively. C: potassium chloride (K) and acetylcholine (A) responses as shown in Strontium Krebs (after previous depletion of calcium). 100 μM lanthanum at arrow. Inhibition is 68% (potassium chloride), and 63% (acetylcholine) for upper record and 44% and 70% for these agonists for lower record. Note that doubling dose of potassium chloride at final contraction increases amplitude of contraction. D: nonpregnant tissues; upper tissue exposed to 40mM potassium chloride on 5 minute cycle. Lower tissue exposed to 8.8×10^{-4}M acetylcholine. Continuous exposure to 100 μM lanthanum after arrows. Tissues did not reach equilibrium with respect to lanthanum inhibition and contractions progressively decline. Inhibition is 33, 30, 46, 55 and 61% respectively for potassium chloride (omitting final contraction) and 30, 35, 45, 57 and 65% respectively for acetylcholine.

minute for potassium chloride and acetylcholine (Figure 27). After exposure to lanthanum, the membrane gradually depolarised to 33.8 ± 1.7 mV (n=24); at 30-60 minutes, small electrotonic potentials were frequently seen between spikes (Figure 28). However, when applied during barium-induced contraction (which was not sensitive to low doses of lanthanum), spikes were converted from notched, prolonged repolarisation, to fast repolarisation types (see Figure 17). Inhibition by lanthanum was only partially reversible. Addition of 20mM calcium to inhibited tissues caused transient inhibition of acetylcholine-induced contractions but transiently returned potassium chloride contractions to the original maximum. On some occasions in both Normal and Potassium chloride Krebs solutions, after washout of lanthanum an irreversible contracture occurred (Figure 29).

When calcium is removed from the external medium, contractions to acetylcholine and potassium chloride declined sometimes in a parallel manner, but usually, contractions to potassium chloride were abolished before those to acetylcholine. However, at a time when contractions to potassium chloride had been abolished, contractions to acetylcholine were never more than 10% of the original maximum. The time required for abolition of all contractions varied considerably (30-60 minutes), probably due to varying degrees of contamination with calcium of the Ca-free solution. When EGTA (10^{-4}M) was used, contractions were abolished in less than 10 minutes. The membrane potential gradually declined in Ca-free solutions, but contractions induced by acetylcholine and potassium chloride were

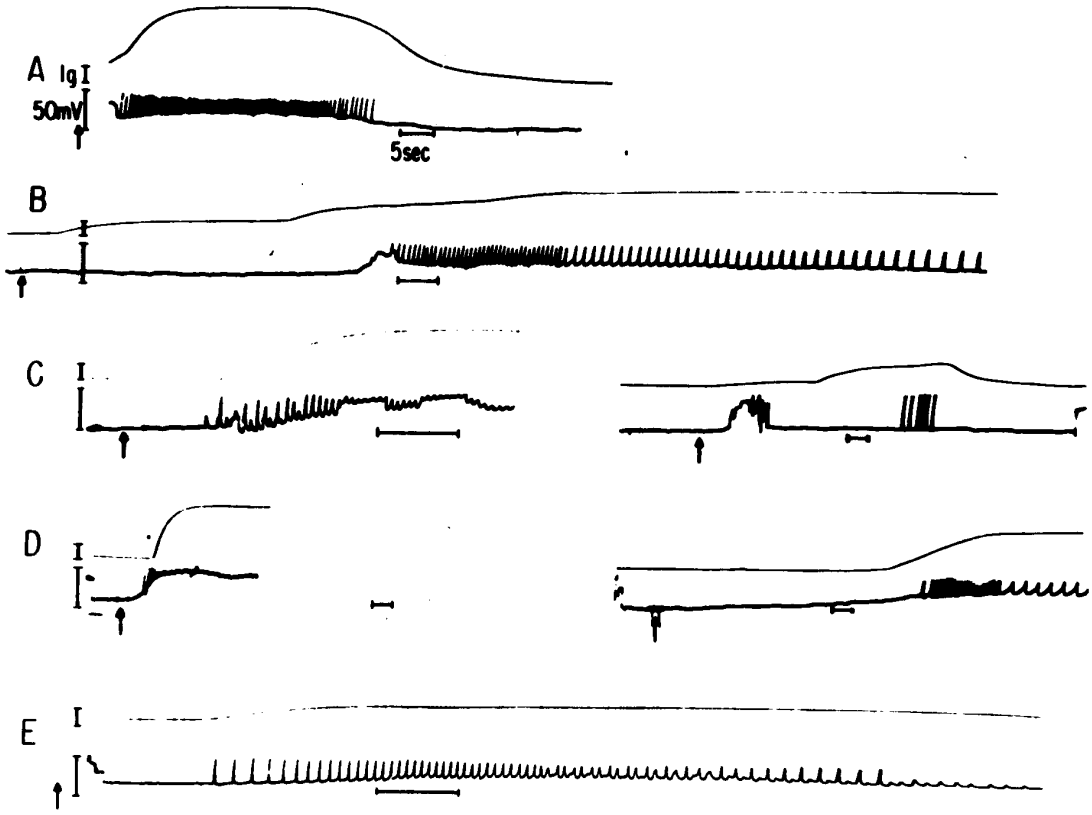

Figure 27. Effect of lanthanum on the electrical response of the longitudinal muscle of the pregnant rat myometrium.
A. control response to 8.8 x 10^{-6}M acetylcholine (spike frequency 270/min).
B. response to 8.8 x 10^{-6}M acetylcholine 3 min in 200 µM lanthanum.
C. responses to: 20mM potassium chloride and 8.8 x 10^{-6}M acetylcholine, 6 min in 200 µM lanthanum.
D. responses to 30mM potassium chloride and 2 mU oxytocin after return to Normal Krebs.
E. 8.8 x 10^{-6}M acetylcholine after partial recovery from lanthanum.

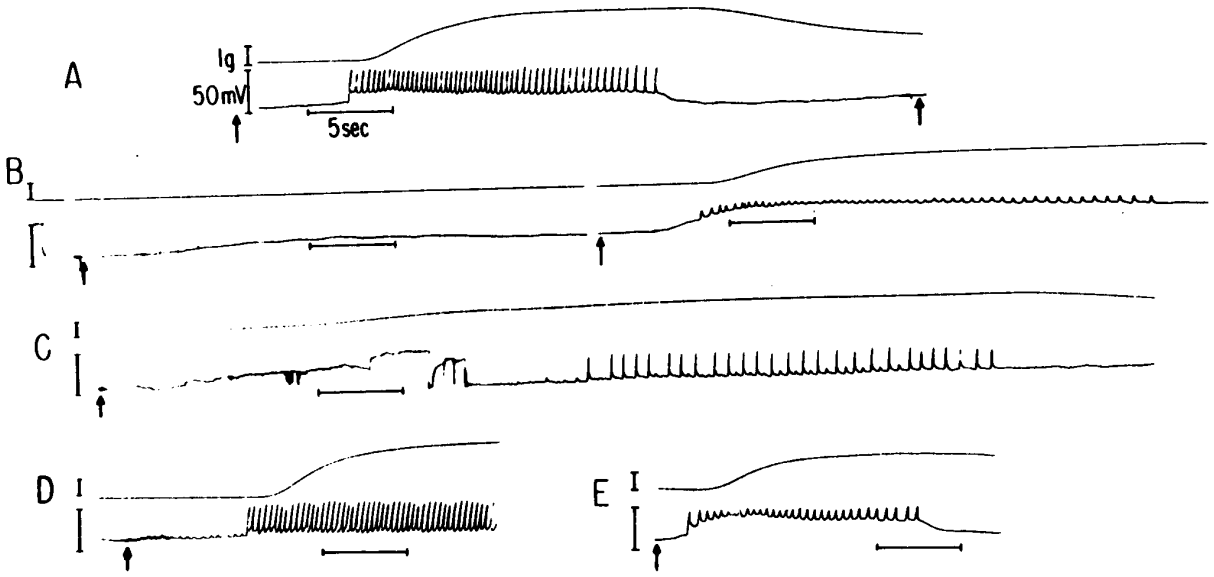

Figure 28. Effects of lanthanum on electrical responses to acetylcholine and potassium chloride. A. control response to 20mM potassium chloride (arrow); lanthanum (1mM) exposure at right (arrow). B. 20mM potassium chloride and 80mM potassium chloride. C. 8.8×10^{-3}M acetylcholine. D. different tissue; 20mM potassium chloride after 20 µM lanthanum E. same tissue as D, 2mM barium after 200 µM lanthanum for 8 minutes.
All time scales 5 sec.

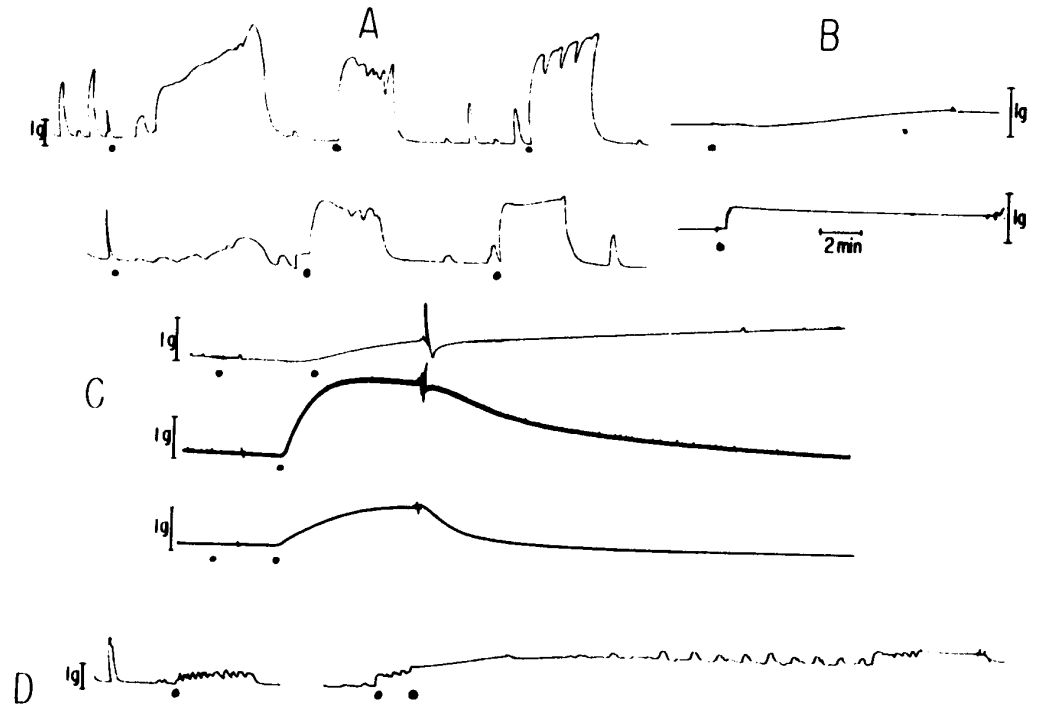

Figure 29. Effects of lanthanum on ion and drug-induced contractions. A.(above) responses to Lithium Krebs, acetylcholine (4.4 x 10^{-6}M) and potassium chloride (20mM). (Below) response to same agonists 3 mins after adding 100 μM lanthanum. % inhibition 87, 36, and 37 respectively. B.(above) response to Lithium Krebs; (below) response to Potassium chloride Krebs, both after 50 μM lanthanum. C. 3 tissues in Ca-free Potassium chloride Krebs. First marker, top to bottom trace, 5mM lanthanum, nil (control), 2 x 10^{-5}M SKF 525-A. 20mM calcium chloride at second marker to all tissues. D. response to 20mM potassium chloride in presence of 200 μM lanthanum; repeat dose in absence of lanthanum and addition of further 10mM potassium chloride. Note contracture continues in spite of wash.

still accompanied by spikes (Figure 30).

