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HORMONAL CONTROL OF UTERINE CONTRACTION

CHARACTERIZATION OF CYCLIC AMP-DEPENDENT MEMBRANE PROPERTIES IN THE MYOMETRIUM

J. FREDERICK KRALL*, JANET L. SWENSEN and STANLEY G. KORENMAN

Department of Medicine, UCLA San Fernando Valley Medical Program, Veterans Administration Hospital, Sepulveda, Calif. 91343 *(U.S.A.)*

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SUMMARY

A mitochondria-free membrane fraction prepared from rat myometrium accumulated $^{45}Ca^{2+}$ in the presence of oxalic acid and ATP. The rate of transport of Ca^{2+} into the membranous vesicles was increased by greater than 50% in the presence of 3′,5′-cyclic AMP, but not by 2′,3′-cyclic AMP or 5′-AMP. Membrane ATPase activity was stimulated by cyclic AMP in a manner similar to Ca^{2+}-transport. ATPase activity was stimulated by Mg^{2+}; slight additional stimulation was obtained in the presence of Na^+ and K^+ but not in the presence of Ca^{+2}. Despite the cyclic AMP sensitivity of membrane ATPase activity, the absence of any effect of inhibitors of Ca^{2+}-transport suggest it has little to do with Ca^{2+} accumulation by the membranes.

Cyclic AMP-induced increase in Ca^{2+}-transport and membrane ATPase activity was duplicated in vivo by incubating uteri in 10^{-4} M isoproterenol prior to membrane isolation. Isoproterenol has been previously shown to increase myometrial cyclic AMP levels, and changes in Ca^{2+}-transport by cell membranes in relation to intracellular cyclic AMP levels may be the mechanism through which hormones modulate uterine contractility.

INTRODUCTION

The role of calcium in the control of muscle contraction is widely recognized [1]. Intracellular Ca^{2+} levels (and hence the contractile state of the muscle cell) are regulated by the membranes of the sarcoplasmic reticulum which alternately sequester and release the cation [2–4]. One mechanism by which Ca^{2+} movements

Abbreviations: Cyclic AMP, adenosine cyclic 3′,5′-monophosphate; EGTA, ethyleneglycol bis(β-aminoethylether)-*N,N′*-tetra acetic acid.

* Send reprint requests to: J. Frederick Krall, Molecular Endocrinology Laboratory, Veterans Administration Hospital, 16111 Plummer Street, Sepulveda, Calif. 91343 (U.S.A.)

within the muscle cell are coordinated is beginning to be resolved. β-Adrenergic catecholamine receptors are located on the sarcolemmna where they are coupled to membrane adenylate cyclase [5, 6]. Occupation of the receptors by β-agonists leads to increased cyclic AMP levels and altered intracellular Ca^{2+} distribution which results in a change in the contractile state [7]. β-Agonist-induced changes in muscle contraction can be reproduced by direct treatment with cyclic AMP or with inhibitors of phosphodiesterase activity, verifying a second messenger role for the cyclic nucleotide in the modulation of muscle contraction [8–10].

Cyclic AMP has been shown to stimulate Ca^{2+}-transport by sarcoplasmic reticulum vesicles isolated from cardiac muscle [11, 12] and from the smooth muscle cells from the colon [13], and so may be representative of the way in which β-adrenergic agonists mediate muscle contraction. Uterine contractility is subject to modulation by steroid and membrane active hormones as well as by catecholamines [14–16], and the report of a cyclic AMP unresponsive sarcoplasmic reticulum in the myometrium raised the possibility of a unique regulatory mechanism [17]. Investigation of the interacting roles of hormones and cyclic AMP in the control of uterine contractility disclosed the presence of a cyclic AMP-dependent protein kinase associated with the myometrial smooth muscle membranes most active in Ca^{2+}-transport (Krall, J. F., Schindler, A. M. and Korenman, S. G., unpublished). Since this enzyme may be analogous to the membrane protein kinase of cardiac muscle [12] in which cyclic AMP-dependent phosphorylation of sarcoplasmic reticulum proteins has been shown to increase Ca^{2+}-transport [18], we were prompted to re-examine the cyclic AMP sensitivity of Ca^{2+} uptake by myometrial membranes.

