PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Adrenergic Influences on Uterine Smooth Muscle

Jean M. Marshall and E. A. Kroeger

Phil. Trans. R. Soc. Lond. B 1973 **265**, 135-148
doi: 10.1098/rstb.1973.0016

Phil. Trans. R. Soc. Lond. B. **265**, 135–148 (1973) [135]
Printed in Great Britain

Adrenergic influences on uterine smooth muscle

By Jean M. Marshall and E. A. Kroeger
Division of Biological and Medical Sciences, Brown University, Providence, R.I. 02912, *U.S.A.*

1. The mechanism of β adrenergic inhibition of pregnant rat myometrium was investigated.
2. Isoproterenol (4×10^{-7} mol/l) caused membrane hyperpolarization of about 12 mV. The magnitude of this hyperpolarization was unaffected by K-free and Cl-free (isethionate substitution) solutions, and was reduced by about 50 % in the presence of ouabain (10^{-3} mol/l) and at 10 °C. Hyperpolarization was calcium-dependent, was partially reduced by 12.5 mmol/l $[Ca^{2+}]_o$ and was abolished when 2.0 mmol/l La was added to the bathing solution.
3. Isoproterenol (4×10^{-7} mol/l) increased tissue cyclic AMP levels with a time course paralleling that of the relaxation both at 37 and at 10 °C. Papaverine (10^{-4} mol/l) also showed similar actions.
4. Tissue calcium content as measured by the lanthanum technique increased during a K induced contracture and decreased when isoproterenol (4×10^{-7} mol/l), papaverine (10^{-4} mol/l), or dibutyryl cyclic AMP (10^{-3} mol/l) relaxed the K contracture.
5. Another relaxant, D-600, a methoxy derivative of verapamil, (10^{-5} mol/l) had no effect on membrane polarization, tissue cyclic AMP or tissue calcium content.
6. It is concluded that there is an active component to the β adrenergic hyperpolarization of the rat myometium, although an electrogenic sodium pump is not likely to be involved. Rather an electrogenic calcium pump, possibly activated by cyclic AMP, is consistent with the data obtained. Moreover, a mechanism in which cyclic AMP stimulates calcium extrusion may underlie the adrenergically mediated relaxation in the myometrium.

Introduction

Adrenergic amines excite or inhibit the uterus depending upon the species and upon the hormonal status of the individual (Miller 1967). This communication concerns only the inhibition as studied in the rat, a species where this effect is especially prominent. The inhibition is characterized by a cessation of spontaneous action potential discharge and contractions, followed by hyperpolarization of the myometrial cell membrane and reduction of resting tension (figure 1). Since these actions are unaffected by tetrodotoxin (10^{-6} g/ml), atropine (10^{-5} mol/l) and α adrenoceptor blocking agents but are abolished by β blockers (Diamond & Marshall 1968), they presumably result from a direct action of the amines on the β adrenoceptors of the myometrial cell.

The present study focuses on two aspects of this inhibition: (1) the ionic mechanism underlying hyperpolarization, and (2) the relation of tissue adenosine 3′,5′-adenosine monophosphate, (cyclic AMP) and of tissue calcium content to relaxation.

Various theories regarding the ionic mechanisms mediating adrenergic hyperpolarization in smooth muscle have implicated passive permeability changes as well as electrogenic ion pumping (Somlyo & Somlyo 1969; Daniel, Paton, Taylor & Hodgson 1970; Axelsson 1971). The passive changes may involve an increase in potassium permeability and/or a decrease in Na permeability (Marshall 1968; Daniel *et al.* 1970). The stimulation of an electrogenic cation pump, presumably through some metabolic intermediate, has been proposed as the mechanism for adrenergic hyperpolarization in vascular smooth muscle (Somlyo, Somlyo & Smieško 1972). The operation of an electrogenic sodium pump has been demonstrated under special conditions

in rat myometrium (Taylor, Paton & Daniel 1970), but its contribution to the resting potential and to adrenergic hyperpolarization is not known. This possibility has been examined in the present study.

Although the processes underlying relaxation are not precisely known, a number of studies have implicated cyclic AMP in these events since this nucleotide is presumed to be the intracellular mediator of the metabolic effects of the adrenergic amines in a variety of tissues, including the uterus (Robison, Butcher & Sutherland 1969; Triner, Overweg & Nahas 1970). A crucial point concerns the correlation of the actual time-course of relaxation with changes n cyclic AMP concentration. Regarding this correlation, however, the evidence is not clear (Polacek & Daniel 1971; Polacek, Bolan & Daniel 1971; Dobbs & Robison 1968). One of the aims of our experiments was to measure the concentration of tissue cyclic AMP at different times during relaxation in an effort to clarify this important point.

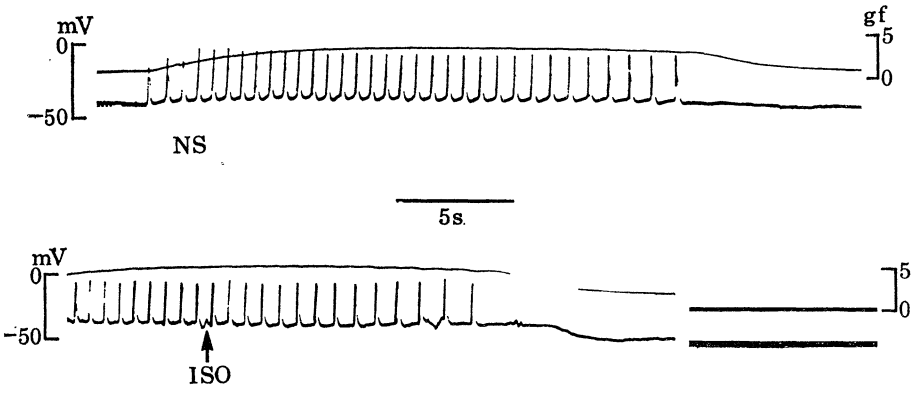

FIGURE 1. Effect of isoproterenol (ISO, 4×10^{-7} mol/l) on pregnant rat myometrium at 37 °C. Representative recording of tension (top tracing) and membrane potential (bottom tracing). Upper panel shows spontaneous, myogenic contraction in normal solution (NS). Lower panel shows effects of isoproterenol (ISO), 4×10^{-7} mol/l, when given during spontaneous contraction. Interval between lower panels 30 s during which time muscle tension fell to zero and membrane hyperpolarized to −55 mV.