When Lithium Krebs was applied without calcium, no contractions, or rarely, only small spontaneous contractions, were elicited (Figure 31). Removal of calcium from Lithium Krebs during a contraction led to a decline in tension which could be restored by addition of calcium (Figure 31). When replacement of sodium by lithium is compared to replacement with potassium, quite different results are obtained in relation to calcium. Isotonic Potassium chloride Krebs caused a contraction which consists of a fast initial phase, followed by a gradual decline to a stable level which will persist for several hours at 37°C. Such a contraction can be elicited when Ca-free Potassium chloride Krebs is applied; removal of calcium during a contraction leads only to a small decline in tension (Figure 31). Tissues in Ca-free Potassium chloride Krebs relax to baseline over a period of 40-60 minutes.

When Ca-free Krebs solution containing acetylcholine or oxytocin is applied during a contraction induced by these agents, there is a gradual decline in tension and contractions induced by oxytocin are abolished. When calcium is added back to the solution, contraction returns (Figure 31).

2. Ion contents

The ion contents of tissues exposed to inhibitory drugs are shown in Table VIII. Values of potassium are not significantly different from controls ($p>0.05$) but tissues appear to gain sodium

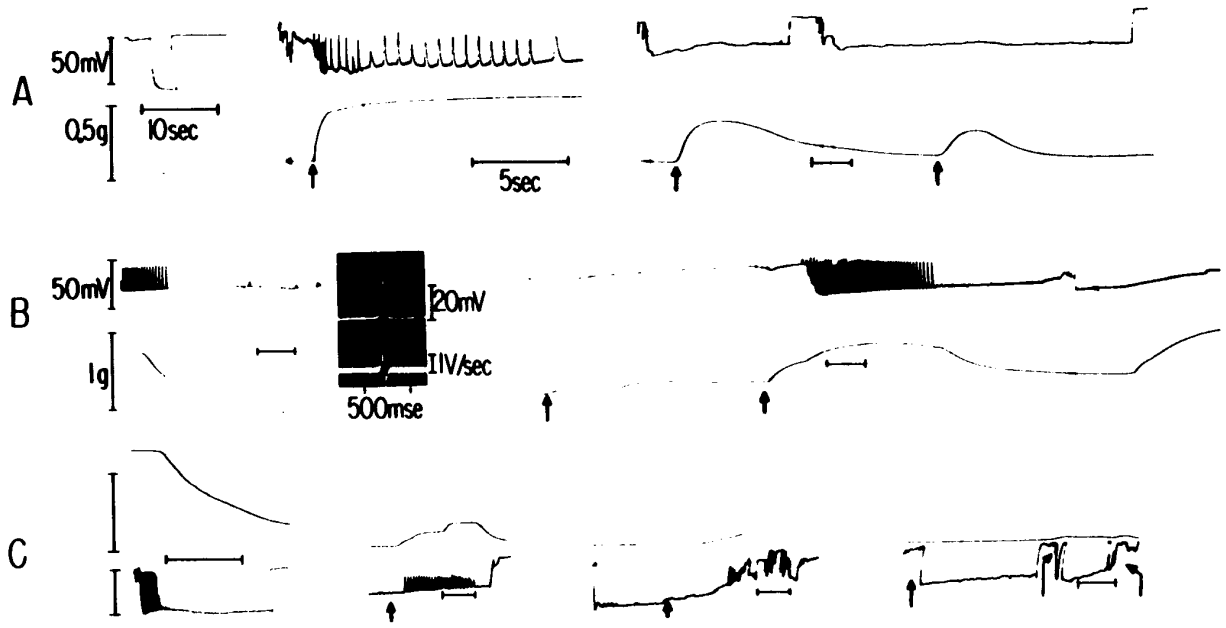

Figure 30. Effect of Ca-free solution on electrical activity. A. control membrane potential; response to acetylcholine (8.8×10^{-6}M) after 7 minutes in Ca-free solution; responses to 40mM potassium chloride and 2.2×10^{-5}M acetylcholine 30 minutes in Ca-free solution. B. (1 to r) trace and oscilloscope record of spikes after 6 minutes in Ca-free Krebs; addition of 4.4×10^{-5}M acetylcholine at each arrow, then 40mM potassium chloride at end of trace. C. (1 to r) before Ca-free solution; 10 minutes in Ca-free solution 8.8×10^{-5}M acetylcholine; 14 minutes in Ca-free solution, 8.8×10^{-5} acetylcholine ; 40mM potassium chloride; spikes arrowed.

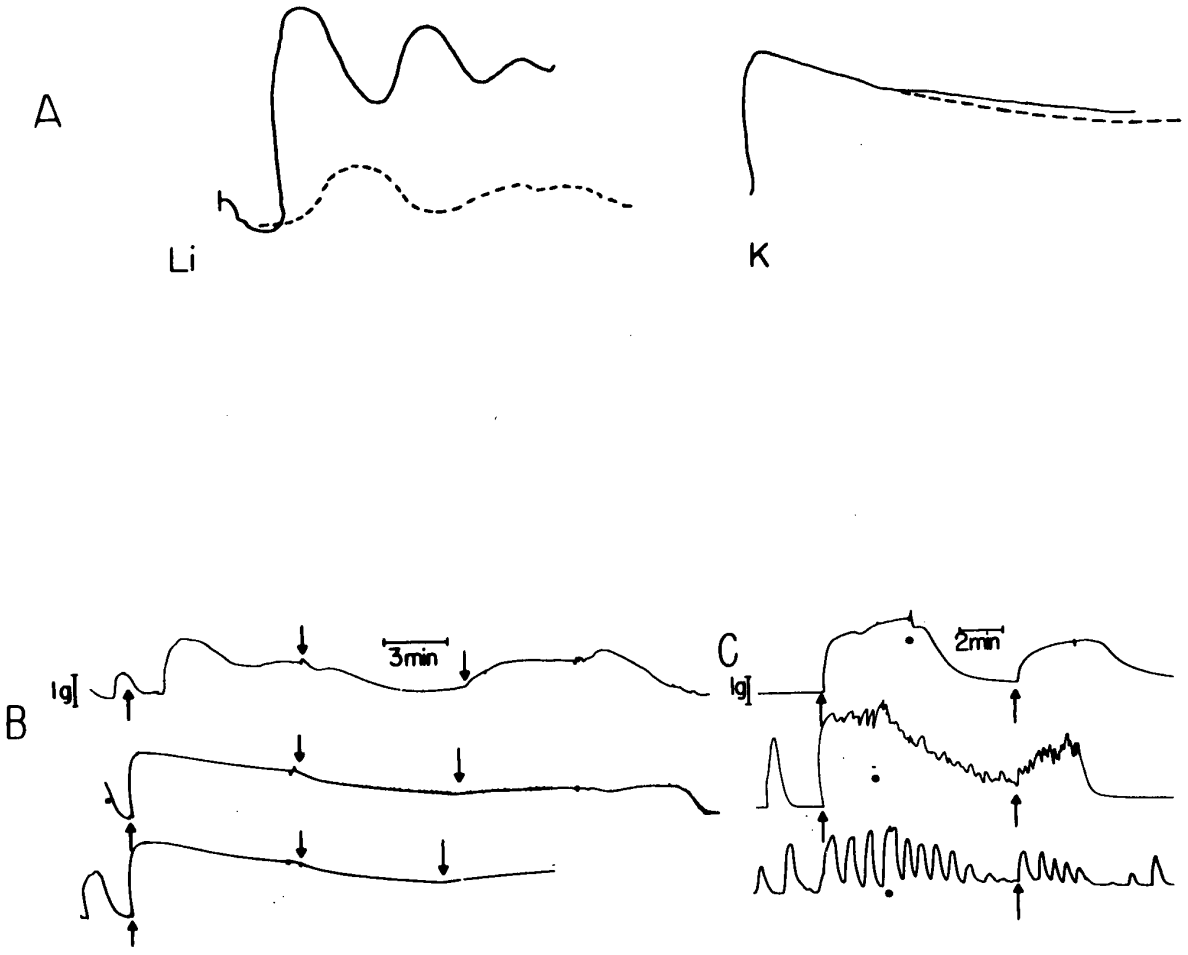

Figure 31. Effects of Ca-free solutions on agonist responses. A. response to Ca-containing (solid line) Ca-free (dashed line) Lithium and Potassium chloride Krebs respectively. B. Top, Lithium Krebs; Centre and bottom, Potassium chloride Krebs at first arrow. Respective Ca-free solution at second arrow, and introduction of, top - 2.47mM strontium chloride, Centre - 2.47mM strontium chloride. Bottom - 2.47mM calcium chloride. C. Top, Lithium Krebs, Centre, acetylcholine (1.3×10^{-5}M). Bottom 100 μU oxytocin at first arrow. Respective Ca-free solution at filled circle and introduction of 2.47mM calcium chloride at 2nd arrow.

TABLE VIII

Effects of Inhibitory Drugs on Ion Content of Rat Myometrium (mmoles/kg)

	$[Na]_t$	$[K]_t$	$[Ca]_t$
$Control_1$ (4)	99.7 3.4	46.8 3.1	--
Isoprenaline (4) $5 \times 10^{-6}M$	121.3 4.4	39.5 4.4	--
Papaverine (4) $2 \times 10^{-5}M$	118.6 6.9	43.2 1.8	--
$Control_2$ (6)	109.9 1.8	37.0 1.4	4.10 0.47
Lanthanum (6) 2mM	96.5 3.3	39.2 2.2	2.08 0.48
SKF 525-A (6) $2.6 \times 10^{-5}M$	113.2 2.3	36.2 1.2	3.93 0.26

*refers to isoprenaline and papaverine; Bicarbonate Krebs Buffered Solution
**refers to lanthanum and SKF 525-A; Tris Buffered Krebs Solution

in the presence of isoprenaline and papaverine. $p<0.05$. Lanthanum decreased the total calcium content of tissues

When the effects of lanthanum on extracellular space were studied, values in excess of controls were encountered. An investigation revealed that in the presence of lanthanum, the inulin space increased. Values obtained for the inulin space after 60 and 120

minutes in ^{14}C inulin are shown in Table IX. Uptake of inulin was carried out in the presence of three doses of lanthanum. Increasing either the length of time of exposure to a dose of lanthanum, or increasing the dose of lanthanum, increased the measured inulin space. When tissues were exposed to 2mM lanthanum for a period of 20 minutes before being placed in inulin, inulin space was not significantly different from controls. When mannitol was used as extracellular marker, the mannitol space was not different for controls and those exposed to 2mM lanthanum. In the presence of 20mM lanthanum, the mannitol space was significantly decreased ($0.02<p<0.05$). Samples of ^{14}C-inulin incubated with and without lanthanum were run on thin layer chromatography and paper chromatography but these techniques failed to conclusively demonstrate any breakdown of inulin.

