A microsomal fraction resembling striated muscle sarcoplasmic reticulum was isolated from uterine smooth muscle. ATP induces calcium accumulation in this fraction. Increased temperature enhances calcium accumulation and calcium-activated ATPase. In the absence of ATP, approximately 55% of the intrinsic calcium exchanges with the 45Ca in the incubation medium. In the presence of ATP, exchange of intrinsic calcium with 45Ca increases by an amount which equals the ATP-dependent calcium binding. In preparations partially preloaded with calcium, a steady state of bound calcium is reached when the ATP is exhausted. Calcium is released under these conditions by prostaglandins E2 and F2, but not by PGF2α. The antibiotic ionophores X537A and A23187, as well as oxytocin, also release calcium previously accumulated under ATP stimulation. None of these agents, with the exception of oxytocin, release intrinsic calcium. Thus, the effect of prostaglandins resembles that of the ionophores, suggesting an ionophoretic action of these prostaglandins. The release of calcium conforms with the in vivo smooth muscle contracting action of these agents.

The transport of calcium through the membranes of the sarcoplasmic reticulum is a well recognized step in the contraction and relaxation cycle of cardiae and skeletal muscle (1). Minute amounts of calcium are required for actomyosin activation in muscle contraction. This calcium is provided both by influx of extracellular calcium and by release from intracellular stores mostly in the sarcoplasmic reticulum. Relaxation is concurrent with a lowering of the intracellular free calcium concentration, the calcium being sequestered by the sarcoplasmic reticulum. Evidence for the role of intracellular calcium in contraction and relaxation of smooth muscle is based largely on the work of Van Breemen et al. (2); this involves the use of lanthanum to block the passage of calcium across the cell membrane. The presence of relatively large amounts of sarcoplasmic reticulum in uterine smooth muscle was established in electron microscope studies. It was shown that the volume of sarcoplasmic reticulum correlates with the dependence of the smooth muscle on intracellular calcium (3). Because the amounts of calcium involved appear to be much smaller than in other muscles, it is only recently that we have been able to demonstrate calcium accumulation on the subcellular level, particularly in uterine muscle (4).

It is thought that in muscle sarcoplasmic reticulum the measured calcium accumulation represents a steady state between active calcium accumulation and passive calcium efflux. Moreover, calcium binding can be reversibly dissociated upon the addition of ATP (5, 6). We have been able to demonstrate energy-linked calcium accumulation in uterine microsomal preparations (4) as well as to show a dose-related inhibition of this calcium accumulation by uterine contractile agents such as certain prostaglandins and oxytocin (7). This phenomenon could be attributed to either an inhibition of ATP-dependent calcium binding or a simultaneous increase in hormone-induced calcium release.

In the present study we have attempted to separate these processes. Because of quantitatively improved calcium accumulation, it has been possible to show, in the present communication, the enhancement of calcium release by contractile agents in the microsomal model. In addition, we have considered the possibility that the prostaglandins act like ionophores.

**HYPOTHESIS FOR IONOPHORETIC ACTION OF PROSTAGLANDINS**

**MATERIALS AND METHODS**

**Method of Preparation**—In brief, the procedure, modified from a previous study (8) is as follows: uteri, obtained at the slaughterhouse from close-to-term pregnant cows, were immediately dissected and the myometrium carefully stripped free of endometrium. The muscle strips were rinsed, immersed in ice-cold buffer (0.3 M sucrose, 5 mM dithiothreitol, 0.02 M Tris, pH 7.2), and transported in an ice bath to the laboratory. The muscle tissue was diced with scissors, minced in a meat grinder, and homogenized first in a Waring Blender for 15 s, then in a Polytron for 5 s, followed by a Waring Blender for 5 s. All operations were carried out in the cold room at 0-4°C. Differential centrifugation was at 2,500 × g for 20 min, 15,000 × g for 20 min, and 40,000 × g for 90 min. The final pellets were suspended in 0.08 M NaCl, 0.005 M sodium oxalate and placed on a sucrose density gradient consisting of layers of 35, 45, and 55% sucrose. After 3 h of centrifugation in a Spinco 25.1 swinging bucket rotor (average force 64,000 × g), the main protein layer was isolated from the 35% sucrose layer (density of 1.136). Protein concentration was determined by the method of Lowry et al. (9). The protein was stored at 4°C and used the following day. The yield obtained was 122 ± 5.6 mg/kg of tissue, averaged for 34 experiments. Previous characterization (8) of this fraction showed no mitochondrial or myofibrilar contamination detectable by biochemical and morphological analyses. The latter showed predominantly vesicular structures, resembling frag-

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ment of sarcoplasmic reticulum of cardiac muscle.