Of course, the ultimate step governing relaxation is the removal of calcium from the contractile elements via a decrease in $[Ca^{2+}]_i$. This might involve an increase in the sequestration of calcium to intracellular binding sites and/or an increase in net efflux of calcium from the cell. These possibilities are examined in the present studies which have employed the lanthanum technique recently developed by van Breemen & McNaughton (1970), for the measurement of tissue calcium content.

In our studies we have compared the effects of isoproterenol, an adrenergic agent highly selective for the β (inhibitory) adrenoceptors, with those of papaverine, a smooth muscle relaxant and an inhibitor of phosphodiesterase (Poch & Kukowitz 1971), and of D-600, a methoxy derivative of verapamil, recently characterized by Fleckenstein, Grun, Tritthart & Byon (1971), as a potent uterine relaxant and 'calcium antagonist'.

METHODS

Small strips of myometrium were isolated from pregnant rats within one day of parturition. The segments were mounted in a 10 ml isolated organ bath maintained at 37 °C and arranged for the recording of membrane potentials and tension as previously reported (Diamond &

Marshall 1969). Membrane potentials and tension were displayed on a dual beam oscilloscope and photographed on 35 mm film. The composition of the various bathing solutions appears in table 1. The rate of flow of solution through the organ bath was about 5 ml/min. The drugs were injected directly into the bath in a volume of 0.1 ml saline. Drug concentrations given in results represent the final bath concentrations in terms of free base.

TABLE 1. COMPOSITION (mmol/l) OF VARIOUS BATHING MEDIA

solution	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	$H_2PO_4^-$	La^{3+}	Ise†	SO_4	tris‡	dextrose	O_2–CO_2%
normal	142	5.9	1.2	2.5	132	23.8	1.2	—	—	—	—	5.5	95–5
K^+-free§	142	—	1.2	2.5	126	23.8	1.2	—	—	—	—	5.5	95–5
Cl-free§	142	3.5	1.2	2.5	—	23.8	1.2	—	119	7	—	5.5	95–5
Ca-free§	142	5.9	1.2	—	127	23.8	1.2	—	—	—	—	5.5	95–5
high Ca	125	2.7	1.2	12.5	173	—	—	—	—	—	23.8	11.0	100–0
La-tris	125	2.7	1.2	1.8	158	—	—	2	—	—	23.8	11.0	100–0
normal tris	125	2.7	1.2	1.8	152	—	—	—	—	—	23.8	11.0	100–0
K-rich tris	—	127	1.2	1.8	151	—	—	—	—	—	23.8	11.0	100–0
La-tris (Ca-free wash)	125	2.7	1.2	—	154	—	—	2	—	—	23.8	11.0	100–0

Isethionate, formula: $HO–CH_2–CH_2–SO_3–Na$.
Tris (hydroxymethyl) amino methane.
These solutions are deficient in the ions indicated to the extent that these ions have not been added to the respective solutions. No pecial procedures or chelating agents have been employed to remove trace amounts of these ions from the solutions.

The experimental procedure was to equilibrate the muscle strips in normal solution for 1 to 2 h until regular spontaneous contractions appeared. Control records were then made of membrane potentials from the same cell before, during and after a spontaneous contraction. Isoproterenol or other relaxant was introduced into the bathing medium and the resulting hyperpolarization was recorded continuously from the same cell. The bathing medium was switched to one in which the ionic environment was altered, temperature changed or metabolic inhibitor added. Continuous records were again made before and during exposure to the relaxant.

The term 'membrane potential' as used throughout the text refers to the potential measured by an intracellular electrode relative to the extracellular fluid and hence the resting membrane potential has a negative sign. However, to conform with previous publications in this field, the absolute values of the membrane potentials are given in the text, tables and figures without designating their sign. Thus, hyperpolarization refers to an increase in potential, depolarization to a decrease.

Tissue cyclic AMP analysis

Tissue levels of cyclic AMP have been measured enzymically by a modification of the method developed by Goldberg, Larner, Sasko & O'Toole (1969). Muscles were mounted isometrically on glass hooks in an isolated organ bath and tension of a representative muscle recorded. After equilibration with normal or drug-containing physiological salt solutions, the muscles were plunged into liquid nitrogen. They were pulverized at −180 °C in the presence of 0.5 mol/l $HClO_4$. Afterwards they were rewarmed to 35 °C and extracted with 0.5 mol/l $HClO_4$, and the extract neutralized with KOH. Nucleotides (ATP, ADP, AMP) which might interfere with the subsequent assay, were removed by double Ba–Zn precipitation and ion exchange chromatography and the cyclic AMP fraction was lyophilized. This fraction was analysed for cyclic AMP by first converting the cyclic AMP to 5′AMP and then to ATP. The ATP was then used to phosphorylate glucose via a cycling reaction. The NADP which was reduced during

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

the subsequent oxidation of glucose-6-phosphate was measured fluorimetrically. A boiled phosphodiesterase blank for each sample was included to correct for stray fluorescence due to the presence of any remaining non-cyclic nucleotides.

Tissue Ca *analysis*

Muscle strips were mounted isometrically on glass hooks in an organ bath and the tension of a representative muscle was recorded. After equilibration in either normal physiological salt solution or in K-rich or drug-containing, tris-buffered solution (table 1) the muscles were removed and washed with a tris-buffered salt solution containing 2 mmol/l $LaCl_3$ to which no $CaCl_2$ has been added. Under these circumstances calcium is removed from the extracellular space while cellular calcium exchange is presumably minimized by lanthanum (van Breemen & McNaughton 1970). After 1 h in this solution the muscles were removed, blotted, weighed and dried. They were then wet-ashed in H_2SO_4 and their calcium content determined by atomic absorption spectrophotometry.