3. ^{42}K fluxes

At a time when isoprenaline causes hyperpolarisation, it fails to increase potassium efflux in polarised (n=12) or depolarised (n=14, with Potassium chloride Krebs) tissues. Influx of ^{42}K is increased by isoprenaline in Normal Krebs, but when electrical events are eliminated in Potassium chloride Krebs, isoprenaline fails to increase uptake as shown in Table X. Isoprenaline caused a delayed increase in potassium efflux. This increase could be elicited by 3',5'-cAMP (Figure 32). Furthermore, when adrenaline was used, either α or β type of efflux response could be elicited depending upon what blocker was used (Figure 33). Eleven tissues were exposed

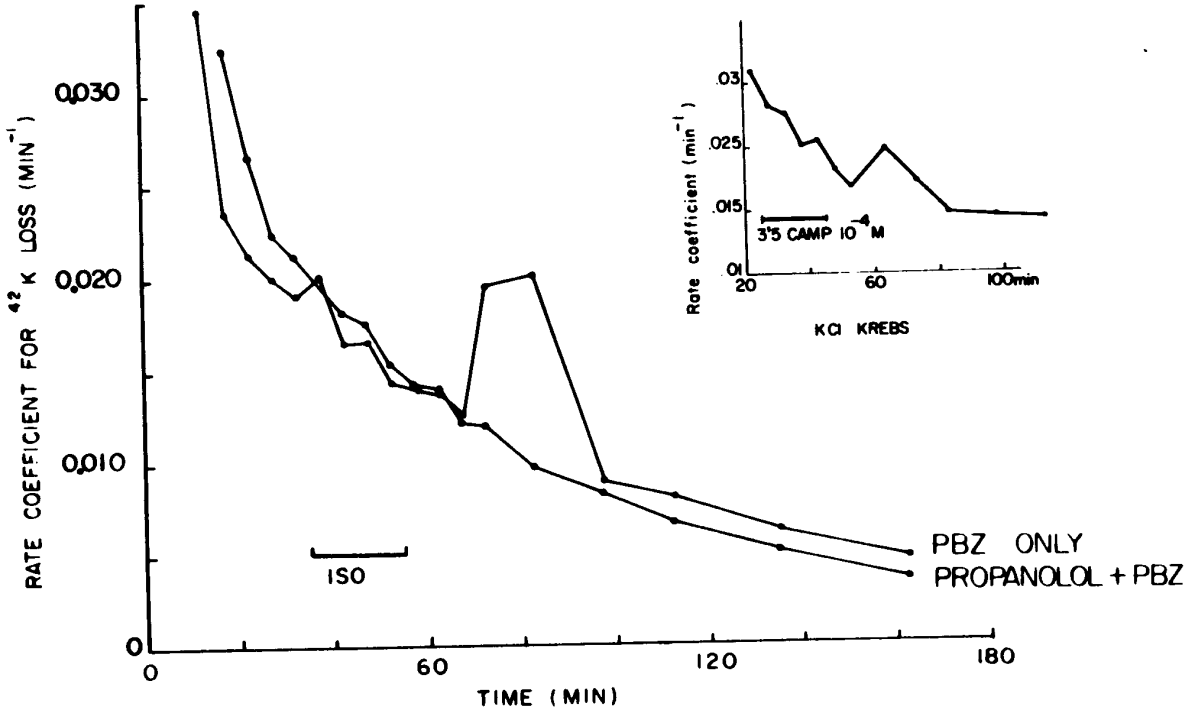

Figure 32. Effect of isoprenaline (5 x 10^{-6}M) on rate coefficient of ^{42}K loss into Potassium chloride Krebs. Inset shows similar effect of 3',5'cAMP (10^{-4}M).
Ordinate: rate coefficient (min^{-1})
Abscissa: time after removal from radioactive solution.

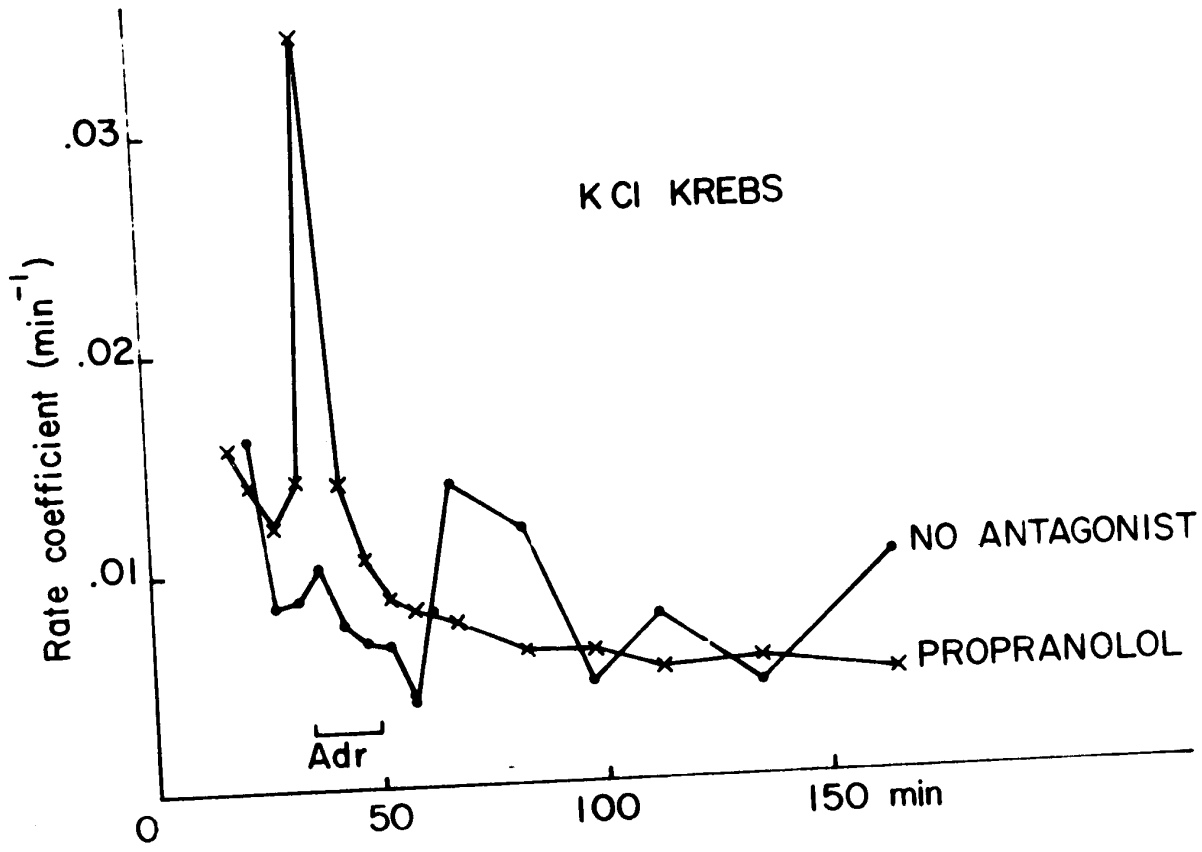

Figure 33. Effect of adrenaline (6×10^{-6}M) on efflux coefficient for ^{42}K loss into Potassium chloride Krebs.
Ordinate: rate coefficient (min^{-1}).
Abscissa: time after removal from radioactive solution

TABLE IX

Effect of lanthanum on inulin and mannitol spaces
(mls/100 g; n=4 for each value)

Extracellular Marker		Control	Lanthanum 200 μM	Lanthanum 2mM	Lanthanum 20mM
Inulin	60 mins†		50.5	67.9	93.7
			2.4	4.9	11.2
	120 mins	44.1	166.2	136.0	166.2
		1.9*	9.6	17.2	11.3
				49.3**	
				2.7	
Mannitol	60 mins	56.5	--	56.1	47.5
		2.6		1.7	1.9
	120 mins	66.0	--	61.5	52.4
		3.3		1.7	2.5

†time of exposure to radiotracer, containing lanthanum (except controls)
*standard error of mean
**preexposure to 2mM lanthanum for 20 minutes and then uptake in absence of lanthanum in the solution.

to papaverine during ^{42}K efflux but 5 of these experiments were confused by previous exposure to isoprenaline. In the remaining experiments, papaverine had no effect in 3 and appeared to decrease efflux by about 20-30% in the other 3. Papaverine did not affect uptake of ^{42}K in polarised or depolarised tissues.

TABLE X

Effect of Isoprenaline (5×10^{-6}M) on the Uptake of ^{42}K by the Longitudinal Muscle of Pregnant Rat Myometrium (mmoles/kg, n=4)

Treatment	Polarised ^{42}K	Polarised K	Depolarised ^{42}K	Depolarised K
Control 10*	7.9 0.6	39 3.9	47.8 4.9	93.6 7.8
Isopren 10	10.0** 0.7	38.2 1.8	42.6 5.2	97.5 3.1
Control 30	18.3 2.3	46.9 5.4	66.9 3.4	100.7 2.6
Isopren 30	27.4** 1.2	43.8 2.9	60.9 3.6	96.8 3.6
Control 60	28.9 0.7	44.9 1.7	93.8 6.8	92.8 1.6
Isopren 60	42.4** 0.4	54.4 4.7	90.4 4.4	94 2.8

*time of exposure in radioactive solution (in mins).
**$p<0.05$

4. ^{45}Ca and ^{89}Sr

Isoprenaline, chlorpromazine, SKF-525A and papaverine failed to affect the uptake of ^{45}Ca in normal Tris Buffered Krebs (Table XI). Values in the presence of drugs are not significantly different from control values ($p>0.05$).

TABLE XI

Isoprenaline, SKF 525-A, Chlorpromazine on ^{45}Ca Uptake
(60 minutes uptake)

Treatment	mmoles/kg	Treatment	mmoles/kg
$Control_1$	2.81 0.15	$Control_2$	4.81 0.37
Isoprenaline $5 \times 10^{-6}M$	3.23 0.16	SKF 525-A $2 \times 10^{-5}M$	5.00 0.43
Papaverine $2.5 \times 10^{-5}M$	2.95 0.21		
Chlorpromazine $2 \times 10^{-5}M$	2.77 0.30		

IIID Further interactions of lanthanum with rat myometrium

1. ^{45}Ca and ^{89}Sr fluxes

When ^{45}Ca and ^{89}Sr uptake by rat myometrial tissue is carried out in the presence of 2mM lanthanum, the uptake is inhibited by 50-60%, as shown in Table XII. Figure 34 shows the effect of lanthanum on efflux when applied during ^{45}Ca and ^{89}Sr efflux into Ca-containing and Ca-free solutions. Lanthanum increases ^{45}Ca efflux into Ca-free solution, but not into calcium-containing solution. It increases ^{89}Sr efflux into Ca-free or Strontium-containing medium. The effects of lanthanum on efflux are compared to those of lithium in Figure 34, because these agents have distinctly different effects

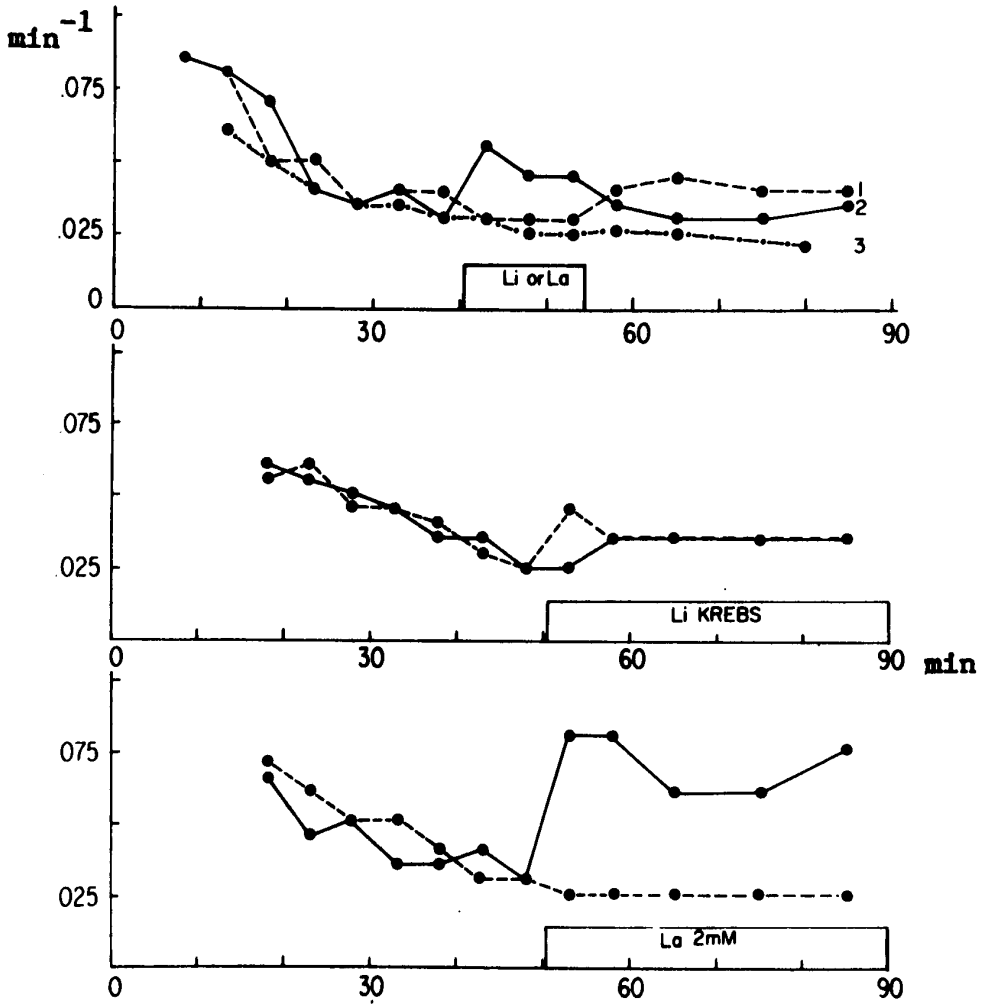

Figure 34. Effect of lanthanum and Lithium Krebs on efflux of ^{45}Ca and ^{89}Sr.

