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Stimulation of Microsomal Calcium Uptake and Protein Phosphorylation by Adenosine Cyclic 3',5'-Monophosphate in Rat Uterus

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SUMMARY

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The molecular mechanism by which N^6-2'-*O*-dibutyryladenosine cyclic 3',5'-monophosphate inhibits oxytocin-induced contraction of the rat uterus was studied. Cyclic AMP enhanced calcium uptake by the microsomal fraction from rat uterus over a range of ATP concentrations. In the presence of 250 μM ATP, which is almost identical with the intracellular concentration, the uptake reaction responded maximally and specifically to cyclic AMP, with an apparent K_a of about 1 μM, the concentration required for half-maximal activation. Uterine mitochondrial calcium uptake could easily be distinguished from the microsomal activity, either by the inhibitory effect of sodium azide or by the lack of effect of cyclic AMP. When microsomes were phosphorylated endogenously with [γ-^{32}P]ATP, phosphorylation of a microsomal protein with a molecular weight of 48,000, referred to as protein A, depended upon cyclic AMP. The correlation coefficient between cyclic AMP-dependent protein A phosphorylation and cyclic AMP-stimulated calcium uptake was 0.968 ($p < 0.01$). These results suggest that the inhibition by dibutyryl cyclic AMP of oxytocin-induced contraction of the rat uterus may be due at least partly to the stimulation of microsomal calcium uptake mediated by cyclic AMP-dependent phosphorylation of protein A.

INTRODUCTION

Uterine contractility is subject to modulation by hormones (1-3). Oxytocin, for instance, induces uterine contraction, whereas stimulation of *beta* adrenergic receptors causes relaxation of smooth muscle with a concomitant increase in cyclic 3',5'-AMP (4, 5). N^6-2'-*O*-Dibutyryl cyclic AMP and inhibitors of cyclic AMP phosphodiesterase mimic *beta* agonists, indicating a possible involvement of cyclic AMP in uterine relaxation (6-8). By analogy with skeletal muscle, intracellular transport of calcium into the sarcoplasmic reticulum has been postulated to play an important role in uterine smooth muscle tone (9, 10). Accordingly, of particular interest to us is the way in which cyclic AMP modulates the tone of uterine smooth muscle at the molecular level, especially with respect to intracellular calcium movement. Although cyclic AMP stimulated calcium uptake by microsomal fractions from other smooth muscles such as rabbit small intestine (11) and rabbit aorta (12), Batra and Daniel (13) reported little effect of cyclic

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AMP on the uptake reaction in uterine smooth muscle. Recently, however, Krall *et al.* (14) reported the activation of microsomal calcium uptake by cyclic AMP in rat uterus, and we (15) reported similar results independently in preliminary form.

The present paper demonstrates the importance of proper concentrations of ATP in the incubation medium in order to obtain a cyclic AMP effect on uterine microsomal calcium uptake, the lack of cyclic AMP effect on mitochondrial calcium uptake, and the close relationship between the stimulation by cyclic AMP of microsomal calcium uptake and that of endogenous phosphorylation of a specific microsomal protein.

MATERIALS AND METHODS

Materials. Cyclic nucleotides and ATP assay kits were obtained from Boehringer/Mannheim-Yamanouchi, and oxytocin, from Yamanouchi. Millipore filters and $^{45}CaCl_2$ (2 mCi/μmole) were purchased from Millipore Japan and New England Nuclear, respectively. [γ-^{32}P]ATP (12 mCi/μmole) was prepared by the method of Post and Sen (16).

Animals. Virgin female Sprague-Dawley rats (200–250 g) in estrus, as determined by vaginal smear examination, were used throughout the experiments. Rats were decapitated, and the uterus was quickly removed and cleaned of fat and connective tissue.

Determination of isometric contraction of isolated rat uterus. The contraction of isolated rat uterus was determined by the method of Mitznegg *et al.* (6). An isolated strip of rat uterus was suspended in Tyrode's solution bubbled with 95% O_2–5% CO_2 at 37°. The solution contained 137 mM NaCl, 2.7 mM KCl, 1.1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.42 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, and 5.5 mM glucose. The strip was gently stretched until a steady baseline tension of 1 g was reached. The contraction was induced by 1 USP munit/ml of oxytocin, and the contractile response was recorded isometrically for at least 15 min by a strain gauge transducer connected to a Nihon-Kohden polygraph, model RM-150. When the effect of varied concentrations of dibutyryl cyclic nucleotides on contraction was studied, oxytocin was removed by washing with Tyrode's solution. Following incubation of the tissue with dibutyryl cyclic nucleotides for 10 min, 1 USP munit/ml of oxytocin was added again, and the contractile response was recorded. The percentage inhibition of oxytocin-induced contraction was calculated by comparing the responses before and after addition of dibutyryl cyclic nucleotides. Ninety minutes after removal of oxytocin and dibutyryl cyclic nucleotides by washing with Tyrode's solution, the uterus could still respond to oxytocin as normally as before.