Results

The effects of K-*deficiency*, Cl-*deficiency*, *ouabain and low temperature on the hyperpolarization produced by isoproterenol* (4×10^{-7} mol/l)

The purpose of these experiments was to test the possibility that an electrogenic sodium pump might be involved in the hyperpolarizing actions of isoproterenol. The operation of such a pump in many excitable tissues is absolutely dependent upon the presence of potassium in the external medium and is short-circuited in the presence of chloride (Rang & Ritchie 1968; Sokolove & Cooke 1971). The pump is completely inhibited by ouabain (10^{-3} mol/l) and by low temperature (Taylor *et al.* 1970).

The results in table 2 indicate that the hyperpolarization caused by isoproterenol is unaffected by K-free and Cl-free media, but both ouabain (10^{-3} mol/l) and low temperature (10 °C) reduce the magnitude of the hyperpolarization by about 50 %. The high concentration of ouabain used in these experiments reflects the well known resistance of rat tissues to this agent.

Table 2. Effect of various solutions and drugs on the resting membrane potential (mV)

solution	normal	K-free	Cl-free (isethionate)	ouabain (10^{-3} mol/l)	10 °C
control	42.5 ± 0.9 (33)	43.9 ± 1.3 (15)	38.7 ± 1.4 (14)	37.8 ± 1.3 (5)	40.1 ± 1.4 (17)
isoproterenol (4×10^{-7} mol/l)	54.3 ± 1.1 (33)	56.4 ± 1.4 (15)	49.7 ± 1.7 (14)	44.6 ± 0.8 (5)	45.1 ± 1.7 (17)
Δ mV	11.8 ± 0.8†	12.5 ± 0.8†	11.0 ± 0.7†	6.8 ± 1.1†	5.0 ± 0.6†

Mean ± s.e.m. listed; number of observations in parentheses.
† Significant at $P < 0.05$ level.

These data imply that the hyperpolarization has a metabolic component, although the results from the K-free experiments suggest that an electrogenic Na pump is not the main mechanism involved. If electrogenic cation pumping was the major contributor to the metabolic component, then the hyperpolarization should have been abolished by ouabain and low temperature.

The low temperature, in addition to diminishing the amount of hyperpolarization, produced several additional effects which are illustrated in figure 2. One prominent feature was the increase in muscle tension which occurred as the temperature fell, with phasic contractions gradually merging into a tonic contracture as the temperature reached 10 °C. Although the magnitude of this cold contracture varied from muscle to muscle it invariably occurred and was not accompanied by a significant depolarization of the cell membrane. While isoproterenol relaxed the muscle and hyperpolarized the membrane at both temperatures, the time course for the development of the maximal effects on both membrane potential and relaxation was about 5 min at 10 °C compared with about 30 s at 37 °C (cf. figure 1). Furthermore, the latency between the addition of isoproterenol to the bathing medium and the beginning of relaxation was about 15 s at 37 °C compared with 2 min at 10 °C. An increased latency of similar magnitude for the onset of hyperpolarization was also consistently seen at 10 °C. When isoproterenol was washed out of the bath the contracture gradually reappeared and the membrane potential returned to control level. As at 37 °C, these effects of isoproterenol at the low temperature were prevented by propranolol (10^{-7} mol/l).

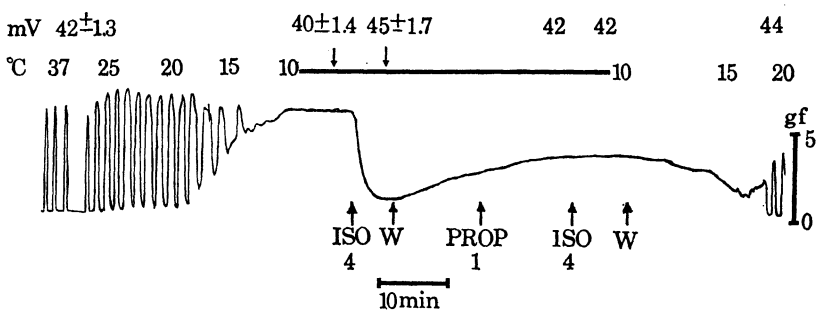

FIGURE 2. Isometric mechanogram from segment of pregnant rat myometrium cooled from 37 to 10 °C. Numbers above mechanogram represent mean values (± s.e.m.) of resting membrane potentials obtained from ten muscles during cooling. Last three values (without s.e.m.) are from one muscle where electrode remained in the cell during rewarming to 20 °C. ISO, isoproterenol; W, wash; PROP, propranolol. The numbers give the concentration $\times 10^{-7}$ mol/l. Latency between introduction of isoproterenol and beginning of relaxation about 2 min, and to complete relaxation about 5 min.

TABLE 3. EFFECTS OF Ca AND La ON THE ACTION OF ISOPROTERENOL ON THE RESTING MEMBRANE POTENTIAL (mV)

$[Ca]_o$/mmol l^{-1}	2.5	0.0	12.5	2.5 (+La 2.0 mol/l)
control	44.1 ± 0.5 (18)	42.6 ± 1.3 (8)	47.0 ± 1.0 (5)	44.6 ± 2.4 (5)
isoproterenol (4×10^{-7} mol/l)	54.4 ± 0.6 (18)	42.6 ± 1.2 (8)	52.6 ± 1.5 (5)	45.6 ± 2.7 (5)
Δ mV	10.3 ± 1.0†	0.0 ± 0.3	5.6 ± 1.4†	1.0 ± 0.7

Mean ± s.e.m. listed; number of observations in parentheses.
† Significant at $P < 0.05$ level.