Top: Efflux of ^{89}Sr into 2.47mM Strontium Krebs.
Curve 1 exposure to Lithium Krebs as shown
2 exposure to lanthanum (2mM)
3.Control

Centre: Efflux of ^{45}Ca into Ca-free Krebs —
and calcium-containing Krebs ·---·
Exposure to Lithium Krebs as shown

Bottom: Efflux of ^{45}Ca into Ca-free Krebs — and calcium-containing Krebs ·---· Exposure to lanthanum 2mM as shown

Ordinate: efflux coefficient (min^{-1})
Abscissa: Time in minutes after removal from radioactive solution.

TABLE XII

Effect of Lanthanum on ^{45}Ca and ^{89}Sr Uptake by Longitudinal Muscle of Pregnant Rat Myometrium

		Time of Uptake (mins)		
		10	30	60
^{45}Ca mmoles/kg	Control	2.61 0.19	3.52 0.39	4.81 0.37
	La 2mM	1.74† 0.13	2.26† 0.07	2.31† 0.09
Ca space mls/100 g	Control	105.5 7.6	142.0 15.7	194.4 13.9
	La 2mM	70.4† 5.4	92.0† 2.9	94.6† 4.6
^{89}Sr mmoles/kg	Control	--	--	3.97 0.26
	La 2mM	--	--	1.62† 0.06

†$p < 0.05$

on contractile activity of the uterus (see Discussion). Lithium increases both calcium and strontium efflux, into either Ca-free or calcium/strontium containing medium. However, only in the case of ^{45}Ca efflux into Ca-free medium does Lithium Krebs cause an immediate increase in efflux. The increase in ^{89}Sr efflux occurs after return to Normal Sodium Krebs, such that this could be interpreted as lithium causing a decrease in ^{89}Sr efflux. When the effect of lanthanum on

^{45}Ca content is investigated in terms of counts per minute remaining in tissues exposed to Ca-free solutions with or without lanthanum (lanthanum throughout the washout period), there is no significant difference between the groups of tissues until 60 minutes (Table XIII). Lanthanum did not affect net calcium content in Ca-free solutions (content 0.721 ± 0.03 for control and 0.710 ± 0.09 for lanthanum treated tissues.

2. The effects of drugs on ^{45}Ca uptake measured after lanthanum wash.

Since lanthanum increases efflux of calcium tissues, possibly by removing calcium from non-specific binding sites, and has been postulated to prevent calcium efflux from cells (van Breemen and McNaughton, 1970), it was utilised in this study as a tool in an attempt to reveal any increase in ^{45}Ca uptake during exposure to drugs. Uptake of ^{45}Ca was carried out in the same way as previously described for experimental and control tissues (Methods, IIF). After 60 minutes in radioactive solution, however, tissues were placed in Ca-free Krebs solution containing 2mM lanthanum, for a further period of 60 minutes, after which tissues were counted and control and experimental tissues compared. Uptake is expressed as mmoles/kg wet weight by dividing count min^{-1} kg^{-1} by counts min^{-1} $mmole^{-1}$ of of the uptake medium. Similar experiments were carried out using uptake of ^{89}Sr and subsequent washout in Ca-free solution containing lanthanum for 10 minutes.

Under these conditions, the effects of drugs on ^{45}Ca or ^{89}Sr

TABLE XIII

Effect of Washout in Ca-free Solution, with or without lanthanum, on ^{45}Ca Content after 60 minutes Uptake (mmoles/kg)

Isotope	Washout medium	Time in washout medium (mins) after end of uptake		
		10	20	60
^{45}Ca	Ca-free	1.46*	0.906	0.466
		0.098	0.032	0.080
	Ca-free + La	1.26	1.12	0.262**
		0.17	0.012	0.013
^{89}Sr	Ca-free	0.750	0.471	---
		0.069	0.043	
	Ca-free + La	1.36	0.509	---
		0.21	0.015	

*means of 4 tissues each with standard error of mean
**$p<0.05$

uptake were, with one exception, not different from those experiments in which uptake was measured without any washout (Section IIIB 4. and Table VII). When tissues were exposed to hypertonic potassium chloride during ^{45}Ca uptake, and then treated with washout in Ca-free lanthanum solution, ^{45}Ca content was greater than controls (Table XIV). The increase in ^{45}Ca content induced by Lithium Krebs was still apparent. However, in an experiment in which uptake of ^{45}Ca was carried out in Lithium Krebs for all tisses, and subsequently washed out in either Ca-free Krebs solution or Ca-free Krebs solution containing 2mM

TABLE XIV

Effects of Drugs on ^{45}Ca and ^{89}Sr Uptake after Lanthanum Wash[†]
mmoles/kg (n=4)

Experiment	mmoles/kg	Experiment	mmoles/kg
1. Control ^{45}Ca	0.386 0.040	4. Control ^{45}Ca	0.098 0.006
KCl Krebs	0.392 0.072	Li	0.135†† 0.009
Li Krebs	0.495†† 0.036	Li + isoprenaline	0.129†† 0.006
2. Control ^{45}Ca	0.194 0.037	Li + papaverine	0.146†† 0.011
KCl 40mM (hypertonic	0.314 0.059	5. Control ^{45}Ca KCl Krebs	0.371 0.06
3. Control ^{45}Ca	0.211 0.023	ACh in KCl Krebs 1.4×10^{-5}M	0.294 0.041
ACh 1.4×10^{-5}	0.198 0.023	Oxytocin in KCl Krebs 1 mU/ml	0.312 0.034
Oxytocin 1 mU/ml	0.193 0.044	6. Control ^{89}Sr	1.45 0.138
ACh during washout	0.200 0.032	Li Krebs	1.58 0.123
Oxytocin during washout	0.184 0.009	KCl 40mM	1.87 0.129
		Ach	1.48 0.18
		Oxytocin 1 mU/ml	1.20 0.16

[†]uptake of ^{45}Ca or ^{89}Sr for 60 minutes followed by 60 minutes (^{45}Ca) or 10 minutes (^{89}Sr) washout in Ca-free solution containing 2mM lanthanum.

††$p < 0.05$ from contol.

lanthanum, the ^{45}Ca content was greater in those not exposed to lanthanum in the washout medium (Table XV). Table XIV also shows (experiment 3) that acetylcholine and oxytocin failed to affect the rate of loss of ^{45}Ca into Ca-free solution containg 2mM lanthanum. A number of possibilities were anticipated in this experiment. It is not certain that lanthanum prevents all cellular efflux. If lanthanum acts merely to remove non-specifically bound calcium, effects of agonists to either increase calcium permeability, release intracellular calcium or prevent efflux might have been revealed under these conditions.

Experiment 4 in Table XIV was designed to determine whether the presence of isoprenaline or papaverine could inhibit the increase in ^{45}Ca uptake due to Lithium Krebs. Such an inhibition was not observed. When lanthanum was present during uptake, it only partially inhibits the increased uptake due to Lithium Krebs (Table XV). Note that in this case, lithium caused a large increase in uptake without washout in lanthanum.

When tissues were exposed to Ca-free solution until there was no response to acetylcholine and then treated with lanthanum for 30 minutes, a total of 1.02 mmoles/kg calcium remained in the tissue. When tissues were exposed to ^{45}Ca for 60 minutes in the presence of 2mM lanthanum and then transferred to Ca-free Krebs solution containing 2mM lanthanum for 30 minutes, ^{45}Ca content was 0.147 ± 0.07 mmoles/kg.

TABLE XV

Effect of Lanthanum during Washout and during Uptake on Lithium Krebs induced Increase in ^{45}Ca Uptake

Treatment			Ca (mmoles/kg)
Uptake (60 minutes)	Wash	Time	
Control	Ca-free	60	0.175 ± 0.008
Li Krebs	Ca-free	60	0.321 ± 0.03
Li Krebs	Ca-free + La 2mM	60	0.230 ± 0.05
Control	--	nil	3.81 ± 0.019
Li Krebs	--	nil	6.25 ± 0.33
Control + La 2mM	--	nil	1.65 ± 0.12
Li Krebs + La 2mM	--	nil	2.63 ± 0.15

3. ^{140}La uptake by myometrium

When tissues were exposed to ^{140}La, uptake was linear with time up to 1 hour (Table XVI). Owing to the very large size of the lanthanum space, it has only been calculated for one value. Lanthanum uptake was slowed by calcium, but not be strontium (Table XVI). Uptake represented an accumulation of lanthanum and for tissues exposed to 200 μM represents an increase in weight of 4.3 μg/mg of tissue, due

TABLE XVI

Uptake of ^{140}La by Longitudinal Muscle of Pregnant Rat Myometrium (mmoles/kg wet weight, n=4)

Concentration lanthanum in uptake medium	Uptake medium	Time of Uptake (mins) 3	10	20	60
20 μM La	Ca-free	0.200*	0.408	1.12	2.16
		0.027	0.020	0.18	0.065
20 μM La	Ca 2.47mM	0.132	0.285	0.393	2.78
		0.024	0.039	0.025	0.4
20 μM La	Sr 2.47mM	0.184	0.343	1.16	3.4
		0.054	0.037	0.23	0.80
200 μM La	Ca-free	--	2.97	4.96	14.4
			0.175	0.30	2.94

*represents a lanthanum space of 1.15×10^4 mls/kg

to uptake of lanthanum. A simple calculation using Avogadro's number shows that lanthanum, assuming that it is not taken up intracellularly and using a surface to weight ratio of 4.1 cm^2/mg, would be bound at a value of 1×10^{16} molecules/cm^2, assuming an even distribution. The ionic radius of the lanthanum atom is 1.15 Å; thus 1.89×10^{15} molecules would cover an area of 1 cm^2 in a monomolecular layer, assuming perfect packing.

When efflux is carried out for ^{140}La after 3 hours loading, the curve is essentially a horizontal line (t 1/2 > 1000'). As

shown in Figure 35, subtraction of the bound fraction yields a curve which in control tissues had a t 1/2 of 234 minutes. Bound fraction had a value of 7-10 mmoles/kg. Tissue space for lanthanum was 2×10^5 mls/kg. After effluxing for 4 hours, only 12% of tissue lanthanum had exchanged. Calcium or strontium increased the efflux of lanthanum, as shown in Figure 35, after a delay of 20-60 minutes. Quantitatively, the uptake of ^{140}La into whole tissues was considered to be incompatible with the hypothesis that lanthanum is bound only at the surface of cells. For this reason, myometrial tissues were subjected to fractionation techniques, after uptake of ^{140}La into whole tissues.

4. ^{140}La content of myometrial fractions

Two experiments were carried out in which non-pregnant myometrial tissue was incubated with ^{140}La and then homogenised and fractionated. In order to ensure that ^{140}La was not merely being taken up by intracellular organelles during homogenisation, one half of the tissues were homogenised in the presence of added, non-radioactive lanthanum. Figure 36 shows the binding of lanthanum in terms of µg/g protein in the fraction of the two portions of uterine tissue. Lanthanum moved the mitochondrial fraction down the sucrose gradient compared to the routinely fractionated myometria. Thus this was more apparent in tissues where non-radioactive lanthanum was added. The protein and ^{140}La data have been combined, to give the total protein and total µM La/g protein for the plasma membrane, endoplasmic reticular and mitochondrial fractions as shown in Table XVII.