Preparation of microsomes and mitochondria. The microsomal and mitochondrial fractions were prepared from rat uterus at 4°. The cleaned uterus (500 mg of protein) was homogenized with a Polytron homogenizer (Kinematica) at setting 7 for two 10-sec periods with an interval of 20 sec, in 9 volumes of 0.33 M sucrose containing 10 mM histidine HCl buffer, pH 6.8, and centrifuged at 900 × g for 15 min. The supernatant was centrifuged at 12,000 × g for 20 min, and the pellet (10.2% recovery of protein) was used as the mitochondrial fraction after resuspension in 10 mM histidine HCl buffer, pH 6.8. The postmitochondrial supernatant was further centrifuged at 105,000 × g for 60 min, and the pellet (6.8% recovery of protein) was used as the microsomal fraction after resuspension in 10 mM histidine HCl buffer, pH 6.8. This microsomal preparation was virtually devoid of succinate dehydrogenase activity, used as a marker enzyme for mitochondria. However, the extent of contamination with plasma membranes was not known. Protein was determined by the method of Lowry *et al.* (17) with bovine serum albumin as a standard.

Standard assay for calcium uptake by microsomes and mitochondria. Microsomes or mitochondria (80–120 μg of protein) were first incubated for 2 min at 25° in a final volume of 0.1 ml containing 120 mM KCl, 2.5 mM Tris-oxalate, 250 μM ATP, 500 μM $MgCl_2$, and 10 mM histidine HCl buffer, pH 6.8. The calcium uptake reaction then was started by addition of

[^{45}Ca]calcium EGTA[1] buffer containing 125 μM $^{45}CaCl_2$ and 391 μM EGTA [the calculated concentration of free calcium (0.4 μCi/nmole of calcium) was 1 μM (18)]. After 1 min of incubation, an 80-μl aliquot of the reaction mixture was filtered through an HA (0.45-μm) Millipore filter disc, which was then quickly washed with ice-cold [^{45}Ca]calcium EGTA-free medium as described by Martonosi and Feretos (19). The ratioactivity on the filter disc was counted with a Packard Tri-Carb liquid scintillation spectrometer, model 3390, in a scintillation solution containing 5 g of 2,5-diphenyloxazole, 100 mg of 2,2'-*p*-phenylenebis(4-methyl-5-phenyloxazole), and 0.33 liter of Triton X-100 in 1 liter of toluene. The radioactivity incorporated into microsomes under these conditions was reduced 73% by addition of 5 μM nonradioactive free calcium, ruling out the possibility that the incorporated radioactivity was due to a radioactive substance which might contaminate the ^{45}Ca-labeled stock solution. The term calcium uptake used in this paper means both the transport of calcium into membrane vesicles and the binding of calcium to membranes.

Assay for endogenous membrane phosphorylation. Phosphorylation of endogenous substrates in the microsomal fraction was assayed by the method of Casnellie and Greengard (20). Microsomes (250–300 μg of protein) were incubated at 20° for 20 sec in 0.1 ml of a reaction mixture containing 50 mM sodium glycerol phosphate (pH 7.5), 10 mM magnesium acetate, and 5 μM [γ-^{32}P]ATP (2 mCi/μmole). The reaction was terminated by addition of 0.05 ml of 3% SDS containing 10% sucrose, 30 mM Tris-HCl (pH 7.5), 2 mM EDTA, 60 mM dithiothreitol, and 50 μM bromphenol blue. Under these conditions about 40% of the added ATP was found at the end of the incubation. A 50-μl aliquot (80–100 μg of protein) of the mixture was subjected to electrophoresis on an SDS–polyacrylamide slab gel (5.6% acrylamide, 1% SDS, 14 cm × 16 cm × 2 mm gel) as described previously (21). The gel was dried with a Hoefer slab gel drier under vacuum, and its radioautographic pattern revealed the protein bands into which ^{32}P had been incorporated during the reaction. The absorbance of the individual peaks on the densitometric tracing was proportional to the amount of ^{32}P incorporated into each band.