The effects of calcium and lanthanum on the hyperpolarizing action of isoproterenol (4×10^{-7} mol/l)

Elevation of external calcium concentration from its normal value of 2.5 to 12.5 mmol/l in itself hyperpolarized the membrane and, in addition, reduced the hyperpolarizing effects of isoproterenol. Elimination of calcium from the bathing medium abolished the effects of isoproterenol. Since it was very difficult to impale a muscle cell with a microelectrode after

equilibration with 2 mmol/l La it was necessary to add La after the electrode was in the cell and maintain the electrode within the cell during the 10 min period needed for La to achieve maximal effectiveness. Lanthanum, 2.0 mmol/l, in the presence of normal calcium concentration, stabilized the membrane potential at its normal resting level and prevented the hyperpolarizing effects of isoproterenol. These results are summarized in table 3.

Correlation of the relaxant effects of isoproterenol (4×10^{-7} mol/l) *and papaverine* (10^{-4} mol/l) *with tissue cyclic AMP concentration*

Since papaverine inhibits phosphodiesterase (Triner *et al.* 1971), while isoproterenol stimulates adenyl cyclase (Robison *et al.* 1969), the net effect of either agent should be an increase in tissue cyclic AMP.

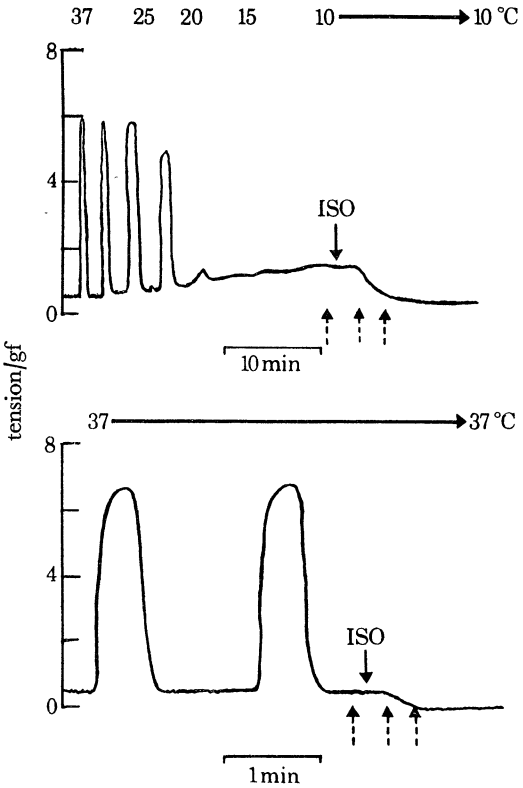

FIGURE 3. Diagrams of isometric mechanograms of isolated myometrial segments at 37 °C and during cooling to 10 °C which serve to illustrate sampling of muscles for cyclic AMP analyses. Note increase in resting tension at 10 °C. ISO, isoproterenol, 4×10^{-7} mol/l. Interrupted arrows show times when individual muscles were taken for analyses; before addition of drug, when relaxation began, and when relaxation was about 80 % complete.

The procedure for this series of experiments was as follows. Several myometrial segments were mounted in an isolated organ bath and their contractions recorded isometrically. After a 2 equilibration period in normal solution at 37 °C, and during a quiescent period between spontaneous contractions, one muscle segment was plunged into liquid nitrogen and prepared for cyclic AMP analysis. Isoproterenol (4×10^{-7} mol/l) was then introduced into the muscle chamber and as soon as relaxation began (within about 15 s) a second muscle was removed for analysis. A third muscle was taken when relaxation was about 80 % complete (in about 30 s).

This same procedure was repeated on muscles at 10 °C, when the latency between the addition of drug and the beginning of relaxation was prolonged and relaxation slowed. Diagrammatical representations of tracings from two of these experiments appear in figure 3 and serve to show the times when the muscles were selected for analysis. A similar set of experiments was repeated using papaverine (10^{-4} mol/l) as the relaxant. With papaverine, however, muscles were taken only after maximal relaxation.

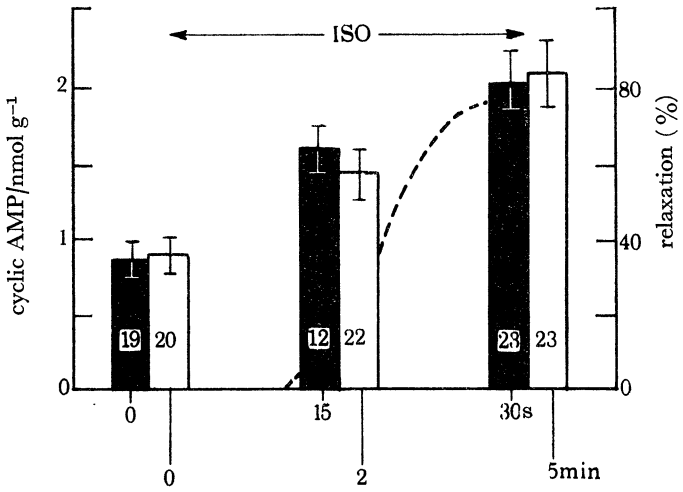

FIGURE 4. Increase in tissue concentration of cyclic AMP, during relaxation in the presence of isoproterenol (ISO), 4×10^{-7} mol/l. Mean values ± s.e.m. at 37 (■) and 10 °C (□). Interrupted line indicates time course of mechanical relaxation. Number at bottom of each column represents number of muscles analysed.

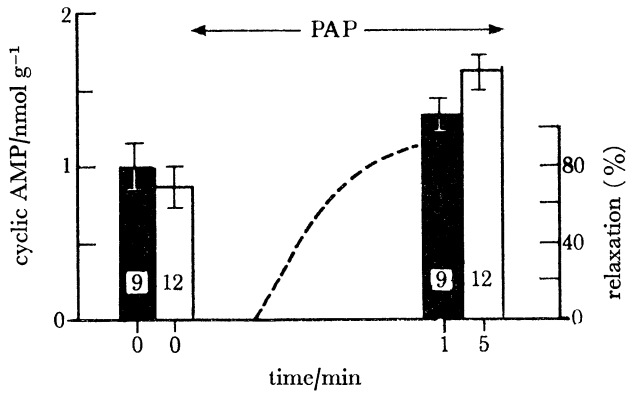

FIGURE 5. Increase in tissue concentration of cyclic AMP as function of percent relaxation in presence of papaverine (PAP), 10^{-4} mol/l. Means ± s.e.m. are given at 37 (■) and 10 °C (□). Interrupted line indicates time course of mechanical relaxation. Number at bottom of each column represents the number of muscles analysed.