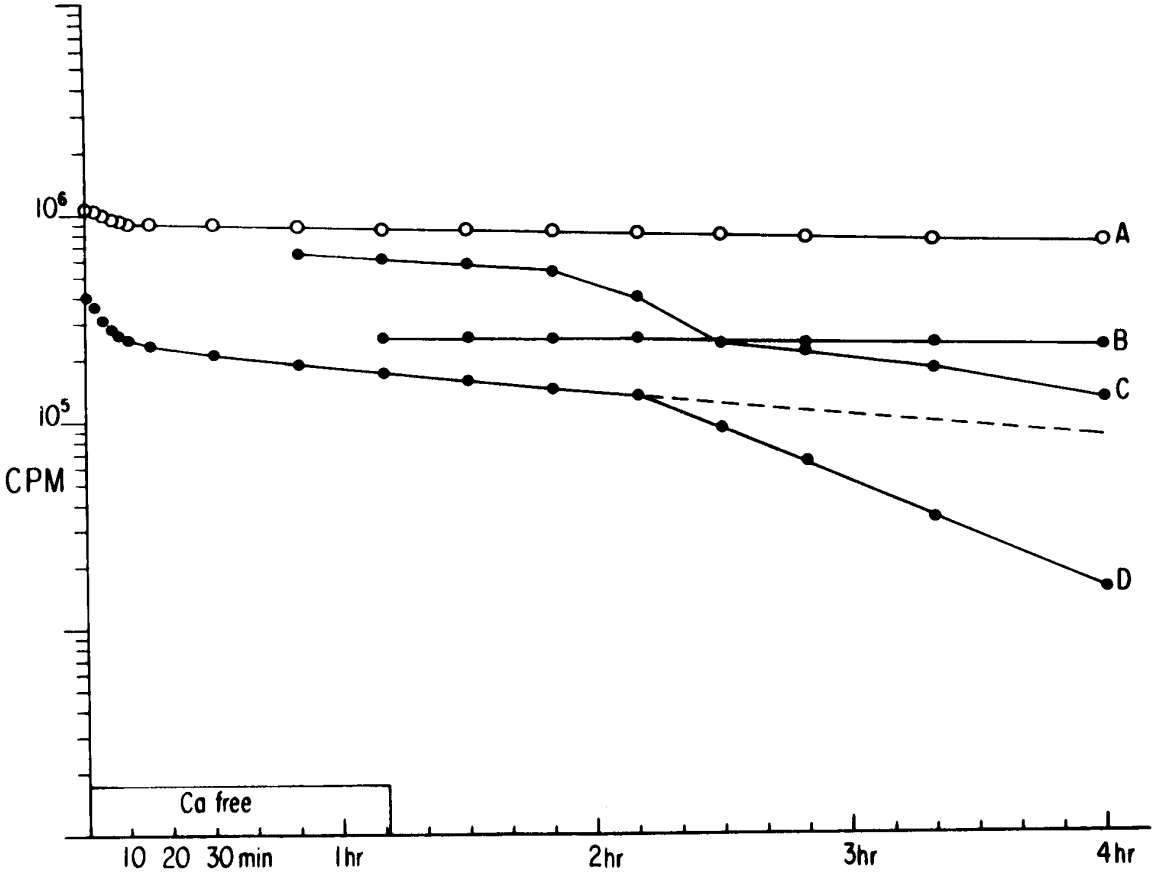

Figure 35. Efflux of ^{140}La from longitudinal muscle of pregnant rat myometrium. A. counts min^{-1} remaining in tissue. B. control efflux, bound fraction subtracted. In Ca-free throughout. C. counts min^{-1} after subtraction of bound fraction; switch to 2.47mM strontium medium as indicated at lower left. counts min^{-1} after subtraction of bound fraction. D. as for C but switch to Ca-containing medium.

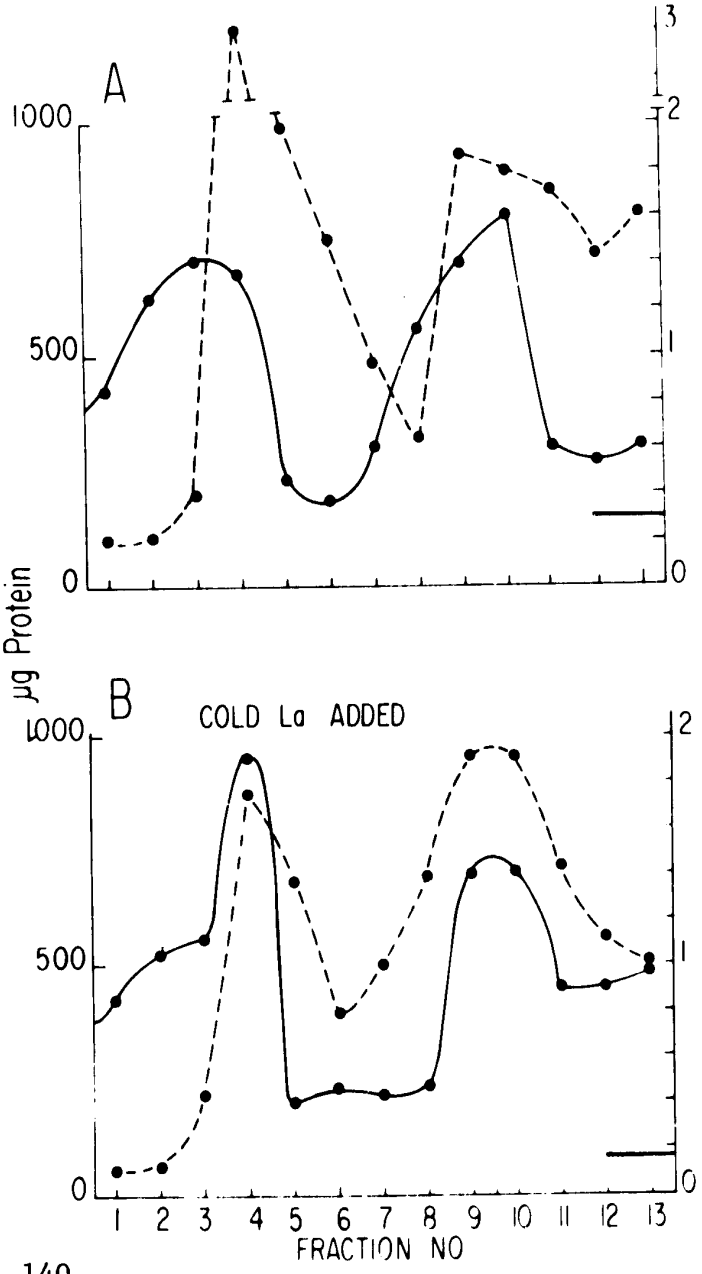

Figure 36. ^{140}La content of fractions of non-pregnant myometrial tissue. A and B represent equal portions of uteri from 8 rats, which had been incubated in ^{140}La for one hour. In B, tissues were homogenised in 0.25M sucrose containing 10 µM non-radioactive lanthanum. Continuous curves: total µg protein in that fraction. Broken curves: µM of lanthanum per g protein. Plasma membrane corresponds to fraction numbers 2, 3 and 4. µmoles lanthanum/g protein of soluble fraction (supernatant after first centrifugation) is shown at bottom right of each plot. Ordinate (left) µg protein per fraction (= 1 ml) and (right) µmoles lanthanum/g protein. Abscissa: fraction number after collection from sucrose gradient.

TABLE XVII

Lanthanum Content of Myometrial Fraction

Fraction	GROUP 1*			GROUP 2		
	µg protein	µM La/g protein	Tube Nos**	µg protein	µM La/g protein	Tube Nos
FI	2040	1.005	2,3,4,	1915	1.205	2,3,4
F2	885	1.13	5,6,7,8	726	1.46	5,6,7
F3	1850	1.79	9,10,11	1595	1.83	8,9,10

FI = Plasma Membrane
F2 = Endoplasmic Reticular
F3 = Mitochondrial

*1 is group of tissues homogenised in 0.25M sucrose containing 10 µM lanthanum.
**fraction numbers to be combined were judged from a continuous plot of the absorbance at 280 mµ, monitored during collection of fractions.

It can be seen that dilution with non-radioactive lanthanum had little or no effect on the amount of lanthanum bound to fractions. Furthermore, the amount of lanthanum bound to the mitochondrial fraction exceeded that of the plasma membrane fraction.

IV. DISCUSSION

IV. DISCUSSION

Tissue Preparation

The longitudinal muscle of the pregnant rat myometrium possesses a number of advantages in comparison to whole or even stripped non-pregnant rat uterus for the study of drug-induced ion movements and their relation to electrical and mechanical events. Firstly, the relatively large size of the cells during pregnancy permits intracellular membrane potentials to be recorded with comparative ease. Secondly, the preparation is sufficiently thin that diffusion delays should be small (Keynes, 1954). Thus effects of changing the external environment should be manifest rapidly; induced changes in permeability to radiotracers during efflux should likewise occur with a minimum of delay. Thirdly, the stripped preparation consists of a pure muscle type and thus should demonstrate a greater consistency in its responses than would a tissue of mixed muscle, and certainly more consistent than muscle mixed with endometrium. Finally, because the uterus is increased in size during pregnancy, enough tissue can be obtained from the same animal for control data and for several experimental procedures. The use of myometrial tissues as thin as 75 μ has been previously reported by Kao (1967). In the present study, the effects of the handling involved in stripping tissue was initially investigated, to ensure that this procedure did not impose undue limitations upon the responses of the preparation. Some degree of cell damage is indicated by the higher sodium, lower potassium and higher calcium values for

fresh, moistened tissues, than those from studies of non-pregnant whole uteri (van Breemen and Daniel, 1966; Daniel and Robinson, 1970). However, the mean membrane potential reported here for stripped tissues, although less than that reported by Marshall (1962), is not greatly different from that reported by Kuriyama and Csapo (1961) and Taylor (1969). The question therefore arises as to whether the downhill movements are due to a general decrease in the active ion transport properties of all cells, or due to a proportion of inviable cells. The increased extracellular space measured in stripped tissues using inulin (compared to those of Casteels and Kuriyama, 1965) would indicate that the latter possibility is more likely. Inviable cells may be assumed to lose the membrane permeability and active ion transport properties, such that they essentially become part of the extracellular space. This would also explain the increased proportion of 'poor' membrane potential values (of only a few mV) recorded from stripped tissues, and the increased size of the fast fraction of ^{42}K efflux. The calculation of intracellular concentrations of sodium and potassium on the basis of the inulin space determined in this study yields values which are in good agreement with previous studies (Casteels and Kuriyama, 1965). The finding that the membrane potential decreased towards the end of gestation in stripped tissues, which is in accord with previous observations (see Introduction IA for references), further indicates that stripping does not reduce cells to a common low level of activity, but rather, damages a proportion of cells. On immersion

in Krebs solution, the stripped tissue loses potassium and gains sodium, but subsequently values remain stable in Bicarbonate Krebs for at least 4 hours.

In view of the above evidence, and since the use of whole uterus is open to considerable criticism it was decided to exploit the advantages of stripped tissues.

Action Potential Processes

The aim of the present investigation was to correlate electrical, mechanical and ionic mechanisms of drug action in the uterus; this inevitably involved establishment of control parameters. A number of observations concerning action potentials recorded from the stripped preparation are relevant. Action potentials with two rising phases have been repeatedly recorded using intracellular microelectrodes. Kao (1967) has discussed how complex spikes can arise from sucrose-gap recordings. When such spikes are recorded from intracellular electrodes, an explanation is more difficult. Records such as Figure 5 of this thesis confirm that complex spikes are due to intercellular interactions.

This presents the apparent paradox: the myometrium must possess low resistance electrical connections between each cell for such marked interactions to occur, yet cells are still firing asynchronously. This could be accounted for, if cells had a relatively long refractory period. However, in Figure 5, the cell was firing at a rate of about 1/sec, and spike frequency in the presence of acetylcholine can rise to 5/sec. There is a surprising paucity

of information concerning the refractory period of smooth muscle cells. Goto, Kuriyama and Abe (1961) reported a value of 0.1-0.2 seconds for mouse uteri, using extracellular stimulation. However, electrical stimulation of uterine muscle fails to evoke spikes when applied between spontaneous contractions unless higher intensities (relative to that required just before a spontaneous train of spikes) are used (Casteels and Kuriyama, 1965). Such cyclic changes in excitability or refractoriness are presumably linked ultimately to the metabolism of cells, and act to regulate the length and frequency of spontaneous contractions. Under normal conditions, such a process would presumably initiate relaxation by slowing action potentials and dissociating cells.

In view of the complex intercellular electrotonic interactions, the actions of drugs on the shape of action potentials are difficult to evaluate. Repolarisation could be due to potassium efflux, inflow of chloride or pumping out of sodium. Barium ions have been reported to slow the falling phase of the action potential of taenia coli (Bülbring and Tomita, 1968b; Kuriyama, 1970). The effect of barium on uterine action potentials was not conclusive. Action potentials with two rising phases and in some cases, plateaus were frequently observed in the presence of barium but these could be due to intercellular interactions. Addition of acetylcholine to a preparation showing slowed repolarization of only a few mV, caused uncoordinated spikes, which presumably implies greater intercellular interactions due to pacemaker formation in a number of areas

of the tissue. Kuriyama (1970) suggests that the spike plateau in response to barium is due to an increase in sodium conductance, as it is abolished in the absence of sodium. In the uterus, both double spikes and delayed repolarisation (of a few mV) have been observed after barium in the absence of sodium; this may mean that these effects of barium are merely a reflection of intercellular interactions. The fact that lanthanum abolishes complex spikes in the presence of barium, could be interpreted as a lanthanum-induced change in ionic permeabilities (Na or K) or a dissociation of cells.

Strontium in the external medium in the absence of calcium rapidly changes the action potential by delaying depolarisation and/or repolarisation, without altering contractions. A relatively short time period was necessary before an effect on the action potential was observed. It has been previously reported (Daniel, 1963; Marshall, 1965; Abe, 1971) that strontium is able to replace calcium and support spontaneous action potentials. Results reported here suggest that strontium is less efficient in this regard. The rapidity of the effect of strontium also tends to support the view that the depolarisation phase of action potentials is due to calcium influx under normal conditions. An alternative explanation would be that strontium fails to substitute for calcium in controlling sodium permeability and hence indirectly affects the inward (sodium) current. Other results in the present study support the hypothesis that the depolarisation process is due to calcium. Thus action potentials have been recorded from stripped preparations immersed

in Lithium Krebs containing 26mM sodium, with little or no decrement to spontaneous action potentials or their maximum rate of rise, for periods of over 30 minutes. When all sodium is removed, action potentials were recorded for periods in excess of 20 minutes. Kao (1967) rejected data of Daniel and Singh (1958) concerning spikes recorded in sodium-free solution, on the basis of the thickness of preparation. Since the thickness of the tissue in the present experiments is comparable to that used by Kao, diffusion of sodium out of the tissue cannot be considered to be delayed due to tissue thickness. [In fact however, Daniel (1970) showed that diffusion delay of sodium in the extracellular space was due, not to a tortuous diffusion path, but to a low diffusion coefficient.] As discussed in the Introduction (Part IA) the problem of sodium removal is compounded by the absence of a satisfactory substitute. Thus, the above data could be interpreted to mean that lithium is able to substitute for sodium and carry the inward current. This is suggested by the failure of lithium to alter ^{22}Na fluxes (Daniel, *et al.* 1970). Sucrose, in these experiments, caused the appearance of prolonged action potentials, especially at the central portion of a spike train, a time when pacemaker-type action potentials are often observed in Normal Krebs. This may be explained as either due to the absence of sodium or a direct effect of sucrose. Since neither lithium (this study) nor choline (Daniel and Singh, 1963) substitution caused such an effect, the latter explanation seems more likely.

Deficiency of calcium in the stripped tissues led to depolarisation, and absence of action potentials. Drug induced stimulation did cause discharge of small spikes in Ca-free solution, after 10-15 minutes. This data supports the hypothesis that calcium is the current carrying ion.

Effects of drugs on Potassium fluxes

One aim of the present investigation was to study the effects of drugs on potassium ion movements, and determine whether such effects could be correlated with electrical and/or mechanical activity. The elution curve of ^{42}K from this preparation can be reduced by graphical analysis to the sum of three exponential functions, each of which can be considered to be some function of the transfer of potassium from one compartment. No attempt was made to give these compartments a physical meaning. Efflux from the slowest fraction is considered to be intracellular potassium, because egress is from a single homogenous compartment [on the basis of parallelism of the count min^{-1} and counts min^{-2} plot, and the slope of unity of the log-log plot of these parameters (Persoff, 1960)]. The rate constant for efflux of ^{42}K from pregnant tissue is approximately twice that observed for non-pregnant tissues by Daniel and Robinson (1971a,b). This could be a reflection of increased passive permeability of the pregnant tissue; such an increase has been suggested from electrophysiological data (Casteels and Kuriyama, 1965) and from the effect of progesterone treatment on potassium uptake and efflux from pregnant rabbit myometrium (Jones, 1970). The "bound" fraction of

^{42}K cannot be considered to be merely due to variation in experimental data, because only rarely was such a fraction absent. If the non-parallelism (albeit a small deviation) were due to experimental error, a "bound" fraction would be expected in only 50% of curves. The value for this fraction was small and variable; its relationship to osmotically inactive intracellular potassium, if such exists, cannot be determined.

The use of ^{86}Rb to investigate permeability of membranes to potassium has been advocated for the heart (van Zweiten, 1968). An identical performance of the ^{86}Rb and ^{42}K has also been demonstrated in the rabbit lens (Paterson, 1970). In contrast, Adrian and Slayman (1966) and Taylor (1969) have demonstrated discriminatory behaviour of muscle membranes for potassium and rubidium. In the results obtained here, the rate constant for egress of rubidium was approximately one half of that of ^{42}K. Nevertheless, drugs were found to affect efflux of ^{86}Rb in a quantitatively similar manner to that of ^{42}K. The effects of drugs have been studied by using each tissue as its own control, by applying the drug during the slowest phase of efflux. Since the diffusion of electrolytes from one compartment to another will be influenced by changes in the electrochemical gradient, most of the tissues used were depolarised with Potassium chloride Krebs, when drugs do not influence the elctrical gradient, if any, across the cell membrane. Under these conditions, effects on potassium ion movements should be easier to interpret and should clarify the results of experiments

using polarised tissues.

The results of the present investigation are not consistent with the view that changes in potassium permeability can account for the electrical events induced by drugs. Three excitatory drugs have been compared. Acetylcholine (and carbachol) increased efflux of ^{42}K in both normal and depolarised tissues, while the effect on influx was variable. The increased efflux in Normal Krebs solution could be explained as being due to induced action potential discharge, secondary to the increased potassium outflow which is thought to occur during the falling phase of the action potential. This cannot be the sole explanation, however, because the increase persists when depolarised tissues are used. Thus, acetylcholine must be considered to increase the passive permeability of the membrane to potassium. Burgen and Spero (1968) used carbachol to stimulate guinea-pig intestine and dissociated the dose-response curves for drug induced potassium efflux and for increase in tension. Thus higher doses (by a factor of 100) were necessary to affect potassium efflux. This was interpreted to mean that acetylcholine is able to act exclusively on pacemaker cells at low doses. In the present study, contractility for isometric tension and efflux were not measured concurrently. Dose-response curves given in Figure 25 are not comparable to the flux responses because of the different buffering system, and tris was shown to decrease tissue sensitivity to drugs. Thus, it is not possible to plot directly comparable dose-response curves and

speculation on this aspect does not seem worthwhile.

Noradrenaline, in the presence of propranolol, increased potassium influx and efflux in depolarised tissues, representing a true increase in permeability of the cell membrane to potassium. Efflux of potassium is primarily a passive process and effects on active transport are unlikely. Since noradrenaline, even in the presence of propranolol, only in some strips of myometrium caused a contraction, and sometimes caused a slight relaxation prior to contraction, the possibility of a residual effect on β-receptors [which are the dominant type of catecholamine receptor in this tissue (see Marshall, 1970)] must be considered. Evidence that α-receptor stimulation is responsible for increased potassium permeability may be summarised: 1) in contrast to β-agonists, noradrenaline does not cause persistent relaxation of the tissue after washout, implying only slight β-receptor stimulation; 2) the increase in potassium efflux is prevented by α-receptor blocking agents; 3) selective β-receptor agonists, in the presence of α-blockade do not increase potassium efflux during the period immediately following exposure to the drug, but cause a distinctly different type of response (*vide infra*); 4) when adrenaline is used, which has both α and β receptor agonist activity, β-blockade converts the response to one similar to that due to noradrenaline.

In smooth muscles, the same transmitter may produce excitation in one tissue and inhibition in another. Such is the case for noradrenaline on the uterus, compared to its effect on intestinal muscle. It has been reported (Jenkinson and Morton, 1967a,b) that

noradrenaline consistently increases potassium permeability in the depolarised taenia coli, and this was considered to be the most likely explanation of the hyperpolarisation in this tissue. The results reported here suggest that α-receptor stimulation mediates a similar increase in potassium permeability in the uterus. (Under these circumstances, it must be considered that phenylephrine, considered to be a pure α-receptor agonist, must be considered not to stimulate α-receptors in this tissue). However, in the absence of concurrent data on mechanical activity, it could be argued that the α-receptor response of an increase in potassium permeability actually accounts for the unexcitability of pregnant tissue to α-receptor agonists. It would be of interest to know whether α-receptor agonists increase potassium permeability in non-pregnant tissues, where α-excitation is more readily demonstrable.

The third excitatory drug used in this study was oxytocin. At doses of 1 and 10 mU/ml, oxytocin failed to increase either efflux or influx of potassium in this tissue, although such doses invariably caused contraction and spike discharge in polarised tissues and caused contraction in depolarised tissues. Marshall (1961, 1964) has investigated hormonal influences on the action of oxytocin on the uterus, and found that progesterone dominated tissues were unresponsive to oxytocin, and did not exhibit spontaneous activity. In the present study, as stated in the methods, animals were killed as near to term as possible. Date of conception was not known accurately but 17-18 day pregnant uteri are readily distinguishable

from uteri at term. Tissues from animals 17-18 days pregnant were not used in experiments involving oxytocin. In any case, virtually all tissues used here were spontaneously active and on this criterion should have been oestrogen-dominated and responsive to oxytocin. In fact, a dose of 1 mU/ml of oxytocin invariably caused contractions and higher doses (5 mU/ml) caused a prolonged contraction.

The findings concerning ^{42}K fluxes have important implications in relation to the action of excitatory drugs on the uterus. Firstly, the increase in potassium permeability seen with acetylcholine is not due to an aritfact caused by mechanical deformation of the extracellular space. Secondly, it suggests that the action of oxytocin is more selective than the general increase in the permeabilities to ions suggested by Marshall (1964). Finally, this result raises again the question as to the repolarisation process in smooth muscle. This has been discussed more fully previously; however, it is convenient here to make a calculation concerning the possibility of observing an increase in potassium efflux due to increased action potential discharge. Assuming that the repolarisation of a 50 mV action potential is due to potassium, calculation of the amount of potassium required to carry the current for repolarisation is possible, in precisely the same way as described for the influx of calcium during depolarisation described in the Introduction. Such a calculation yields a figure of 7×10^{-12} moles potassium per action potential or 3×10^{-10} moles

potassium/mg/min for efflux assuming a persistent mean spike discharge of 100 per minute. This can be compared to the values of efflux calculated from values of rate constants and sizes of compartments from Table II. For compartments B and C, approximate values for efflux are 5×10^{-10} moles potassium/mg/min and 1.2×10^{-10} moles/mg/min respectively (using $M_o = kcv$, where M_o is outflow of potassium, k is the rate constant, c the initial concentration in that compartment, and v is the volume of cells). These limits are of the same order of magnitude as the figure above for the estimate of efflux due to repolarisation. Hence it is not unreasonable that action potential discharge would be manifest as an increase in potassium efflux.