Determination of ATP concentration in rat uterus. The amount of ATP in rat uterus was determined by the method of Bücher (22), using an ATP assay kit.

Assay for succinate dehydrogenase. The activity of succinate dehydrogenase was determined by following the decrease in optical density at 600 nm of 2,6-dichloroindophenol at room temperature under aerobic conditions, using a modification of the method of Bonner (23). The reaction mixture, in a total volume of 1.0 ml, contained 100 mM sodium phosphate buffer (pH 7.2), 10 mM NaCN, 83 μM 2,6-dichloroindophenol, 33 μM sodium succinate, and enzyme fraction (100–150 μg of protein).

RESULTS

Antagonistic effect of dibutyryl cyclic AMP on rat uterine contraction induced by oxytocin. Rat uterine contraction induced by 1 USP munit/ml of oxytocin was antagonized by dibutyryl cyclic AMP in a dose-dependent fashion. The apparent K_i value, the concentration required for half-maximal inhibition, was about 3 mM as estimated from the dose-dependence curve illustrated in Fig. 1. Neither dibutyryl cyclic GMP nor butyric acid produced significant inhibition under these conditions.

Properties of calcium uptake by uterine microsomes. The rate of calcium uptake was considerably increased by 5 μM cyclic AMP over a range of ATP concentrations, with an optimum of 250 μM ATP (Fig. 2). Above this optimum concentration, the stimulation by cyclic AMP sharply decreased, and there was virtually no effect at 2.5 mM ATP. In these experiments, the ratio of the concentration of added ATP to that of $MgCl_2$ was kept at 0.5. It is of particular interest that the amount of ATP in rat uterus was determined to be 0.269 ± 0.010 μmol/g of wet tissue, almost identical with the concentration of ATP required for the maximal stimulation of micro-

[1] The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetracetic acid; SDS, sodium dodecyl sulfate.

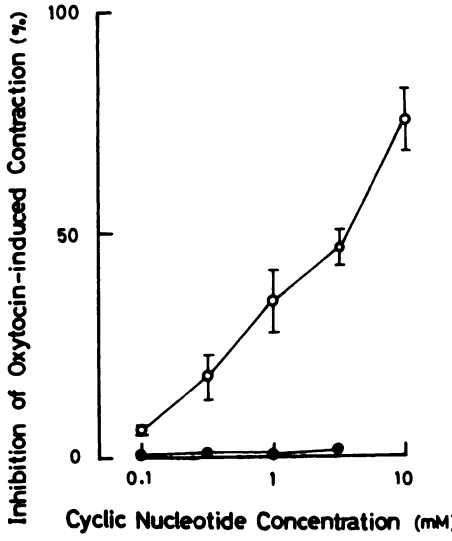

FIG. 1. *Dose-dependent effect of dibutyryl cyclic AMP* (○) *or dibutyryl cyclic GMP* (●) *on oxytocin-induced contraction of rat uterus*

Contractions were determined as described in MATERIALS AND METHODS, except for the presence of the indicated concentrations of cyclic nucleotides. Each point represents mean percentage inhibition of oxytocin-induced contraction of rat uterus, and each vertical bar shows the standard error for four experiments.

somal calcium uptake by cyclic AMP. In subsequent experiments, therefore, the incubation mixture contained 250 μM ATP unless otherwise stated.

Several experiments were carried out in an attempt to rule out the possibility that calcium uptake by the microsomal fraction was due to contaminating mitochondria. Although the rate of microsomal calcium uptake was found to be about one-third that of the mitochondrial fraction, the microsomal preparation was virtually devoid of succinate dehydrogenase, a marker enzyme for mitochondria (Table 1). The uptake reactions by both organelles were easily distinguished from one another by the following two tests. First, cyclic AMP markedly stimulated calcium uptake by microsomes but not by mitochondria. Second, sodium azide inhibited considerably and selectively the rate of mitochondrial calcium uptake.

Figure 3A illustrates the time course of calcium uptake in the absence and presence of 5 μM cyclic AMP. The reaction proceeded linearly for 1 min and leveled off at about 5 min of incubation. The effect of the amount of protein on calcium uptake is shown in Fig. 3B; uptake increased linearly up to at least 150 μg of protein. Stimulation by cyclic AMP was evident at any incubation time or any protein amount tested. The incubation temperature also influenced calcium uptake. In the presence of 5 μM cyclic AMP, the rate of calcium uptake at 4° was only 10% of the activity measured at 25°. However, the cyclic AMP dependence of calcium uptake was virtually unchanged.

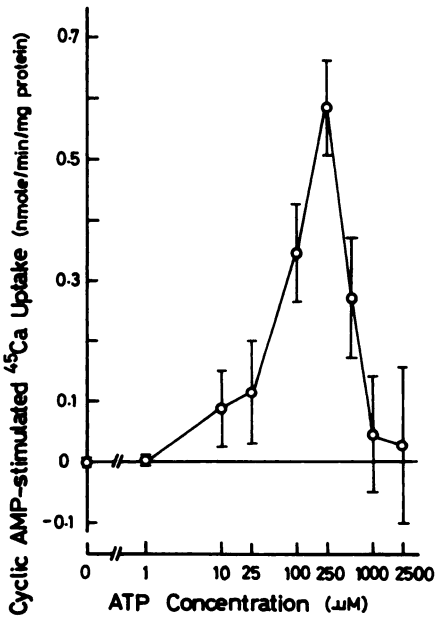

FIG. 2. *Stimulation of microsomal calcium uptake by cyclic AMP in the presence of varied concentrations of ATP*

Incubations were carried out under the standard conditions, except for varying the concentrations of ATP and $MgCl_2$ in the absence and presence of 5 μM cyclic AMP. The ratio of the concentration of added ATP to that of $MgCl_2$ was always kept at 0.5. Each point represents the mean cyclic AMP-dependent value over the basal microsomal calcium uptake, and each vertical bar shows the standard error for six experiments. The basal calcium uptake was dependent upon ATP concentration, with an apparent K_m of 520 μM. When 250 μM ATP was present in the incubation, the rate of calcium uptake in the absence of cyclic AMP was 0.613 ± 0.096 nmole/min/mg of protein.

TABLE 1

Difference in calcium uptake between microsomal and mitochondrial fractions

Calcium uptake was determined with each fraction (80–120 μg of protein) under the standard conditions, except for the presence of 5 μM cyclic AMP and/or 1 mM NaN_3. Results are means ± standard errors for six experiments. Succinate dehydrogenase activity was assayed as described in MATERIALS AND METHODS and is expressed as micromoles of 2,6-dichloroindophenol reduced per minute per milligram of protein. The percentage recoveries of succinate dehydrogenase activity in mitochondrial and microsomal fractions were 72.1% and 2.3%, respectively.

Fraction and addition	^{45}Ca uptake	Succinate dehydrogenase
	nmoles/min/mg protein	*μmoles/min/mg protein*
Microsomes		
No addition	0.568 ± 0.044	0.513
5 μM cyclic AMP	1.11 ± 0.10	
1 mM NaN_3	0.737 ± 0.092	
5 μM cyclic AMP + 1 mM NaN_3	1.25 ± 0.06	
Mitochondria		
No addition	1.93 ± 0.22	9.57
5 μM cyclic AMP	1.47 ± 0.12	
1 mM NaN_3	0.697 ± 0.123	
5 μM cyclic AMP + 1 mM NaN_3	0.748 ± 0.068	
Cell sap		
No addition	<0.05	0.615

The effects of several nucleotides on calcium uptake are summarized in Table 2. Cyclic AMP and its butyryl derivatives considerably enhanced the uptake reaction, whereas other cyclic nucleotides, 5'-AMP, and adenosine failed to stimulate the reaction. The increase in calcium uptake by cyclic AMP was dose-dependent between 0.1 and 100 μM, with an apparent K_a value of about 1 μM, the concentration required for half-maximal activation (Fig. 4). Cyclic GMP had no significant effect over the same range of concentrations.

Relationship between membrane phosphorylation and calcium uptake. In order to study the mechanism by which cyclic AMP stimulated calcium uptake by uterine microsomes, endogenous cyclic AMP-dependent phosphorylation was studied. As described by Casnellie and Greengard (20), cyclic AMP stimulated specifically the phosphorylation of a microsomal protein referred to as protein A (mol wt 48,000), whereas cyclic GMP stimulated selectively the endogenous phosphorylation of two proteins, referred to as protein G-I (mol wt 130,000) and protein G-II (mol wt 100,000). The incorporation of ^{32}P into protein A through an acyl bond was ruled out by the finding that the incubation of 12.5% trichloroacetic acid-precipitated ^{32}P-labeled microsomes for 10 min at 30° with

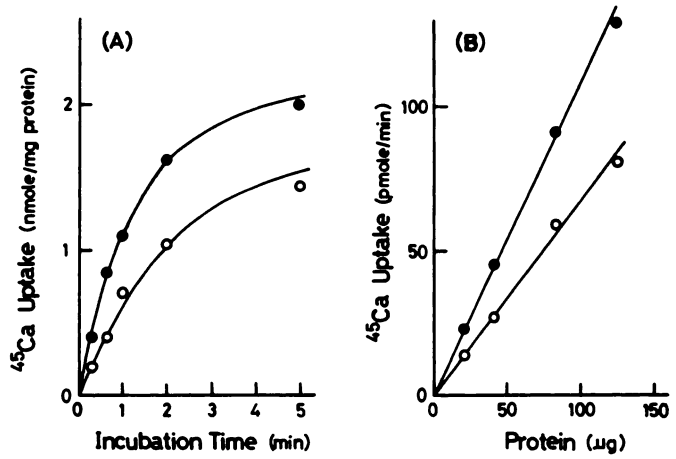

FIG. 3. *Calcium uptake in the absence* (○) *and presence* (●) *of 5 μM cyclic AMP as a function of incubation time (A) or protein amount (B)*

Incubations were carried out under the standard conditions, except for varying the incubation time or the amount of microsomal protein.

0.8 M hydroxylamine in 0.1 M sodium acetate buffer, pH 5.3, failed to remove the incorporated radioactive phosphate from protein A. When cyclic AMP-stimulated endogenous protein A phosphorylation was plotted against cyclic AMP-stimulated calcium uptake over the basal level at identical concentrations of cyclic AMP, it was found that the correlation coefficient was 0.968 ($p < 0.01$) (Fig. 5), indicating that both stimulated activities are closely correlated.

TABLE 2

Effects of several nucleotides on microsomal calcium uptake

Incubations were carried out under the standard conditions, except for the presence of the indicated nucleotide or nucleoside. Results are means ± standard errors for six experiments.

Addition	Concentration	^{45}Ca uptake	p
	μM	*nmoles/min/mg protein*	
None		0.625 ± 0.093	
Cyclic AMP	5	1.24 ± 0.15	<0.01
Cyclic GMP	10	0.631 ± 0.087	NS[a]
Cyclic IMP	10	0.597 ± 0.105	NS
Cyclic UMP	10	0.598 ± 0.083	NS
N^6-Butyryl cyclic AMP	100	0.835 ± 0.083	<0.05
N^6-2′-O-Dibutyryl cyclic AMP	100	1.13 ± 0.22	<0.01
5′-AMP	10	0.603 ± 0.078	NS
Adenosine	10	0.611 ± 0.103	NS

[a] Not significant.

DISCUSSION

The present data demonstrate that cyclic AMP stimulates calcium uptake by a uterine microsomal fraction but not by mitochondria. Upon stimulation of calcium transport into microsomes, the calcium concentration in cytosol may decrease, thereby causing a concomitant decrease in contractility of the muscular contractile protein, as in the case of skeletal muscle (24, 25). In contrast with our present results, Batra and Daniel (13) reported that cyclic AMP failed to stimulate calcium uptake by microsomes from uterine smooth muscle. Since calcium uptake is maximally stimulated by cyclic AMP in the presence of 250 μM ATP, approximately the intracellular concentration, but is not increased significantly above 1 mM ATP, the lack of cyclic AMP dependence in their experiments may be attributed to the utilization of 5 mM ATP in the incubation. Recently, however, Krall *et al.* (14) reported briefly the stimulation by cyclic AMP of microsomal calcium uptake in rat uterus even in the presence of 4 mM ATP, although it was not shown whether

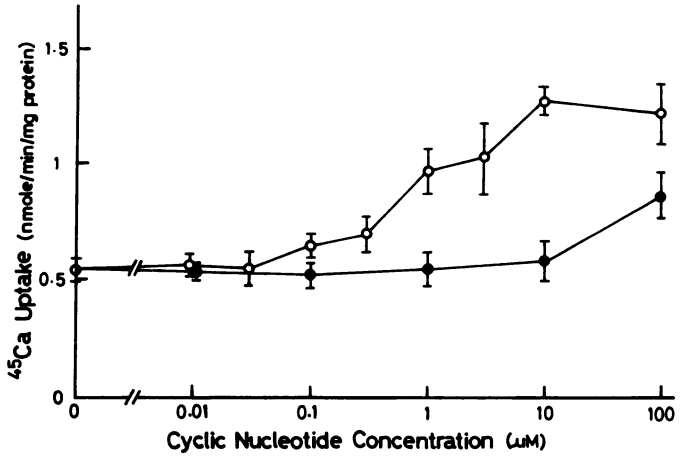

FIG. 4. *Effect of cyclic AMP* (○) *or cyclic GMP* (●) *on microsomal calcium uptake*

Incubations were carried out under the standard conditions, except for the presence of the indicated cyclic nucleotide. Each point represents mean calcium uptake, and each vertical bar shows the standard error for six experiments.

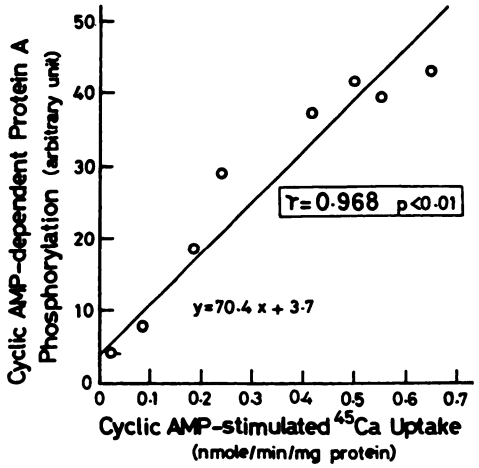

FIG. 5. *Relationship between cyclic AMP-dependent protein A phosphorylation and cyclic AMP-dependent calcium uptake*

Experimental conditions were the same as described in MATERIALS AND METHODS, except for varying the concentration of cyclic AMP. The incorporation of radioactive phosphate into protein A was measured quantitatively by densitometric analysis of the radioautograms, and the optical density was expressed in arbitrary units as described by Ueda *et al.* (21) as well as Casnellie and Greengard (20). The scale of arbitrary units used was the same for all experiments. The correlation coefficient (r) and the line of best fit were computed by the method of least mean squares.

the uptake reaction responded specifically to cyclic AMP. In their experiments, the rate of calcium uptake was constant for about 30 min, in contrast with our results as well as those of Batra and Daniel (10, 13), in which the reaction reached a plateau in 5 min. The reason for these discrepancies is not known at present.

It has been postulated that diverse biological effects of cyclic AMP may be mediated by stimulation of protein phosphorylation (26–29). In support of this hypothesis, the present study indicates clearly for the first time a close correlation between phosphorylation of membrane-bound protein A [mol wt ~48,000 (20)] of rat uterine microsomes and microsomal calcium uptake, which may trigger the relaxation of uterine smooth muscle. Protein A is widely distributed in various kinds of smooth muscle (20) and, although not definitively characterized, it may be a regulatory subunit of cyclic AMP-dependent protein kinase (30).

Maximum stimulation of microsomal calcium uptake by cyclic AMP occurs at 250 μM ATP; yet 5 μM ATP, at which concentration virtually no stimulation of calcium uptake occurs, was used to measure the phosphorylation of protein A. The difference in ATP concentration, however, does not appear to affect our conclusion regarding the relationship between the two activities, since we have found in preliminary experiments that increasing the concentration of ATP from 5 to 250 μM does not significantly alter the stimulation of protein A phosphorylation by 5 μM cyclic AMP. We have not studied the effect of ATP concentration on protein A phosphorylation in detail because of the difficulty of preparing large amounts of ATP with a specific radioactivity high enough for routine radioautographic measurements of the phosphorylation of protein A. It is generally understood that high concentrations of ATP decrease the effect of cyclic AMP on protein phosphorylation (31). This may be partly responsible for a marked decrease in the sensitivity of uterine microsomal calcium uptake to cyclic AMP in the presence of ATP concentrations higher than 1 mM.

Tada *et al.* (32–34) have suggested that calcium uptake by sarcoplasmic reticulum in cardiac muscle may be modulated through increased phosphorylation of a membrane-bound protein (referred to as phospholamban; mol wt ~22,000) catalyzed by exogenously added cyclic AMP-dependent protein kinase from either cardiac or skeletal muscle. It would be of interest to determine whether uterine smooth muscle also contains a protein like cardiac phospholamban.

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