The results of both sets of experiments are shown in figures 4 and 5. Note that the control levels (time zero) for cyclic AMP were the same at 37 and at 10 °C despite the appreciable muscle tension at the lower temperature, indicating that the tension, *per se*, does not influence cyclic AMP concentration. During the action of isoproterenol and papaverine, however, the increase in tissue cyclic AMP concentration follows the time course for relaxation at both temperatures. The absolute increase in cyclic AMP concentration is the same at both temperatures and correlates positively with the percent relaxation.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Propranolol, 10^{-7} mol/l, blocked the effect of isoproterenol but not that of papaverine on cyclic AMP levels.

The effects of isoproterenol (4×10^{-7} mol/l), *papaverine* (10^{-4} mol/l) *and dibutyryl cyclic AMP* (10^{-3} mol/l) *on tissue* Ca *content*

If an acceleration of calcium efflux is a prominent factor in the reduction of intracellular calcium concentration during relaxation, then the tissue calcium content should fall at this time. These experiments were designed to examine this possibility.

Muscle segments were suspended isometrically in isolated organ baths and equilibrated for 2 h in normal physiological salt solution. During equilibration muscles were selected at 60, 90 and 120 min for Ca analysis using the lanthanum technique, care being taken to remove the muscles from the bath when they were quiescent. The bathing medium was then switched to one containing 127 mmol/l K^+ which caused a sustained contracture of the muscles.

The rationale for using the K contracture in these experiments was twofold. First, we wished to verify on uterine muscle the previous results of van Breemen & Lesser (1971) on vascular muscle, i.e. that the Ca content increased during K contracture. Confirmation of their findings gave us confidence in our use of the La technique. Secondly, we wished to test the effects of the relaxants against a background of elevated muscle tension and Ca content.

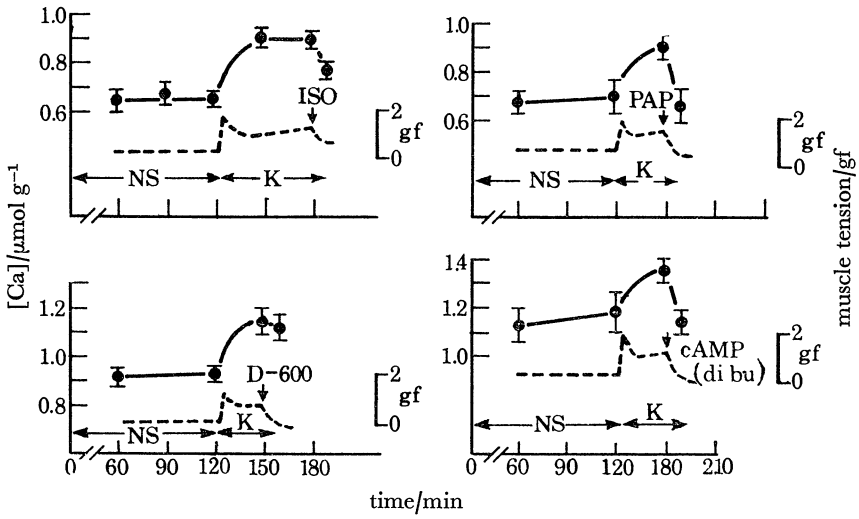

FIGURE 6. Tissue calcium content (solid lines) in normal solution (NS), 127 mmol/l K^+ (K) and in the presence of various relaxants. Interrupted line, diagram of isometric mechanogram. ISO, isoproterenol, 4×10^{-7} mol/l; D-600, methoxy derivative of verapamil, 10^{-5} mol/l; PAP, papaverine, 10^{-4} mol/l; cAMP di bu, dibutyryl cyclic AMP, 10^{-3} mol/l.

Individual muscles were selected for analysis at 30 and sometimes also 60 min later, at times when the contracture was maximal. The various relaxants were then added to the bath, and 10 min later the muscles were removed for analysis when the relaxation was stable. Since previous measurements of cyclic AMP had shown that this compound might play a role in relaxation (cf. figures 4 and 5), an analogue, dibutyryl cyclic AMP which appears to cross the cell membrane more readily than cyclic AMP, was also tested with regard to its effects on tissue Ca.

The results of these experiments appear in figure 6, where the isometric mechanograms are also shown along with the mean ($\pm$s.e.m.) values of tissue calcium concentrations. The

calcium content was significantly increased during the K contracture in agreement with the previous findings for vascular muscle (van Breemen & Lesser 1971). A decrease in Ca content accompanied the relaxant effects of isoproterenol, papaverine and dibutyryl cyclic AMP. The relaxant effect of dibutyryl cyclic AMP was not shared by other nucleotides (e.g. 5′AMP, ADP, ATP).

Comparison of the relaxant effects of D-600 with those of isoproterenol and papaverine

D-600 at a concentration of 10^{-5} mol/l, was equally effective to that of isoproterenol (4×10^{-7} mol/l) and papaverine (10^{-4} mol/l), in relaxing a K contracture but had no effect on either the resting membrane potential or tissue content of cyclic AMP (figure 7). Furthermore, D-600 relaxed the K induced contracture without altering tissue Ca content figure 6).