To summarise the effects of excitatory drugs on potassium movements, cholinergic drugs and α-adrenergic agents increase potassium permeability, and these effects occur in the absence of electrical events; oxytocin does not affect potassium permeability. The relationship of these effects to electrical and mechanical effects are not readily obvious. An increase in potassium permeability would be expected to hyperpolarise the membrane and prevent spike discharge. Since the major portion of the increase in permeability is not a consequence of electrical changes (action potentials or depolarisation), this appears to be a direct effect of acetylcholine. An increase in P_K under these circumstances may be considered to be either a secondary side-effect of drug-induced increased ionic permeabilities of the membrane, or a step in the sequence of events leading to excitation. If part of the

repolarisation process is due to increased g_K, then a more rapid increase caused by acetylcholine could be envisioned as permitting faster spike discharge and enhancement of the induced contraction.

The possible mechanisms by which inhibitory drugs cause relaxation has been discussed in the Introduction (Part ID Spasmolytics). Marshall (1968) postulated that one cause of the hyperpolarisation observed in response to β-receptor agonists on the myometrium would be an increase in potassium permeability, which would move the membrane potential towards the potassium equilibrium potential. The equilibrium potential calculated for potassium in stripped myometrial tissue was 67 mV for the lowest mean $[K]_t$ obtained (36.4 mmoles potassium/kg wet weight).

Isoprenaline, acting exclusively on β-receptors in the presence of α-receptor blockade, caused a mean hyperpolarisation of 10.3 mV, and this occurred within 2-3 minutes of application. However, isoprenaline (and adrenaline), at the same concentration and under the same conditions of α-blockade, failed to increase the loss of potassium from either polarised or depolarised tissues at a similar time after application. 20-25 minutes after exposure to isoprenaline, ^{42}K efflux was increased, regardless of whether or not the drug was present. Such an effect could be due to a delayed increase in permeability, in turn due to either metabolic changes, or (in Normal Krebs only) return of mechanical activity and concommitant extrusion of ^{42}K in extracellular fluid accumulated

during relaxation. The delayed increase in potassium permeability was also induced by 10^{-4}M dibutyryl cAMP, which suggests that the effect of isoprenaline on efflux is mediated via the increase in cAMP levels of the cell. The long delay in the effect on efflux compared to that for the activation of adenyl cyclase (see Introduction ID) by catecholamines, suggests a very slow intermediate step or a sequence of many intermediate steps. Isoprenaline did increase the uptake of potassium from Normal Bicarbonate Krebs, but failed to do so in depolarised tissues. The latter observation means that increased influx cannot be accounted for on the basis of the delayed increase in passive permeability seen during efflux. However, calculation of the effect of a hyperpolarisation shows that influx would be expected to be increased by a factor of about 1.5 (using $\log_{10} \frac{M_i}{M_o} = \frac{\xi_K - \xi_m}{61}$ and assuming efflux does not change).

Thus the results concerning isoprenaline, which failed to alter efflux in depolarised tissues, are not compatible with the hypothesis that hyperpolarization is mediated by an increased potassium permeability.

The absence of effect of isoprenaline on the sodium pump in uterine smooth muscle, as observed by Daniel *et al.* (1970) has been discussed in the Introduction (Part ID). Of the three possible mechanisms of action to cause hyperpolarisation suggested by Marshall (1968) *viz.* increased P_K, increased sodium pumping and increased fixation of calcium, increased calcium fixation resulting in decreased

P_{Na} appears to be the only plausible explanation. Direct evidence for such an effect is lacking. The observations concerning an increased input resistance in the presence of isoprenaline deserve further investigation. A similar finding - hyperpolarisation and decreased membrane conductance - has been reported by Siggins, Oliver, Hoffer and Bloom (1971) for cerebellar cells, but they offerred no explanation for this effect. In uterine tissue it could reflect increased binding of calcium and a resulting decreased sodium conductance. Some evidence for sodium/calcium competition has been obtained from the use of lithium substitution (*vide infra*).

Papaverine in this study may have caused a small decrease in potassium efflux but the effect was variable and the data is not sufficiently clear to draw any conclusions concerning the effect of the drug on potassium permeability.

Effects of drugs on calcium movements and the relation to contractile responses

Another aim of the present experiments was to study the influence of drugs upon the movements of calcium, and to correlate such effects to mechanical activity. The theories of excitation-contraction coupling in smooth muscle cells may be simplified as: 1) calcium is released from a store, and 2) calcium enters from the extracellular space. The most direct way of studying which of these two mechanisms is acting during drug responses, is to measure ^{45}Ca exchange in tissues in the presence and absence of drugs. The conflicting observations concerning calcium fluxes in smooth muscle have been treated in the Introduction

(Part ID). In this study, measurement of ^{45}Ca uptake and efflux parameters in stripped uterine preparations was carried out as part of the preliminary experiments. A number of comments are worthwhile. Uptake of ^{45}Ca and total calcium content into stripped tissues was considerably greater than that reported by van Breemen and Daniel (1966). This is mostly due to the use of a higher concentration of calcium in the external medium. Thus the value quoted by Schatzmann (1961) for taenia coli incubated in 2.8mM calcium solution of 4.2 mmoles/kg wet weight is close to that obtained for the longitudinal muscle of the pregnant rat myometrium in a similar environment. van Breemen (1965) also found similar values for rat uterus in 2.5mM calcium solution (3-4 mmoles/kg depending on length of time in Krebs). A portion of the increased calcium content may have been caused by damage during the stripping procedure. Increased calcium content of smooth muscle due to dissection has been previously reported by van Breemen (1965), Bauer, Goodford and Hüter (1965), and Altura and Altura (1970). Preliminary experiments revealed that calcium values measured either by spectrophotometry or by exchange with ^{45}Ca were variable between animals, but were reproducible for portions of any one uterus. Parameters of ^{45}Ca fluxes established here were not different from those previously reported for uterine muscle (van Breemen and Daniel, 1966; Krejci and Daniel, 1970a,b). Thus uptake and efflux could not be represented by a single exponential function. When attempts were made to analyse efflux using an objective computer program, values for fitting the curve as the sum

of three exponentials gave inconsistent results, and even after subtraction of a bound fraction, the curve failed to show evidence of efflux from a single homogenous compartment even after 2 hours. These observations question the value of attempting to analyse efflux curves into three or more fractions in smooth muscle (see for example Lüllman, 1970). The presence of calcium in the external medium was found to increase the rate of loss of tracer from tissues; this has been extensively dealt with by Krejci and Daniel (1970b).

In view of the fact that Daniel (1963) has shown that strontium can not only substitute for calcium in supporting contractions to drugs, but also that this supportive role is lost very much more rapidly after removal of strontium from the medium than after removal of calcium, it is surprising that so very few studies on strontium isotope fluxes have been carried out in smooth muscle. In this study, values for the uptake and loss of ^{89}Sr differed only slightly from those of ^{45}Ca. The lack of correlation between loss of these isotopes into Ca-free solution and the rate of loss of contractile activity may be explained either as a reflection of the complete failure of the efflux curves to represent physiologically meaningful compartments, or that the fraction of calcium related to contractile events is negligible compared to the amount of non-specific calcium binding within the tissue. Probably both explanations apply.

Measurement of the uptake of ^{45}Ca and ^{89}Sr in the presence and absence of drugs, not surprisingly, did not, with one exception,

reveal any drug-induced increase in rate of exchange. It is notable that acetylcholine, in depolarised tissues, failed to increase ^{45}Ca uptake. This is in contrast to the results of van Breemen and Daniel (1966b). Due to the fact that Potassium chloride Krebs was observed to decrease uptake of ^{45}Ca (due to the effects of contraction, Krejci and Daniel, 1970a), their absolute values for acetylcholine-treated tissues were less than controls in Normal Krebs solution. In the studies reported here, ^{45}Ca was permitted to equilibrate with the extracellular space prior to addition of acetylcholine. Since contractions to acetylcholine were small and transient in depolarising Krebs solution, this does not necessarily mean that acetylcholine does not permit entry of extracellular calcium. Uptake of ^{45}Ca in Potassium chloride Krebs was decreased at 10 and 30 minutes after exposure but not at 60 minutes. Presumably at this time, when exchange of ^{45}Ca is slow, the effect of contraction on diffusion is no longer significant in this preparation where diffusion distances are very much smaller than in whole uteri.

In the presence of complete removal of sodium and replacement with Lithium Krebs, tissues contracted and under similar conditions took up more ^{45}Ca. Such an effect could be expected if lithium failed to substitute for sodium and compete with calcium for sites in the membrane, as has been postulated by Niedergerke (1963) for the heart. The increased efflux of ^{45}Ca in response to Lithium Krebs is difficult to interpret in the absence of knowledge of the location

of such calcium. However, since efflux was increased in Ca-free solution, when lithium did not cause contraction, the increased efflux is not related to calcium concerned with excitation-contraction coupling. The effect of lithium on the tension of uterine muscle supports the idea that this ion induces an increase in calcium influx from the extracellular fluid. Thus, lithium induced contractions are uniquely dependent upon external calcium. The decline in response to lithium on frequent exposure is presumably due to the replacement of intracellular potassium and sodium which occurs in Lithium Krebs (Daniel, *et al.*, 1970). The reason why Lithium Krebs fails to increase strontium uptake could be due to a decreased affinity of strontium for sites at which calcium and sodium normally compete. Strontium will support Lithium Krebs induced contraction but not as well as calcium does. A small increased uptake of ^{89}Sr would not be significant relative to nonspecific binding of this isotope. The response to replacement of sodium with potassium differs from that described for lithium, since it persists for extended periods of time (more than 20 minutes) in Ca-free solution.

Responses to repeated doses of acetylcholine and hypertonic potassium chloride also persist for periods of more than 20 minutes in Ca-free Krebs solution and the amplitude of contractions to the two agonists decline at approximately the same rate. The initial phase of the response to acetylcholine is, relative to

lithium contraction, insensitive to a decrease in external calcium concentration. Removal of calcium during the latter part of the acetylcholine-induced contraction causes relaxation. This is interpreted to mean that acetylcholine initially causes release of calcium from a store but the subsequent part of a response, if any, is due to calcium influx. At 37°C, acetylcholine and carbachol never cause persistent contraction of the myometrium anyway. After the initial response, the tissue relaxes and subsequently exhibits high frequency spontaneous-type contractions (low spike frequency relative to the initial acetylcholine-induced maximum spike frequency). This shape of contraction is distinct from the shape of Potassium chloride induced contraction which at higher doses consists of a contracture. This could be explained as the ability of potassium to cause a persistent release of calcium from a store. It is of importance to note that these experiments were carried out at 37°C. Daniel (1963) observed contracture in the presence of Potassium Sulphate Krebs at 37°C but tissues relaxed back to baseline at 24°C. The reverse was true for acetylcholine-induced contractions.