MATERIALS AND METHODS

Preparation of microsomes

Uteri from ovariectomized Sprague-Dawley rats (200 g) were removed to ice cold Eagles Medium (GIBCO), trimmed of excess fat and connective tissue, cut open lengthwise and rinsed well. The uteri were then incubated in fresh medium at 37 °C for 30 min with continuous shaking. When β-adrenergic stimulation was investigated, the preincubated uteri were incubated for an additional 10 min in fresh medium or fresh medium supplemented with 10^{-4} M isoproterenol (Isuprel, Winthrop). Following incubation, uteri were washed with ice cold saline and the endometrium removed by scraping.

The scraped myometrium was washed with cold homogenization buffer (0.25 M sucrose, 0.01 M Tris · HCl, pH 8.0), diced with scissors, and homogenized in 10 volumes of homogenization buffer with a Polytron (Brinkman Industries) at a setting of 3 for three 10-s bursts interspersed with 20-s cooling periods. The homogenate was filtered through 3 layers of cheese cloth and centrifuged at $20\,000 \times g$ for 10 min. The supernatant was decanted and the pellet resuspended with a teflon-glass homogenizer in 10 volumes of fresh homogenization buffer. Following another 10 min centrifugation at $20\,000 \times g$, the two supernatants were combined and centrifuged at $50\,000 \times g$ for 60 min. The resulting $20\,000$–$50\,000 \times g$ pellet, resuspended in one volume of homogenization buffer, constituted the myometrial membranes.

Membrane protein content was determined according to the method of Lowry et al. [19] following precipitation, washing and hydrolysis at 90 °C in 0.4 N perchloric acid.

Determination of calcium uptake

ATP-dependent calcium uptake was determined as the amount of Ca^{2+} transported into membrane vesicles and precipitated as calcium oxalate according to the method of Harigaya and Schwartz [20]. Each assay tube contained in 1 ml of 0.02 M Tris · HCl (pH 8.0): 10 μmol $MgCl_2$; 5 μmol sodium oxalic acid; 5 μmol sodium azide, 4 μmol ATP (Sigma); 10 nmol $CaCl_2$ and 15 μCi $^{45}CaCl_2$ (New England Nuclear, 13 mCi/mg). Except where noted, tubes were preincubated at 37 °C for 5 min and the reaction started by the addition of 25 –60μg of protein. Incubation was continued for 20 min. The reaction was stopped by filtering the contents of each tube through a cellulose nitrate filter (Millipore Corp., HA 0.45). The filters were washed 3 times with 2 ml of distilled water. Radioactivity associated with membrane protein on the filters remained unaltered by successive washes with distilled water until a total of more than 10 ml had passed over the filter. After washing with a total of 16 ml, 80 % of the radioactivity was still retained by the filter.

Control tubes with the complete incubation mixture but without membrane protein were included in each assay. In the absence of protein, less than 0.04 % of the isotopic calcium in the incubation mixture remained bound to the filter following washing. Filters were dissolved and counted in Bray's scintillation cocktail. ATP-dependent uptake of calcium was obtained by subtracting values obtained in the absence of ATP.

Determination of ATPase activity

Hydrolysis of ATP was followed as the amount of inorganic phosphorous liberated in the presence of membrane protein. Each assay tube contained in 0.5 ml of 0.02 M Tris · HCl (pH 8.0): 0.5 μmol $MgCl_2$, and 2 μmol ATP (Sigma) plus the additions indicated in the text. The reaction was started by the addition of 10–25 μg of microsomal protein and the temperature increased from 2 to 37 °C. Incubation was continued for 3 min at 37 °C and terminated by the addition of 0.125 ml of ice cold 25 % trichloroacetic acid (Fisher). After 30 min on ice, acid insoluble residues were removed by centrifugation and organic phosphorous content of the supernatant was determined according to the procedure of Fiske and Subbarow [21]. Values obtained in the absence of protein were subtracted from each experimental point to correct for non-enzymatic hydrolysis of ATP as well as phosphorous contamination of the reagents. No acid insoluble phosphorous was detected when microsomes were incubated in the absence of exogenous ATP.

RESULTS

The 20 000–50 000 $\times g$ particulate fraction of rat myometrium is the post-mitochondrial fraction most active in Ca^{2+}-transport activity (Krall, J. F., Schindler, A. M. and Korenman, S. G., unpublished). Observation by electron microscope reveal this fraction as a heterogeneous collection of membranes, but free of either ribosomal or obvious mitochondrial contamination (Fig. 1). The insensitivity of Ca^{2+}-transport to inhibition by sodium azide is another indication that the membrane fraction is mitochondria-free (Fig. 2). Ca^{2+}-transport by the membranes is dependent upon oxalic acid and ATP, although appreciable amounts of Ca^{2+} are bound to the membranes in the absence of a high energy source (Table I).

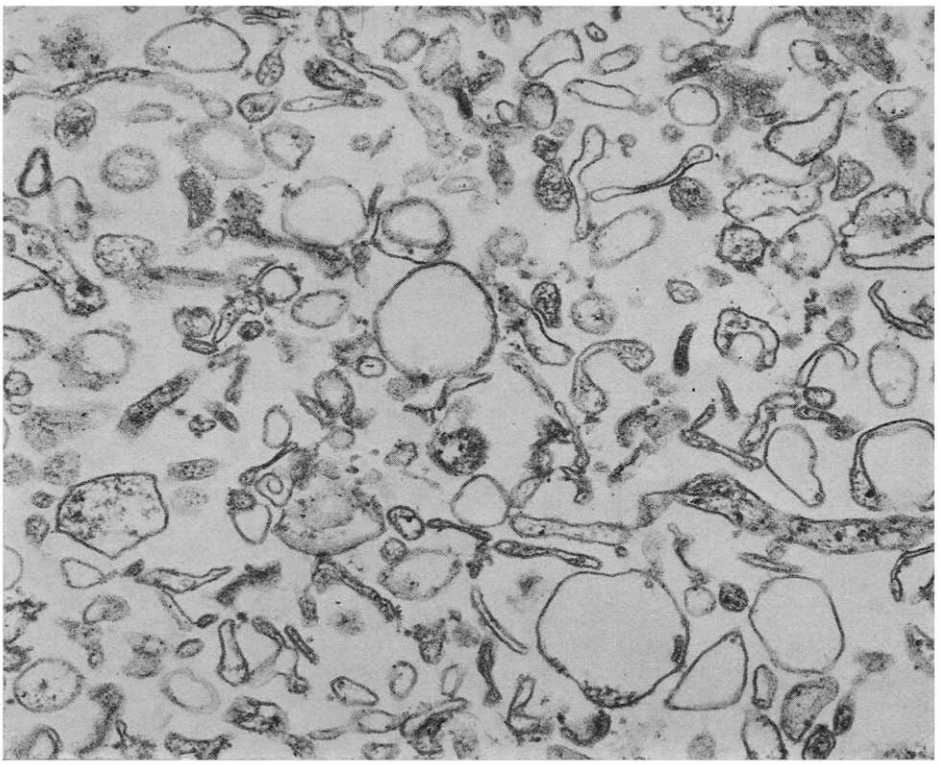

Fig. 1. Electron micrograph of the 20 000–50 000 $\times g$ microsomal pellet. The trilaminar membrane structure can be discerned in several places. Magnification, 50 000 $\times$.

TABLE I

CALCIUM UPTAKE BY MICROSOMES

Myometrial membranes were incubated according to the procedure outlined in Materials and Methods, but with the indicated additions or omissions. All nucleotide monophosphates were at a concentration of $5 \cdot 10^{-6}$ M. Each value is the mean of duplicate determinations in two experiments $\pm$S.D.

Assay conditions	Ca^{2+}-transport activity (nmol Ca^{2+}/mg · min)
Complete	1.53 $\pm$ 0.06
Minus ATP	0.33 $\pm$ 0.01
Minus oxalate	0.71 $\pm$ 0.02
Plus 3′,5′-cyclic AMP	2.28 $\pm$ 0.16
Plus 3′,5′-cyclic AMP minus ATP	0.86 $\pm$ 0.02
Plus 2′,3′-cyclic AMP	1.65 $\pm$ 0.05
Plus 5′-AMP	1.59 $\pm$ 0.08

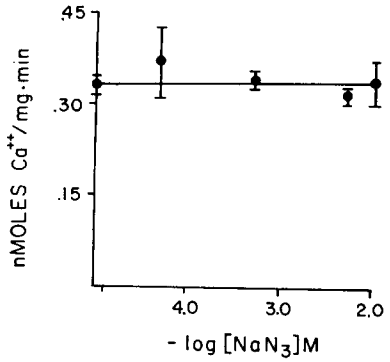

Fig. 2. Effect of sodium azide on calcium uptake. ATP-dependent calcium uptake was determined in the presence of the indicated NaN_3 concentrations according to the procedure outlined in Material and Methods. Vertical lines represent S.D. of four determinations.

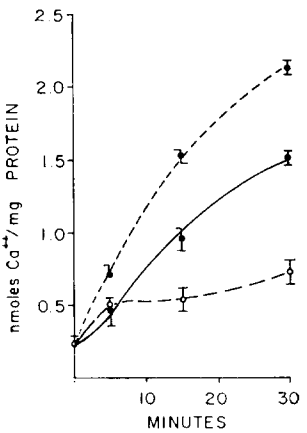

Fig. 3. Effect of cyclic AMP on Ca^{2+}-uptake by membranes. Ca^{2+}-transport activity was determined according to the procedure outlined in Materials and Methods, except that the 5 min preincubation step was omitted. ○– – –○, without ATP; ● — ●, plus ATP; ●– – –●, plus ATP and $5 \cdot 10^{-6}$ M cyclic AMP. Vertical lines represent S.D. of four determinations.

Myometrial membranes prepared from ovariectomized rat uteri showed increased rates of Ca^{2+}-transport when incubated in the presence of cyclic AMP (Table I and Fig. 3). The effect is specific for 3′,5′-cyclic AMP and did not occur in the presence of 2′,3′-cyclic AMP or 5′-AMP (Table I). Increased Ca^{2+}-transport occurs over a physiological range of cyclic AMP concentrations (Fig. 4).

To elucidate the mechanism by which cyclic AMP stimulates Ca^{2+}-transport, we investigated its effects on membrane ATPase activity. ATP hydrolysis by myometrial membranes was stimulated by cyclic AMP in a manner similar to the way that Ca^{2+}-transport was increased: i.e. the rate of hydrolysis was increased (Fig. 5) over a physiological range of cyclic nucleotide concentrations (Fig. 4). The predominant ATPase activity associated with the myometrial membranes was stimulated by Mg^{2+} but not Ca^{2+} (Fig. 6B). Ca^{2+} in the presence or absence of Mg^{2+} had no effect at

concentrations between 10^{-6} M and 10^{-4} M (not shown), but was slightly inhibitory at concentrations greater than 10^{-3} M (Fig. 6B). Since myometrial membranes contain substantial intrinsic levels of Ca^{2+} [22], we used the Ca^{2+} chelating compound EGTA to investigate whether the membranes contained a Ca^{2+}-sensitive ATPase which was maximally activated by endogenous levels of the cation. Membrane ATPase activity was inhibited by over 50% by EGTA (Fig. 6A). However, activity which was suppressed by EGTA could not be restored by the addition of Ca^{2+} in quantities sufficient to raise the free Ca^{2+} concentration to as high as $2.5 \cdot 10^{-2}$ M in the presence of chelator (Fig. 6B). Slight additional stimulation of membrane ATPase activity was obtained in the presence of Na^+ and K^+, but K^+ was largely inhibitory to Ca^{2+}-transport (Fig. 7).

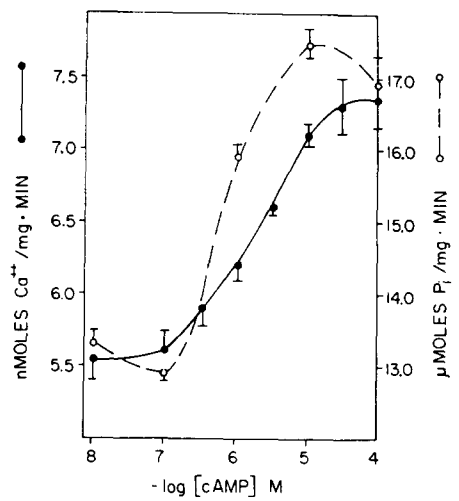

Fig. 4. Effect of cyclic AMP concentration on Ca^{2+}-uptake (●) and membrane Mg^{2+}-ATPase activity (○). Membranes were incubated with increasing concentrations of the cyclic nucleotide according to the procedure outlined in Materials and Methods. Vertical lines represent S.D. of three determinations.

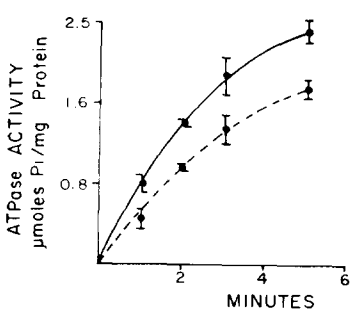

Fig. 5. Effect of cyclic AMP on membrane Mg^{2+}-ATPase activity. Membranes were incubated in the absence (○) or presence (●) of $5 \cdot 10^{-6}$ M cyclic AMP and the reaction was stopped at the times indicated as outlined in Materials and Methods. Vertical lines indicate S.D. of three determinations.

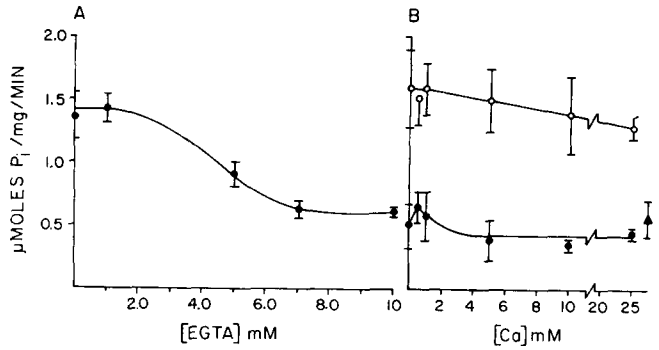

Fig. 6. Effect of Ca^{2+} and Mg^{2+} on membrane ATPase activity. (A) Membranes were incubated according to the procedure outlined in Materials and Methods but without Ca^{2+} and with the indicated concentrations of EGTA .(B) Membranes were incubated with the indicated concentrations of Ca^{2+} (plus Mg^{2+}) and in the absence (○) or presence (●) of 10 mM EGTA. ▲, ATPase activity in the absence of Mg^{2+}, Ca^{2+}, or EGTA. Free Ca^{2+} concentration in the presence of EGTA was calculated according to Batra and Daniel [27]. Vertical bars represent S.D. of three determinations.

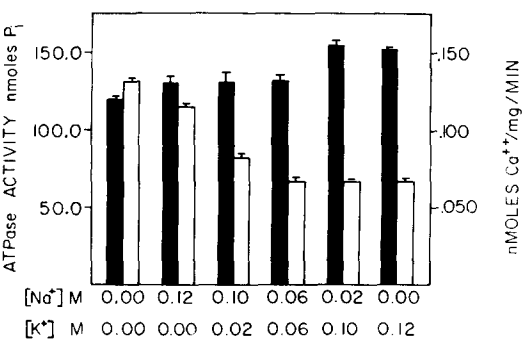

Fig. 7. Stimulation of Mg^{2+}-ATPase activity by Na^+ and K^+. Membranes were incubated according to the procedures outlined in Materials and Methods but in the presence of the indicated concentrations of Na^+ and K^+ to maintain constant ionic strength [40]. Closed bars, ATPase activity; open bars, calcium uptake. Vertical lines represent S.D. of three determinations.

Myometrial membranes routinely transported between 100 and 800 μmol of Ca^{2+} per mol of ATP hydrolyzed. Compared to the purer Ca^{2+}-transporting membranes of skeletal and cardiac muscle, which transport roughly 2 mol of Ca^{2+} per mol of ATP hydrolyzed [1], our rat myometrial Ca^{2+}-transport system was grossly inefficient. We therefore used well characterized inhibitors of membrane ATPase activity to evaluate the role of membrane Mg^{2+}-ATPase in Ca^{2+}-transport

Salyrganic acid, a potent inhibitor of Ca^{2+}-transport in muscle [23, 24], was roughly 100-times more effective in inhibiting Ca^{2+}-transport by the myometrial membranes than was ouabain (Fig. 8), an inhibitor of $(Na^+ + K^+)$-ATPase [25]. Neither inhibitor had an effect on membrane Mg^{2+}-ATPase at concentrations inhibitory to Ca^{2+}-transport (Fig. 8). It appears, therefore, that membrane Mg^{2+}-ATPase activity in our preparation is largely unrelated to Ca^{2+}-transport.

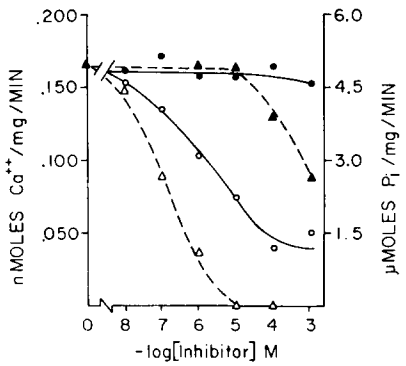

Fig. 8. The effects of ouabain (circles) or salyrganic acid (triangles) on calcium uptake (open symbols) and membrane Mg^{2+}-ATPase activity (closed symbols). Membranes were incubated as outlined in Materials and Methods. Each point is the mean of duplicate determinations.

TABLE II

EFFECTS OF ISOPROTERENOL ON Ca^{2+}-TRANSPORT AND Mg^{2+}-ATPase ACTIVITY OF MYOMETRIAL MEMBRANES

Membranes were prepared from uteri incubated in the absence or presence of 10^{-4} M isoproterenol and assayed for the indicated activity as outlined in Materials and Methods. Each value is the mean of 3–4 determinations ±S.D.

10 min Uterine incubation	Transport nmol Ca^{2+}/mg · min	Mg^{2+}-ATPase activity nmol P_i/mg · min
Control	0.16±0.01	204.2±8.5
$1 \cdot 10^{-4}$ M isoproterenol	0.23±0.01	337.3±0.0

To reproduce in vivo the cyclic AMP effects on Ca^{2+}-transport and Mg^{2+}-ATPase activity which were obtained in vitro, we incubated uteri from ovariectomized rats in the absence or presence of 10^{-4} M isoproterenol for 10 min. Isoproterenol is a β-agonist previously shown to raise myometrial cyclic AMP levels [26]. Microsomes prepared from these uteri showed agonist induced elevations of both Ca^{2+} transport and Mg^{2+}-ATPase activity (Table II).

DISCUSSION

Carsten [22] characterized the sarcoplasmic reticulum of the myometrium as an important Ca^{2+} storage site within the uterine smooth muscle cell. Calculations based upon the capacity of this organelle to sequester Ca^{2+} have led to the conclusion that it can redistribute intracellular calcium to the extent required to alternately trigger contraction and cause relaxation [22, 27]. Mitochondria have also been shown to transport and bind significant amounts of Ca^{2+} in the myometrium [28]. Electron microscope observation of the 20 000–50 000 $\times g$ particulate fraction used in the current investigation reveals a heterogeneous population of membranes but no morphologically distinct mitochondria. Absence of mitochondrial contamination is substantiated by the resistance of ATP-dependent Ca^{2+}-uptake to sodium azide,

shown to be a potent inhibitor of Ca^{2+}-uptake and binding by mitochondria but not sarcoplasmic reticulum in the myometrium [22, 27]. Besides mitochondria, the sarcolemma of the uterine smooth muscle cell has also been shown to be capable of Ca^{2+}-transport [29, 30]. Ca^{2+}-transport by the sarcolemma has been postulated to occur independently of Na^+ and K^+ transport [29], even though Ca^{2+} influx in myometrium is associated with high levels of K^+ [30, 31]. Demonstration of $(Na^+ + K^+)$-ATPase activity in our membrane preparations indicates the presence of sarcolemma [4], but the inhibition of Ca^{2+}-transport at elevated K^+ concentrations implies that little Ca^{2+} is accumulated by plasma membrane vesicles in these preparations. The primary source of Ca^{2+}-transport activity in these membrane preparations is, therefore, probably sarcoplasmic reticulum vesicles.

Ca^{2+}-transport activity was highly variable between preparations. Activities ranged from 0.15 to over 5 nmol calcium transported per mg protein per min. The extreme variability may be due to the fact that subsequent purification of the membranes most active in Ca^{2+}-transport was not attempted after the 20 000–50 000 $\times g$ pellet was obtained. The purification of skeletal muscle sarcoplasmic reticulum membranes by sucrose density gradient centrifugation has shown that the method was capable of separating apparently intact vesicles which were nevertheless damaged and incapable of concentrating calcium [32]. The variability we observed may be due to failure to separate membranes damaged during homogenization from those more active in Ca^{2+}-transport.

It was previously demonstrated that activation of the myometrial adenylate cyclase system is accompanied by the translocation of cytosol protein kinase to the membranes of the smooth muscle cells [26]. We have recently characterized the myometrial protein kinase which is present in the membranes (Krall, J. F., Schindler, A. M. and Korenman, S. G., unpublished). The observation that it is both tightly bound to the membranes and activated by cyclic AMP may mean that the phosphorylation of membrane proteins leads to increased Ca^{2+}-uptake in the presence of the cyclic nucleotide. The phosphorylation of a protein of the sarcoplasmic reticulum of cardiac muscle by cyclic AMP-dependent protein kinase has been shown to lead to increased Ca^{2+}-transport [11, 18].

Cyclic AMP-stimulated calcium uptake by cardiac sarcoplasmic reticulum was accompanied by increased Ca^{2+}-activated membrane ATPase activity [18]. Since Ca^{2+}-ATPase is the sarcoplasmic reticulum "calcium pump protein" [33], the effect of cyclic AMP on membrane ATPase is important in understanding the mechanism controlling calcium uptake in the myometrium. The cardiac glycoside ouabain has been shown to bind to a limited number of sites on the plasma membrane [34], inactivating $(K^+ + Na^+)$-ATPase in a process accelerated by the natural substrates of the enzyme [35]. Salyrganic acid is a mercuramide which has been shown to inhibit calcium uptake in striated and smooth muscle by inactivating Ca^{2+}-ATPase [22, 24]. Results obtained with these inhibitors indicate that membrane Mg^{2+}-ATPase has little function in ATP-dependent Ca^{2+}-uptake by our preparation, despite the cyclic AMP-dependence of ATP hydrolysis. In fact, Mg^{2+}-ATPase is only one of many muscle components which are cyclic AMP responsive [36].

The sensitivity of Ca^{2+}-uptake to salyrganic acid suggests, however, the presence of a Ca^{2+}-ATPase activity. Stimulation of the already high myometrial membrane ATPase activity by addition of exogenous Ca^{2+} has been difficult to demon-

strate [22, 28], perhaps because the activity is already maximal in these preparations due to high levels of intrinsic membrane Ca^{2+}. Because of that possibility, inhibition of membrane ATPase activity by EGTA was suggested to reflect a decrease in Ca^{2+}-ATPase activity as free Ca^{2+} levels were reduced by chelation [22]. We have found, however, that membrane ATPase activity reduced by EGTA cannot be restored by Ca^{2+}, making it unlikely that a myometrial membrane Ca^{2+}-ATPase has been detected thus far. Possible in vitro effects of EGTA other than Ca^{2+} chelation have been discussed by Katz et al. [37].

Batra [28] and Batra and Daniels [27] have characterized myometrial microsomes which bound Ca^{2+} but failed to transport it in an oxalic acid-dependent manner. Electron micrographs of their preparations showed fractured membranes rather than intact vesicles, so the preparations could not be expected to sequester Ca^{2+} in concentrations great enough to form insoluble calcium oxalate [20]. Membranes used in the investigation reported here, in contrast, are intact vesicles which accumulate Ca^{2+} in a manner dependent upon both ATP and oxalic acid. Previous failure to demonstrate cyclic AMP-dependent Ca^{2+}-uptake or binding by myometrial microsomes may have been due to the condition of the vesicles, although the incubation conditions for Ca^{2+}-binding have also been criticized [10]. Alternately, the importance of incubating excised uteri to lower intracellular cyclic AMP levels to values obtained in vivo has been stressed previously [38]. Failure to do so may preclude a detectable cyclic AMP response. Our demonstration of cyclic AMP dependent Ca^{2+}-transport by myometrial membranes indicates that cyclic nucleotide-mediated mechanisms which effect Ca^{2+}-transport in intestinal smooth muscle and cardiac muscle [5, 13] are also present in myometrium. Moreover, since myometrial contractility is modulated by steroid and peptide hormones as well as by catecholamines [14–16], the uterus may represent an instance in which the complex interaction of hormones can be understood in terms of the altered distribution of intracellular Ca^{2+} in response to changing cyclic nucleotide levels [39].

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