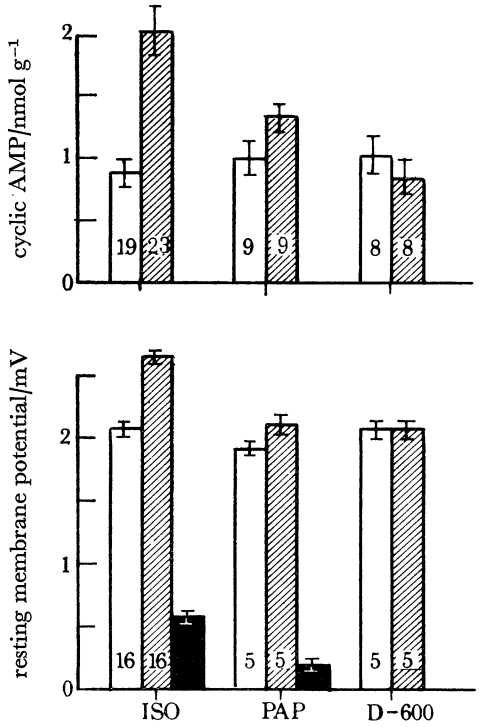

FIGURE 7. Comparison of the actions of isoproterenol (ISO; 4×10^{-7} mol/l), papaverine (PAP; 10^{-4} mol/l) and D-600 (10^{-5} mol/l) on resting membrane potential and tissue cyclic AMP concentration in myometrial segments from pregnant rats. Means ± s.e.m. from number of individual muscles noted at bottom of each bar. □, control; ▨, drugs; ■, change in membrane potential.

DISCUSSION

Two components of inhibition, hyperpolarization and relaxation, mediated by the activation of β adrenoceptors in the myometrium of the rat have been investigated in the present study. The hyperpolarization is shown to have a metabolic component and is also related to extracellular calcium concentration. The relaxation is correlated with an increase in tissue cyclic AMP concentration and with a decrease in tissue calcium content.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Hyperpolarization

Although passive changes in potassium and sodium permeabilities may play a role in the ionic mechanisms underlying the change in membrane potential (Marshall 1968; Daniel *et al.* 1970) a metabolic component is also a prominent feature.

The participation of active, metabolic processes in adrenergically mediated hyperpolarization is evidenced by (1) the decrease in magnitude of the hyperpolarization in the presence of ouabain and at 10° C, and (2) the increase in latency of onset and time-course of development of the potential change at the low temperature. An active metabolic, process which might account for the hyperpolarization is an electrogenic cation pump.

Electrogenic sodium pumps have been demonstrated in the taenia coli (Casteels, Droogmans & Hendrickx 1971), and in the myometrium (Taylor *et al.* 1970), but they are not a common feature of the adrenergically mediated hyperpolarization in these muscles. In the taenia hyperpolarization is an α action, accounted for by changes in potassium and chloride conductances. In this muscle the β action which may have a metabolic component, does not involve membrane potential changes but rather a suppression of spontaneous pacemaker discharge (Bülbring & Tomita 1969*a*, *b*). An electrogenic sodium pump whose Na–K coupling ratio varies with extracellular potassium concentration has been suggested as the mediator of the effects of β adrenergic agents on the membrane potential of smooth muscle cells in the pulmonary artery of the rabbit (Somlyo & Somlyo 1971).

The participation of an electrogenic sodium pump in the adrenergically mediated hyperpolarization in the rat uterus seems unlikely in view of the present findings as well as those reported earlier (Marshall 1968). In these latter experiments uterine muscles were bathed in Na-free solutions (replacement with isomotic sucrose, tris chloride or LiCl) for 3 to 4 h until all spontaneous activity disappeared. Under these circumstances the hyperpolarization was still evident. Therefore it is conceivable that the hyperpolarization involves some other cation pump. The implication of calcium in this regard is attractive since the hyperpolarization is Ca-dependent. Lanthanum which is believed to inhibit calcium flux across the cell membrane (van Breemen & McNaughton 1970) also prevents the hyperpolarization. These findings, together with those described below for changes in tissue cyclic AMP and calcium contents, suggest that an outwardly directed calcium current, stimulated by cyclic AMP, contributes to the hyperpolarizing actions of β adrenergic agents on the uterus.

Relaxation

Cyclic AMP

Although there is convincing biochemical and pharmacological evidence implicating cyclic AMP as an intracellular mediator of the β adrenergic actions in smooth muscle (Mitznegg, Hach & Heim 1970; Triner *et al.* 1971), there is little direct evidence of a correlation between the response of the muscle and a change in tissue concentration of cyclic AMP. Indeed the only workers who measured total cyclic AMP content of the rat uterus as a function of time reported that an increase in cyclic AMP occurred some minutes after the onset of relaxation (Polacek *et al.* 1971; Polacek & Daniel 1971). These investigators were unable to measure an increase in tissue cyclic AMP content in the presence of relaxing doses of papaverine. Therefore they concluded that cyclic AMP does not contribute to the processes subserving relaxation. This evidence against a correlation between cyclic AMP levels and the relaxation produced by

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

these drugs might reflect a lack of sensitivity in the assay methods employed. Their other observation that in epinephrine-pretreated muscles the addition of propranolol restored mechanical activity before cyclic AMP levels had decreased to control levels may be explained by a competition between relaxant effects of cyclic AMP and alpha stimulatory actions of epinephrine.

In contrast to these previous findings, our results showed a significant increase in tissue cyclic AMP concentration at a time when the muscle was just beginning to relax in response to isoproterenol. The tissue levels of cyclic AMP were elevated by about the same amount at corresponding points in relaxation at 37 and 10 °C, although the time course of this increase was greatly prolonged at the lower temperature. The results for papaverine were similar to those for isoproterenol with the exception that they were not prevented by a β blocker.

The cellular mechanism whereby cyclic AMP causes relaxation is not clear. At least two possibilities come to mind; (1) prevention of calcium binding to the contractile proteins, and (2) reduction in concentration of ionized calcium within the cell. The first suggestion appears unlikely since cyclic AMP has no effect on the contraction of glycerinated intestinal muscle (Takagi, Takayanagi & Tomiyama 1971). Therefore it appears that cyclic AMP might enhance calcium sequestration within the cell and/or calcium efflux from the cell. Our results suggest that the latter action may be particularly prominent, possibly mediated by the phosphorylation of a protein (Robison & Sutherland 1970) involved in the outward transport of calcium. A calcium pump in sarcoplasmic reticulum from heart muscle has been shown to be stimulated by cyclic AMP (Shinebourne & White 1970).