In vascular muscle, good evidence is available to indicate elevation of $[K]_o$ and norepinephrine release can cause inward movement of loosely bound calcium and tightly bound calcium respectively (Hinke, 1965; Wende and Peiper, 1970). On the basis of the data above it seems that acetylcholine and

potassium-induced contractions depend upon the same pool of calcium in the rat uterus. In terms of the sequestered superficial model of calcium influx (Daniel, 1965) the following explanation of contractile phenomena may be made:

1. Spontaneous contractions are controlled by superficial binding sites.
2. Acetylcholine (and oxytocin) causes a transient release from superficial and sequestered sites, enhanced by action potential discharge. Subsequent contractions are controlled by the superficial site and associated with spike discharge.
3. Potassium chloride has a similar initial excitatory action as in 2. Subsequent contraction is due to persistent depolarisation, releasing calcium from sequestered or superficial sites and permitting entry of extracellular calcium.
4. Lithium substitution for sodium is controlled by calcium entry from the extracellular space.

Having established some working model it should be possible to see whether it is supported by actions of inhibitory drugs on excitatory events. The approach to the elucidation of the mechanism whereby calcium is transferred towards the contractile proteins has been two-fold. Firstly, an effort has been made to localize the actions of drugs by the use of substances which have been reported

to interfere with one of the steps leading to the final response. Secondly, the so-called "lanthanum technique" (van Breemen and Lesser, 1970) has been used in an attempt to reveal changes in calcium fluxes induced by drugs.

A number of articles have been published concerning compounds which are considered to antagonise the binding of calcium at sites in membranes: chlorpromazine (Frankenheim and Shibata, 1968); desimipramine (Hrdina and Ling, 1970); local anesthetics (Bondani and Karler, 1970); SKF 525-A (Suarez and Bianchi, 1970; Kalsner, Nickerson and Boyd, 1970) and lanthanum (Weiss and Goodman, 1969; Weiss, 1970; van Breemen, 1969).

Three of these compounds, chlorpromazine, SKF 525-A and lanthanum have been used in this study. The effects of these three agents on acetylcholine, potassium chloride and calcium (in Ca-free Potassium chloride Krebs) induced contractions have been studied. In vascular smooth muscle, SKF 525-A can inhibit contractions induced by potassium without significantly affecting those due to norepinephrine. Calcium-induced contractions in Potassium chloride Krebs were also abolished in vascular muscle (Kalsner *et al.*, 1970). SKF 525-A was considered to prevent the potassium-induced inward movement of extracellular or superficial calcium. In skeletal muscle, Suarez, Kurtz and Bianchi considered that SKF 525-A acted in a similar manner to local anaesthetics to decrease the excitability

of the post-junctional membrane. On the frog rectus abdominus, SKF 525-A potentiated the contraction due to exposure to zero-calcium + EDTA. This was explained as potentiation of competitive curarizing substances due to depression of excitability of the post-junctional membrane. SKF 525-A in uterine muscle was found to decrease spike frequency in response to both acetylcholine and potassium chloride, but was able to antagonize acetylcholine contractions to a greater extent than those due to potassium chloride; it could relax acetylcholine induced contractions but not those due to Potassium chloride. SKF 525-A antagonised calcium-induced contractions. Thus the mechanism of action of SKF 525-A on uterine muscle is compatible with an action to decrease the excitability of the smooth muscle membrane (i.e. stabilization) and prevent some step related to calcium movement through the membrane. The rapidity of onset of effect in uterine muscle may reflect the greater lability of sequestered calcium in uterine muscle compared to vascular muscle. This may in turn be a reflection of the intimate relationship of calcium pools and the rapid membrane potential changes induced by action potentials. Vascular muscle, and especially aortic muscle, does not appear to depend to such an extent upon rapid membrane potential changes.

Chlorpromazine (10^{-5}M) prevented the mechanical but not the electrical response to potassium chloride and prevented increased ^{45}Ca uptake in response to potassium chloride in the taenia coli.

(Frankenheim and Shibata, 1968). Thus chlorpromazine can too be considered to prevent the movement of calcium across the cell membrane. In rat uterine muscle, chlorpromazine shifts the dose-response curve to calcium to the right, but moves those of acetylcholine and potassium chloride downwards. An effect on cholinergic receptors cannot be eliminated. However, these results do not nullify the proposal that potassium chloride and acetylcholine activate the same pools of calcium.

The effects of lanthanum on contractions of vascular smooth muscle lend support to the proposal that norepinephrine and potassium mobilise separate pools of calcium (van Breemen, 1969; van Breemen and McNaughton, 1970) and suggests that calcium movement through membranes is strongly inhibited by lanthanum. In the present studies, no discrimination between acetylcholine and potassium could be demonstrated. This result is in contrast to that of Goodman and Weiss (1970) who claimed to demonstrate a good discrimination between these agents by using lanthanum. The cause of such a large and important discrepancy in results may be due to one of three factors: a) The difference in tris concentration. When 26mM tris was present rather than 4mM, in these experiments, it was found that there was a decreased sensitivity of acetylcholine-induced contractions to lanthanum. This effect was relatively small however and probably cannot account for the conflicting results; it may be due to the depolarising action of tris. b) the use of grossly supramaximal doses by Goodman and Weiss means that receptor desensitisation

to acetylcholine could have been a factor in the absence of lanthanum. As the dose-response curve is shifted to the right with lanthanum, the response would eventually become a just-maximal response and then decline with further lanthanum exposure. In these studies, just-maximal doses were used throughout, or dose response curves were obtained. Their data, in the absence of knowledge of the relationship to the dose-response curve, is difficult to interpret. c) Probably the most important difference between the results of Goodman and Weiss and those reported here is that of temperature; their results were obtained at room temperature. As already stated, temperature has profound effects on the responses to both acetylcholine and potassium. Temperature has also been shown to be important in determining membrane resistance and its relationship to the concentration of calcium in the membrane (Tomita, 1970). Cooling may increase the amount of calcium in the membrane.

Very low doses of lanthanum (10 μM) inhibit spontaneous contractions by approximately 90%. Such an effect could be due to removal and binding of lanthanum at the calcium superficial sites. Inhibition of contractions due to acetylcholine and potassium could be due to a number of actions. Lanthanum (20 μM) slows the frequency of action potential discharge in response to these agonists. A decrease in excitability due to decreased sodium and potassium conductances in nerve have been previously observed (Takata, Pickard, Lettrin and Moore, 1966; Hafeman, 1969). Since contractions supported by strontium exhibit approximately the same

sensitivity to lanthanum, it must be assumed that the primary effect of lanthanum, at low doses, is on excitability and on the superficial sites. The uptake of lanthanum is linear with time and represents accumulation within the tissue; increasing doses of lanthanum could be expected to progressively displace calcium from all sites related to contraction. The removal of calcium at superficial sites may be considered to be different from the action of EDTA which merely chelates the calcium, in that lanthanum binds at these sites with high affinity.

The lanthanum technique of measuring ^{45}Ca content of tissues which have been incubated in radioactive tracer and then washed out in Ca-free solution containing lanthanum has been advocated as a means of revealing intracellular ^{45}Ca uptake by van Breemen (1969) and van Breemen and Lesser (1971). This technique makes a number of assumptions which cannot be verified directly. Firstly, it is assumed that lanthanum prevents all fluxes through the cell membrane. This cannot be regarded as an entirely valid assumption, at least for the doses used in this study. Thus calcium can still contract tissues in Ca-free Potassium chloride Krebs solution in the presence of 2mM lanthanum. Secondly, it is assumed that lanthanum does not remove intracellular calcium.

In uterine muscle, this technique revealed that hypertonic potassium chloride (40mM) increased ^{45}Ca uptake. If the above assumptions concerning the technique are valid, this effect should represent a true ^{45}Ca influx into cells during exposure to hyper-

tonic potassium chloride. Increased ^{45}Ca uptake has been observed in intestinal muscle with hyperosmotic, but not with isotonic, potassium chloride by Urakawa, Karaki and Ikeda (1968). In the intestine, the response to isotonic potassium chloride does not persist. In the uterus, hypertonic (40mM) and isotonic potassium chloride cause contracture which persists for over 60 minutes. Thus the reason why isotonic potassium chloride does not cause an increase in ^{45}Ca content is obscure. Using the lanthanum technique, experiments failed to detect any effect of isoprenaline on either ^{45}Ca uptake or on the increased uptake due to Lithium Krebs. In view of the observations concerning the validity of this technique (*vide infra*) this does not nullify the postulated action of isoprenaline as increased calcium binding. Evidence that lanthanum does not enter cells is based on microscopic studies. Indeed, lanthanum has been used as a specific marker for the delineation of nexuses (McNutt and Weinstein, 1970). However, lanthanum under these conditions is in the colloidal state. Scott, McNutt and Weinstein (1970) in fact state that lanthanum is "usually" excluded from the intracellular compartment. Lanthanum in the ionic form has been observed within mitochondria of uterine muscle cells (Garfield, unpublished). In view of the importance of the assumption that lanthanum does not penetrate into cells, the uptake of ^{140}La has been studied. The large amounts of lanthanum demonstrated to be taken up questions the validity of this assumption. A simple calculation of the packing of lanthanum molecules required at the

cell membrane to account for such uptake yields an absurd figure (see Results Part IIID). This calculation cannot be taken as proof of intracellular uptake, owing to the assumptions made concerning the surface area/volume ratio. Dessouky (1969) has shown that the membrane of pregnant uterine tissue exhibits an increased number of pinocytotic vesicles, which would greatly increase the surface area of cells. Experiments in which non-pregnant tissues were exposed to ^{140}La and then fractionated indicated that intracellular uptake of lanthanum is likely. The absence of effect of dilution of the ^{140}La in the homogenate with non-radioactive lanthanum and the efflux data for ^{140}La from whole tissues do not support the possibility that ^{140}La is taken up from the homogenate. The likelihood of intracellular penetration of lanthanum invalidates the implicit and explicit assumptions concerning the mechanism of action of lanthanum and the "lanthanum technique" for measuring ^{45}Ca uptake.

V. SUMMARY AND CONCLUSIONS

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It is useful to summarise the conclusions of this study in terms of the questions posed at the outset. The answers are not complete; in view of the complexity of the mechanism of action of drugs in uterine muscle, to some extent this was anticipated.

1. What changes in net ion movements and unidirectional fluxes do drugs and ions produce?

The present studies show that acetylcholine and noradrenaline increase passive fluxes of potassium through the cell membrane. Oxytocin fails to do so. Isoprenaline does not increase potassium permeability until 20-30 minutes after application. Increased movements of calcium have not been demonstrated in the presence of these agonists. Changing the ionic environment of the tissue leads to calcium influx when lithium is substituted for sodium.

2. Can the demonstrated effects on ion movements be correlated with the electrical events which occur during drug action?

Movements of potassium induced by drugs are not considered to represent the primary site of drug action, since they fail to account for the electrical events. In the case of acetylcholine and potassium ion, the increased permeability to potassium may enhance the primary effect, in increasing the rate of repolarisation of spikes.

3. Do the changes in ion movements mediate the observed changes in contractility of the tissue?

Of the agonists which consistently cause contraction of the uterus, only potassium and acetylcholine increase permeability to potassium. Changes in the passive permeability to potassium do not correlate with changes in tension. Calcium movements induced by acetylcholine and potassium have been shown to originate from the same sources, under the conditions used. The effects of lanthanum, SKF 525-A and chlorpromazine, which inhibit calcium ion movements through the cell membrane support the idea that the calcium utilised by acetylcholine and potassium is derived from membrane-bound stores.

In addition to the answers outline above, an investigation into the actions of lanthanum has been conducted. It has been shown that the intracellular penetration of this ion is a likely event.

A number of recommendations may be made as the result of this study concerning further research:

1. It is imperative that further studies of drug action in relation to ionic permeabilities using tracers utilize a technique which permits constant monitoring of tension.

2. An investigation into the effects of temperature on the actions of acetylcholine and other agonists, may clarify the relationship to calcium movements; this may be studied indirectly using specific agents thought to antagonise such calcium movements.

3. Further investigation into the properties of lanthanum in relation to intracellular penetration is clearly necessary.

4. A thorough investigation of the effects of isoprenaline on membrane resistance may reveal decreased sodium conductance. Voltage clamping in smooth muscle should provide a means of studying such effects.

VI. BIBLIOGRAPHY

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