**Calcium Binding Centrifugation Method** - ATP-dependent calcium binding was assayed in incubation mixtures containing an excess of ATP. Reactions were started by addition of protein and terminated after specified times of incubation by plunging the tubes into an ice bath. In the centrifugation method (10) as modified by us (4), the tubes were centrifuged at 165,000 x g for 30 min, in a Spinco Ti-50 rotor, the pellets were washed and homogenized in 20 mM imidazole buffer, and the centrifugation was repeated, all at 4°. The pellets were then homogenized in deionized water, and protein and calcium were determined. In order to measure initially bound (intracellular) calcium, samples without added ATP were carried through the entire procedure. The intracellular calcium is not removable by repeated washings with buffer (8). Analysis for calcium was in deproteinized solutions containing 0.5% trichloroacetic acid, 0.5% LaCl₃, and 5 mM CsCl. A Perkin-Elmer atomic absorption spectrophotometer was used.

**Filtration Method** - For kinetic studies tubes were incubated at 37° in an incubation medium containing 4Ca and limited amounts of ATP. The reaction was terminated by filtering aliquots through 0.45 μm pore size Millipore filters with prefilters as outlined previously (4). Aliquots of the filtrate were counted in a Beckman LS-100 liquid scintillation system using Redi Solve VI. The calcium bound or released was computed from the difference between counts in the incubation medium and the filtrate, using appropriate controls not containing protein. Correction for exchange of 4Ca with intrinsic cold calcium was made.

**Calcium Exchange** - Exchange of intrinsic calcium with 4Ca added in the incubation medium was evaluated using the method and computation of Carvalho (10), as outlined by us previously (4). Analyses for calcium were done by atomic absorption spectroscopy on the pellets and supernatants after centrifugation. Radioactivity was determined on aliquots of the same solutions used for calcium analyses. From these data the specific activity (counts per min per nmol of calcium) of bound and free calcium was calculated. The fraction of exchangeable calcium under a variety of experimental conditions is calculated from the equation (10):

\[
\text{Fraction of exchangeable calcium} = \frac{\text{Specific activity of calcium in pellet}}{\text{Specific activity of calcium in solution}}
\]

Because some of the calcium in the intracellular calcium pool is exchangeable, the 4Ca counts added represent the sum of the calcium in the incubation medium plus that portion of intrinsic calcium which is exchangeable. The calculations have to be modified to that extent.

Calcium exchange was measured under three experimental conditions: (a) in the presence of ATP-dependent calcium binding; (b) in the absence of ATP, i.e., under conditions of calcium exchange with the intrinsic calcium pool only; (c) in the absence of ATP, but in the presence of prostaglandin E₃ (PGE₃), i.e., under conditions where PGE₃ might affect calcium exchange with the intrinsic calcium pool.

A change in exchangeable calcium could be demonstrated from one of these conditions, and the appropriate correction would have to be made in all experiments involving the 4Ca Millipore filtration method.

**ATPase Measurements** - ATPase activity was measured on filtered aliquots after precipitation in 5% trichloroacetic acid. Liberation of inorganic phosphate was measured (11).

**Materials** - All chemicals were reagent grade. ATP (Sigma Chemical Co.) and sucrose, special enzyme grade (Schwarz-Mann), were used. ATP was freed of traces of calcium by treatment with Dowex 50W-X8-H⁺ as previously described (12). Oxytocin was obtained from Calbiochem. Prostaglandins were generously supplied by the Upjohn Co. Prostaglandin E₃ (PGE₃) and F₂ₐ (PGF₂ₐ) were in aqueous solution. Prostaglandin F₂ₐ (PGF₂ₐ) was dissolved in 90% alcohol. Controls of PGF₂ₐ contained a comparable amount of alcohol.

**Computations and Statistics** - ATP-dependent calcium binding was computed as the difference in calcium in the presence and absence of ATP. All experiments were performed in duplicate. A minimum of six experiments were performed and averaged for statistical evaluation. The standard error of the mean was calculated.

---

1 The abbreviations used are: PG, prostaglandin; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N'-tetraacetic acid; SR, sarcoplasmic reticulum.
TABLE I

Exchange of intrinsic calcium

<table>
<thead>
<tr>
<th>Calcium in pellet</th>
<th>ATP-stimulated calcium binding</th>
<th>Calcium exchanged</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/mg protein</td>
<td>In pellet a</td>
</tr>
<tr>
<td>1. Complete medium</td>
<td>28.7 ± 1.7</td>
<td>6.8 ± 1.2</td>
</tr>
<tr>
<td>2. ATP omitted</td>
<td>21.9 ± 1.4</td>
<td>0</td>
</tr>
<tr>
<td>3. ATP omitted, 50 μg/ml PGE₂ added</td>
<td>22.7 ± 1.6</td>
<td>0</td>
</tr>
</tbody>
</table>

a The difference between 1 and 2, p < 0.0005; between 1 and 3 p < 0.02; between 2 and 3 p < 0.10, i.e. not significant.

b The differences between 1, 2, and 3 are not statistically significant.

Fig. 2. Calcium-stimulated ATPase at 37° and 25°. Incubation medium: 20 mM imidazole buffer, pH 7.0, 2 mM ATP, 2 mM MgCl₂, 10 mM KCl, 7 μM CaCl₂, and 0.2 mg/ml of microsomal protein. ○, calcium omitted, 1 mM EGTA added.

Fig. 3. Dose-response curve for calcium release by PGF₂α. At 10 and at 50 μg of PGF₂α, six experiments were performed, at 30 μg, four experiments, and at 100 μg, 2 experiments.

Fig. 4. Calcium release. Incubation medium: 20 mM imidazole buffer, pH 7.0, 0.15 mM ATP, 0.15 mM MgCl₂, 10 mM KCl, 20 μM CaCl₂, and 0.5 mg/ml of microsomal protein. Prostaglandins E₂, F₂α, F₆α added at arrows. Prostaglandin concentration in the incubation medium: 50 μg/ml.

Calcium Release—Prior studies have shown that the net calcium accumulated in the presence of ATP is reduced in the presence of either PGE₂ or PGF₂α (13). Since the net calcium accumulated represents the equilibrium between active calcium binding and passive calcium release, it was not clear whether these PGs affect the binding phase or the release phase. Since the binding phase depends on the presence of ATP in the incubation medium, conditions were set in the following experiments to limit the ATP concentration in order to limit the binding of calcium. The Ca Millipore filtration method was used in these experiments. In the presence of 0.15 mM ATP, ATP-dependent calcium binding is limited. The steady state is reached in less than 4 min and maintained for at least another 4 min. At 4 min, the test compound is added, causing an immediate release of previously bound calcium. Under the condition of limited ATP concentration only 5.6 ± 0.9 nmol of calcium is taken up per mg of protein. Initial experiments were performed with PGF₂α to construct a dose-response curve for calcium release (Fig. 3). Prostaglandin (50 μg/ml) was chosen for further experiments because this was the lowest dose which consistently produced measurable calcium release. At 50 μg/ml, PGE₂ released approximately 50% of the accumulated calcium (Fig. 4). PGF₂α released less calcium than PGE₂ at the same PG concentration. Statistical analysis showed that both the PGE₂- and the PGF₂α-stimulated calcium release were significant. The effect of PGF₂α was not significant.

Unpublished work.
Corrections for volume changes were made in the calculation. Correction was also made for the exchangeable calcium, inasmuch as the $^{45}$Ca label was taken to represent the sum of the added calcium (20 mM) plus the exchangeable calcium which amounted to 11.9 nmol of Ca$^{2+}$/mg of protein in these experiments. The results graphed in Figs. 3 to 6 reflect the ATP-dependent calcium binding above and distinct from calcium exchange, i.e. the zero point is taken as zero net binding. In the absence of ATP the measured apparent calcium binding (removal of counts from the medium) is due to calcium exchange with the intrinsic calcium and to nonspecific binding to the microsomal protein. PGE$_2$ had no effect on these.

Oxytocin, another uterine contractile agent previously found to inhibit ATP-dependent calcium accumulation (7), also caused a statistically significant calcium release from partially preloaded microsomal preparations (Fig. 5). In addition, at a concentration of 50 microunits/ml, oxytocin released some of the intrinsic calcium, as seen in Fig. 5.

The antibiotic ionophores X537A (Hoffman La Roche) and A23187 (Eli Lilly and Co.) are known to inhibit calcium binding and to release calcium from skeletal and cardiac muscle sarcoplasmic reticulum previously having accumulated calcium due to ATP stimulation (14, 15). In Fig. 6 we show that at appropriate concentrations these ionophores completely release previously accumulated calcium from uterine microsomal preparations. They have no effect on intrinsic calcium levels.

**Discussion**

This study examined the calcium binding and transport properties of a vesicular microsomal fraction largely derived from the membranes of the sarcoplasmic reticulum of the myometrium. One would like to compare the calcium-binding properties with those of similar microsomal fractions from other smooth muscles. Comparisons are difficult, however, because ATP-dependent calcium binding depends on experimental conditions, particularly it increases with the calcium concentration in the medium (16). At lower calcium concentration than used by others our calcium binding appears to be higher than that reported in other smooth muscles such as guinea pig ileum (17), bovine aorta (16, 18), and rat aorta (19).

Improvements in preparative procedures have led to greater ATP-dependent calcium binding and lower yields, compared with our previous studies (4, 8). Calcium accumulation per kg of tissue has almost doubled over our previous results and is now 2074 nmol/kg of tissue in 1 min. In other muscles, calcium uptake in SR fractions is coupled to a calcium- and magnesium-activated ATPase. We have demonstrated a calcium- and magnesium-activated ATPase in the uterine microsomal fraction. Since this ATPase is only a small fraction of the total ATPase, and the systematic error is relatively high for the expected small differences, it appears that the calcium- and magnesium-activated ATPase can be demonstrated convincingly only when relatively large amounts of ATP are split, i.e. at the 16-min point. Accordingly, at the lower level of ATP splitting, at 35°C, it could not be detected. The temperature sensitivity of the various ATPases is unknown. It is not possible to calculate precise Ca/P ratios for initial velocities, which has been done for calcium uptake (in the presence of oxalate) in other muscles, because we cannot detect significant CaATPase activity until the reaction has proceeded for 16 min. At that point the net calcium accumulation has slowed considerably. The simplest interpretation for our results is that there is a limited capacity for calcium binding while the CaATPase remains active. Nevertheless, the results suggest that the ATP splitting could well provide the energy for calcium transport. This calcium transport is temperature-dependent and thus compatible with an enzymatic reaction.

Since the intrinsic calcium is almost as high as the ATP-dependent calcium binding in the microsomal fraction containing sarcoplasmic reticulum, it was of interest to determine whether calcium exchange was a factor obscuring results of calcium binding studies. Our finding that more than half of the intrinsic calcium exchanges with calcium added in the incubation medium, warrants appropriate corrections to
be made in all computations based on experiments using the \(^{45}\text{Ca}\) Millipore filtration method. The magnitude of this correction is a function of the microsomal fraction used and may vary with the state of purification and with the preparative procedure (20). Thus, in our older preparations, substantially less calcium was found to be exchangeable (6, 20).

Our results show two pools of intrinsic calcium, one exchangeable and one not exchangeable. The next question is, which pool does the calcium bound under ATP stimulation join?

If we assume that ATP-dependent calcium binding is much faster than exchange, this bound calcium must be completely exchangeable to explain our experimental result, namely that the increase in exchangeable calcium is equal to the amount of calcium bound. However, we know that calcium binding is not instantaneous. Furthermore, our results from kinetic studies show calcium exchange virtually complete in 0.5 min, whereas ATP-dependent calcium binding continues. If exchange is slower than calcium binding, it is unlikely that the system would have reached equilibrium at the time of sampling. If it had not reached equilibrium, we should have obtained a significantly smaller amount of calcium exchangeable than calcium bound. But our results showed the two figures to be equal.

An alternative explanation is that exchange is much faster than ATP-dependent calcium binding. In this case, exchange occurs with the intrinsic calcium pool. The calcium in the medium is then taken up in the appropriate ratio. If this mechanism applies, we would not know whether this calcium remains freely exchangeable after it has been sequestered. Intermediate conditions between these two hypotheses may exist. Thus our results do not allow us to conclude whether this calcium remains in the exchangeable pool or how it is compartmentalized. Further experiments are in progress to elucidate this point.

PGE\(_2\) and PGF\(_{2\alpha}\) are known uterine contractile agents (21). PGF\(_{2\alpha}\) is a physiologically inactive analogue. We have previously shown their overall inhibition of calcium accumulation to be consistent with their biological activity on the uterus. Calcium release was now found with PGE\(_2\), to a lesser degree with an equal dose of PGF\(_{2\alpha}\) and not significant with PGF\(_{2\alpha}\), again consistent with their physiological action. However, the difference in the effect of PGE\(_2\) and PGF\(_{2\alpha}\) was not found to be of statistical significance. In contrast, our previous experiments (7) confirmed the greater potency of PGE\(_2\) relative to PGF\(_{2\alpha}\), observed in vivo.

The prostaglandin concentration in sheep myometrium in labor has been reported to be as high as 400 ng/g of wet tissue (22). Since prostaglandins are synthesized within microsomes inside the cell the concentration inside or adjacent to the microsomes would be much higher than in the cytoplasm or the extracellular fluid, which all are included in the wet weight of tissue. Thus our prostaglandin concentration probably is within the physiological range.

Prostaglandins would be well suited for a function in calcium transport, since they are water- and lipid-soluble and form calcium complexes. They may transport calcium across biological membranes, such as those of the SR vesicles, by forming calcium complexes which are highly soluble in the lipid layers of these membranes. By alternately combining with calcium ion at a membrane interface, diffusing across the membrane as a calcium complex, releasing the calcium ion at the opposite interface, and diffusing back uncomplexed, they could function as efficient calcium carriers. Substances that modify membrane permeability by virtue of carrying ions across biological membranes are called ionophores. The best known examples of ionophores are the bacterial polypeptides which also cause smooth muscle contraction at the concentrations used in our study (23). The ionophores X537A and A23187 indeed behaved very similar to prostaglandins in our model system, i.e. they caused release of calcium from microsomal preparations under the same conditions as employed for prostaglandins. Neither prostaglandins nor the ionophores had an effect on the intrinsic calcium pool; this is in accordance with observations on ionophores in skeletal SR (24). In contrast, we found that oxytocin did release calcium from the intrinsic calcium pool. Thus, the mode of action of oxytocin may differ from that of prostaglandins and ionophores.

In our system, the effect of prostaglandins or of the ionophores cannot be due to changing enzyme activation by binding and thereby removing activator calcium from solution, because the release of calcium is not enzyme-activated. Furthermore, although prostaglandins, like ionophores, form calcium complexes, they are weak calcium complexers. In our laboratory, the association constant of PGE\(_2\) was found to be 326 \pm 18.1,\(^2\) while PGF\(_{2\alpha}\) forms no calcium complex. This association constant is not high enough to remove significant amounts of calcium from solution at the low PG concentration used. And our results of this investigation show that a mere increase in the calcium concentration gradient does not in itself cause release of calcium.

Thus, it appears that the prostaglandins work in a manner similar to the ionophores, facilitate release of calcium from the microsomal vesicles down a transmembrane gradient. Experiments in progress in this laboratory have shown a weak ionophoretic action for prostaglandins in an in vitro model system as used for antibiotic ionophores (25). The results of these experiments will be published elsewhere. It is suggested that the uterine contracting prostaglandins can function as calcium ionophores. The extent to which the ionophoretic action plays a significant role in the physiologic process remains to be elucidated.

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REFERENCES
Effects of prostaglandins and oxytocin on calcium release from a uterine microsomal fraction.
M E Carsten and J D Miller


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