Calcium

Calcium movements have been notably difficult to correlate with contractile phenomena in smooth muscle (Goodford 1970; Krejci & Daniel 1970). The lanthanum technique, however, has given promising results in this regard in vascular muscle (van Breemen, Farinas, Gerba & McNaughton 1972; van Breeman & McNaughton 1970). Lanthanum has a much higher affinity for tissue calcium binding sites than does calcium, and therefore it displaces extracellular calcium while preventing calcium flux across the cell membrane. Thus if a muscle is washed in a lanthanum solution containing no calcium, extracellular calcium will be removed and intracellular calcium will be 'locked in'. We were gratified to find that the increase in tissue calcium observed during a K contracture in the myometrium was quantitatively similar to that reported by van Breemen & Lesser (1971) for vascular muscle. Of greater relevance was the finding that this increase was reversed by agents which elevate tissue cyclic AMP and relax the muscle.

Three questions regarding these changes in tissue calcium require consideration. (1) Is the increase in tissue calcium measured during a K contracture sufficient to activate the contractile apparatus? (2) Is there adequate cellular energy to move calcium outward across the cell membrane? (3) Could the outward flow of calcium ions, if mediated by an electrogenic pump, provide sufficient current for the membrane hyperpolarization?

With regard to the first question, assuming that the uterus contains about 5 mg of actomyosin per gram of wet muscle (Csapo 1950; Needham & Schoenberg 1967) and that the amount of Ca^{2+} required for complete activation is 1.3 μmol/g actomyosin (Bianchi 1969), then 0.0065 μmol Ca/g of muscle is needed to activate the contractile elements maximally. This value is in agreement with previous estimates for cow myometrium, 9 to 18 μmol/kg (Carsten 1969); rat myometrium, 5 μmol/kg (Krecji & Daniel 1970); aota, 7 μmol/kg (van Breemen &

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

McNaughton 1970). We measured an increase of about 100 μmol Ca/kg of muscle, more than enough to provide adequate 'activator' calcium. Indeed this is the expected result since there are many intracellular structures other than the contractile proteins which have an affinity for calcium (Carsten 1969). These 'calcium sinks' may sequester most of the calcium measured as net gain in the present experiments. Only a small fraction of the total calcium which exchanges between the external medium and the various intracellular binding sites and depots may actually represent 'activator' calcium (Hurwitz & Suria 1971).

Since the electrochemical gradient for ionized calcium across the myometrial cell membrane is overwhelmingly inward (Goodford 1970), extrusion of calcium from the cell demands energy. If 1 mol of ATP is needed for the extrusion of 1 mol of calcium then 0.075 to 0.100 μmol of ATP are needed per gram of muscle. The myometrium contains about 3.2 μmol/g of ATP and phosphocreatine (Volfin, Clauser & Gautheron 1957), and this amount is more than sufficient for active transport of calcium across the cell membrane from inside of the cell to outside. In fact, active calcium extrusion would consume less than 4 % of the available stores of ATP and phosphocreatine.

Could the outward flow of Ca^{2+}, in addition to causing relaxation, also provide current for membrane hyperpolarization? Assuming that the membrane resistance of the rat myometrium is similar to that for taenia coli of the guinea-pig, 30 to 60 kΩ cm² (Tomita 1966), then in order to produce a 12 mV hyperpolarization (cf. tables 2 and 3), an electrogenic Ca pump must supply an outward current of 0.2 to 0.4 μA/cm². This current corresponds to 60 to 120×10^{-12} mol Ca min^{-1} cm^{-2} of tissue. If the surface area of 1 g of muscle is 6×10^3 cm² (Goodford 1970), then 0.36 to 0.72×10^{-6} mol Ca min^{-1} g^{-1} of tissue must move outward. Since the hyperpolarization was maintained throughout relaxation (a maximum of 10 min until isoproterenol was removed from the bath), 3.6 to 7.2 μmol of Ca/g would have to be extruded from the cell to provide *all* of the hyperpolarizing current. This amount is much larger than the 0.1 μmol Ca/g decrease in tissue calcium content measured with the lanthanum technique. Thus an electrogenic calcium pump cannot be the *exclusive* contributor to membrane hyperpolarization. Calcium, however, does play some role in this process as indicated by our findings on the effects of changes in $[Ca^{2+}]_o$.

D-600 caused no significant change in tissue calcium content, cyclic AMP content, or membrane polarization, although its inhibitory effects (measured against a K contracture) were just as pronounced as those of isoproterenol and papaverine, and are antagonized by elevated extracellular calcium concentration (Fleckenstein *et al.* 1971).

The fact that no change in cellular calcium content accompanied the action of D-600 could mean that this agent was inhibiting calcium influx rather than stimulating efflux. Relaxation would then result from a re-binding of free intracellular Ca^{2+}. Under these conditions no change in tissue calcium would be detected with the lanthanum technique.

Thus a mechanism in which cyclic AMP stimulates calcium extrusion provides an explanation of the adrenergically mediated relaxation in the myometrium. The fact that some relaxants do not affect the cyclic AMP system indicates that relaxation can be effected by a variety of mechanisms.

This work was supported by a U.S. Public Health Service Grant, HE-10187.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

References (Marshall & Kroeger)

Axelsson, J. 1971 *A. Rev. Physiol.* **33**, 1–30.
Bianchi, C. P. 1969 *Fedn Proc.* **28**, 1624–1627.
Bülbring, E. & Tomita, T. 1969*a* *Proc. R. Soc. Lond.* B **172**, 89–102.
Bülbring, E. & Tomita, T. 1969*b* *Proc. R. Soc. Lond.* B **172**, 103–119.
Carsten, M. E. 1969 *J. gen. Physiol.* **53**, 414–426.
Casteels, R., Droogmans, G. & Hendrickx, H. 1971 *J. Physiol., Lond.* **217**, 297–313.
Csapo, A. 1950 *Am. J. Physiol.* **160**, 46–52.
Daniel, E. E., Paton, D. M., Taylor, G. S. & Hodgson, B. J. 1970 *Fedn. Proc.* **29**, 1410–1425.
Diamond, J. & Marshall, J. M. 1969 *J. Pharm. exp. Therap.* **168**, 13–20.
Dobbs, J. W. & Robison, G. A. 1968 *Fedn Proc.* **27**, 352.
Fleckenstein, A., Grun, G., Tritthart, H. & Byron, K. 1971 *Klin. Wschr.* **49**, 32–41.
Goldberg, N. D., Larner, J., Sasko, H. & O'Toole, A. G. 1969 *Analyt. Biochem.* **28**, 523–544.
Goodford, P. J. 1970 Ionic interactions in smooth muscle. In *Smooth muscle* (ed. E. Bülbring, A. F. Brading, A. W. Jones and T. Tomita), pp. 100–121. Baltimore: Williams and Wilkins.
Hurwitz, L. & Suria, A. 1971 *A. Rev. Pharmacol.* **11**, 303–326.
Krejci, I. & Daniel, E. E. 1970 *Am. J. Physiol.* **219**, 263–269.
Marshall, J. M. 1968 *Fedn Proc.* **27**, 115–119.
Miller, J. W. 1967 *Ann. N.Y. Acad. Sci.* **139**, 788–798.
Mitznegg, P., Hach, B. & Heim, F. 1970 *Life Sci.* **9**, 975–981.
Needham, D. M. & Schoenberg, C. F. 1967 The biochemistry of the myometrium. In *Cellular biology of the uterus* (ed. R. M. Wynn), pp. 291–352. New York: Appleton–Century–Crofts.
Poch, G. & Kukovetz, W. R. 1971 *Life Sci.* **10**, 133–144.
Polacek, I., Bolan, J. & Daniel, E. E. 1971 *Can. J. Physiol. Pharmacol.* **49**, 999–1004.
Polacek, I. & Daniel, E. E. 1971 *Can. J. Physiol. Pharmacol.* **49**, 989–998.
Rang, H. P. & Ritchie, J. M. 1968 *J. Physiol., Lond.* **196**, 183–221.
Robison, G. A., Butcher, R. W. & Sutherland, E. W. 1969 On the relation of hormone receptors to adenyl cyclase. In *Fundamental concepts in drug-receptor interactions* (ed. J. F. Danielle, J. F. Moran and D. J. Triggle), pp. 59–75. New York: Academic Press.
Robison, G. A. & Sutherland, E. W. 1970 *Circulation Res.* (suppl. 1), **27**, 147–161.
Shinebourne, E. & White, R. 1970 *Cardiovasc. Res.* **4**, 194–200.
Sokolove, P. G. & Cooke, I. M. 1971 *J. gen. Physiol.* **57**, 125–163.
Somlyo, A. & Somlyo, A. V. 1969 *Fedn Proc.* **28**, 1634–1648.
Somlyo, A. & Somlyo, A. V. 1971 Electrophysiological correlation of the inequality of maximal vascular smooth muscle contraction elicited by drugs. In *Vascular neuroeffector systems* (ed. J. A. Bevan and R. F. Furchgott), pp. 216–226. Basel: S. Karger.
Somlyo, A. P., Somlyo, A. V. & Smieško, V. 1972 Cyclic AMP and vascular smooth muscle. In *Physiology and pharmacology of cyclic AMP* (ed. R. Paoletti and G. A. Robison). New York: Raven Press.
Takagi, K., Takayanagi, I. & Tomiyama, A. 1971 *Jap. J. Pharmacol.* **21**, 477–483.
Taylor, G. S., Paton, D. M. & Daniel, E. E. 1970 *J. gen. Physiol.* **56**, 360–375.
Tomita, T. 1966 *J. theor. Biol.* **12**, 216–227.
Triner, L., Nahas, G. G., Vulliemoz, Y., Overweg, N. I. A., Verosky, M., Habif, D. V. & Ngai, S. H. 1971 *Ann. N.Y. Acad. Sci.* **185**, 458–476.
Triner, L., Overweg, N. I. A. & Nahas, G. G. 1970 *Nature, Lond.* **225**, 282–283.
van Breemen, C., Farinas, B. R., Gerba, P. & McNaughton, E. D. 1972 *Circulation Res.* **30**, 44–54.
van Breemen, C. & Lesser, P. 1971 *Microvasc. Res.* **3**, 113–114.
van Breemen, C. & McNaughton, E. 1970 *Biochem. Biophys. Res. Commun.* **39**, 567–574.
Volfin, P., Clauser, H. & Gautheron, D. 1957 *Biochem. biophys. Acta* **24**, 137–140.

Discussion

H. O. Schild (*London*): May I congratulate Dr Marshall on a beautiful paper. I would like to ask three brief questions.

(1) Does she think that isoprenaline relaxation is connected intimately with hyperpolarization in view of the fact that it can be obtained in the fully K-depolarized uterus?

(2) I am not sure of the implications of the lanthanum method, but is it conceivable that there is both an internal uptake mechanism for calcium, followed by extrusion?

(3) Is it possible to differentiate between the effects of isoproterenol and papaverine on cyclic AMP by means of β blockers?

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

DR MARSHALL:

(1) Hyperpolarization is not a pre-requisite for the relaxant action of isoprenaline, as Professor Schild has shown some years ago, and as we have confirmed in the K-depolarized muscle in this communication.

(2) We feel that the reduction in tissue Ca content found to accompany the relaxant action of isoprenaline results primarily from a stimulation of an active extrusion of calcium, with also perhaps a small reduction in Ca influx. The lanthanum technique cannot distinguish changes in distribution of Ca within the cell, hence we cannot tell on the basis of our experimental results if there was also a re-distribution of Ca within the cell.

(3) The increase in cyclic AMP seen in the presence of isoprenaline is prevented by propranolol, while that seen in the presence of papaverine or dibutyryl cyclic AMP is not.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES