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The Effects of Epinephrine, Prostaglandins, and Their Antagonists on Adenosine Cyclic 3′,5′-Monophosphate Concentrations and Motility of the Rat Uterus

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SUMMARY

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Interactions of substances which induce contractions (prostaglandins, oxytocin) and of epinephrine, which induces relaxation, with the adenylate cyclase system were examined in estrogen-treated rat myometrium. Epinephrine and prostaglandin E_1 (PGE_1) (also PGE_2) stimulated adenylate cyclase activity. The response to both agents was very rapid, being detectable at 30 sec. Propranolol inhibited the activation of adenylate cyclase by epinephrine but not that by PGE_1. A single adenylate cyclase appeared to be involved in stimulation by both agonists. $PGF_{1\alpha}$, $PGF_{2\alpha}$, and oxytocin had no significant effect on adenosine cyclic 3′,5′-monophosphate (cAMP) concentrations. All the E and F prostaglandins analyzed elicited contractions in rat uteri; therefore PGE_1 stimulated contractions although elevating cAMP levels. Epinephrine antagonized contractions evoked by $PGF_{2\alpha}$ and oxytocin as well as by PGE_1. The relaxing effect of epinephrine could be mimicked by dibutyryl-cAMP and inhibited by propranolol. There seemed to be a good correlation between the stimulation of adenylate cyclase by epinephrine and its effect on motility, implicating a role for cAMP in smooth muscle relaxation. Such a correlation could not be demonstrated for PGE_1. Two prostaglandin antagonists, polyphloretin phosphate and 7-oxa-13-prostynoic acid, blocked the prostaglandin E- and F-induced contractions but did not alter the ability of PGE_1 to raise cAMP levels. The results suggest that interaction with adenylate cyclase is not a prerequisite for the prostaglandins to induce contractions in uterine smooth muscle. The differences in the action of epinephrine and PGE on uterine motility may reflect compartmentalization of the cAMP formed in response to these agents.

INTRODUCTION

The strong smooth muscle-stimulating activity of the prostaglandins was one of the first pharmacological effects ascribed to this class of compounds. The biological aspect of this activity has been well documented in a general review by Bergström *et al.* (1). However, only recently have biochemical studies been reported on the mechanism of prostaglandin action on smooth muscle preparations, particularly the myometrium. These have dealt either with specific structural

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requirements for prostaglandin activity (2) or with prostaglandin-induced modifications of specific metabolic pathways, including the formation of adenosine cyclic 3′,5′-monophosphate (3–6).

As a result of various investigations (7–10), a possible role for intracellular cAMP[1] in the regulation of uterine tone and responsiveness to physiological stimuli has been suggested, i.e., activation of adenylate cyclase by *beta* adrenergic agonists, resulting in inhibition of contractility. In a previous communication (3), however, we reported that prostaglandins of the E series (PGE_1, PGE_2) stimulated rat uterine contractions but were also potent stimulators of the adenylate cyclase system. In that study adenylate cyclase activity of the myometrium was measured as the accumulation of intracellular [^{14}C]cAMP subsequent to labeling of the ATP pool with [^{14}C]adenine. The PGE effect was difficult to explain, in the light of the hypothesis that cAMP is the intracellular transducer of uterine muscle relaxation. However, with the technique used in our experiments, the existence of various ATP pools with different specific activities could not be completely ruled out, and it appeared necessary to obtain accurate estimations of intracellular cAMP levels under various stimulatory conditions.

In the present work the time course and extent of formation of total cAMP (as well as accumulated [^{14}C]cAMP) and of the ultimate mechanical response elicited by epinephrine and by prostaglandins E and F have been systematically compared in estrogen-treated rat uteri. The results confirm and extend our previous observations. A good correlation was found between epinephrine activation of adenylate cyclase and epinephrine-induced relaxation; on the other hand, experiments performed with the prostaglandin antagonists polyphloretin phosphate and 7-oxa-13-prostynoic acid provide evidence that the dual PGE_1 effects on rat myometrium (i.e., stimulation of adenylate cyclase and contraction) are not causally related. A preliminary report of a portion of these results has been presented (11).

[1] The abbreviations used are: cAMP, adenosine cyclic 3′,5′-monophosphate; PG, prostaglandins; DB-cAMP, N^6,O^2-dibutyryladenosine cyclic 3′,5′-monophosphate; PPP: polyphloretin phosphate; PY_1, 7-oxa-13-prostynoic acid.

MATERIALS AND METHODS

Chemicals

cAMP was obtained from P-L Biochemicals Inc., and theophylline, from Merck (Darmstadt).

Dibutyryl-cAMP and L-epinephrine bitartrate were purchased from Calbiochem, and oxytocin (Pitocin), from Sigma. Prostaglandins E_1, E_2, $F_{1\alpha}$, and $F_{2\alpha}$ were a generous gift from Dr. J. E. Pike of the Upjohn Company.

Propranolol and phentolamine hydrochloride were kindly supplied by Laboratoires CIBA, Paris, and estradiol benzoate (Benzo-Gynoestryl 1), by Laboratoires Roussel, Paris. Polyphloretin phosphate was a gift from Dr. B. Högberg (Leolab) to Dr. B. Vargaftig. 7-Oxa-13-prostynoic acid PY_1 (EC 1.148) was provided through the courtesy of Dr. J. Fried, University of Chicago.

Cellulose ester membrane filters (HA 0.45 μ, 24 mm) were Millipore products Dowex AG50W-X8 (200–400 mesh) was obtained from Bio-Rad, and plastic-backed, thin-layer cellulose sheets, from Eastman Kodak.

All reagents used were products of Prolabo (reagent grade).

[8-^{14}C]Adenine hydrochloride (44.5 mCi/mmole) was obtained from the Commissariat à l'Energie Atomique, Saclay, France, an [^{3}H]cAMP (23 Ci/mmole), from New England Nuclear Corporation.

Animals

Young virgin female rats (Wistar), 6 weeks old, were treated with 30 μg estradiol for 2 days and used on the following day.

Tissue Preparation

Rats were killed by decapitation, and their uteri were immediately removed and immersed in Krebs bicarbonate buffer, pH 7.4, at 4°. The organs were freed from adhering fat and connective tissue. In most experiments, unless otherwise stated, the

endometrium was removed by cleaving the uterus and scraping and washing away the less resistant endometrial tissue in buffer at 4°. Histological sections of these preparations (performed thanks to Dr. G. Vassort and Mrs. M. F. Sire) showed the samples to consist of layers of both longitudinal muscle and circular muscle, contaminated with 30–40% of the endometrium as compared to histological sections of the intact uterus. In some experiments the myometrium was separated by stripping, and in this case the histological sections showed the samples to consist almost exclusively of longitudinal muscle; stripping had removed all the endometrium together with most of the circular muscle layers. The latter myometrial preparation was referred to as "stripped myometrium." Myometrial preparation from 15–20 rat uteri were cut into small segments, which were pooled and divided into different equal parts for the following incubations.

Incubation Techniques

Incubations were carried out in Krebs bicarbonate buffer (12) at 37° (gas phase, 95% O_2–5% CO_2) under constant agitation. The conditions used depended on whether the aim of the experiment was to investigate adenylate cyclase activity through [^{14}C]-cAMP formation from labeled [^{14}C]ATP or to evaluate changes of total intracellular cAMP levels.

Incorporation of [^{14}C]adenine in uterine strips and measurements of [^{14}C]cAMP accumulation. Between 80 and 100 mg of uterine strips were incubated in 2 ml of Krebs bicarbonate buffer in the presence of 2 μCi of [^{14}C]adenine for 30 min at 37° (3). The tissue strips were then transferred into another incubation vessel containing 2 ml of fresh buffer without labeled substrate in the absence or presence of 10 mM theophylline and the different agents at the indicated concentrations. After varied incubation times the reaction was stopped by immersing the tissue strips in 2 ml of cold 7% trichloracetic acid containing 0.20 μmole of unlabeled cAMP added as a carrier.

Tissue incubation for subsequent estimation of cAMP levels. About 50 mg of myometrial strips were added to 1.5 ml of Krebs bicarbonate buffer and incubated for 30 min under the conditions described above. Incubations were further continued with or without the addition of theophylline (final concentration, 10 mM) and the various substances to be tested. Reactions were stopped at different times (see RESULTS AND DISCUSSION) by the addition of 0.2 ml of cold 50% trichloracetic acid and immediate homogenization of the samples at 0° in ice. Levels of cAMP were not significantly different in experiments in which uterine strips after the various incubations, were frozen in liquid N_2 prior to extraction with cold trichloracetic acid or were directly extracted with cold trichloracetic acid at 0°, provided that the homogenization was as fast as possible after the addition of trichloracetic acid.

Tissue Extraction

After one or the other incubation, uterine strips in trichloracetic acid were homogenized (with an Ultra-Turrax homogenizer) three times for 15 sec at 0° and centrifuged at 15,000 × *g* for 40 min. After addition of 0.1 ml of 1 N HCl, the supernatant solution was extracted six times with twice its volume of ether saturated with cold water, evaporated to dryness, and used either for the purification of accumulated [^{14}C]cAMP or for total cAMP assay. The centrifuged pellets were dissolved in 2 ml of 1 N NaOH for protein determination (13).

Purification of [^{14}C]cAMP Accumulated in Extracts

The basis of the method has been described in a previous publication (3). The evaporated, trichloracetic acid-soluble extracts were resuspended in 0.5 ml of 0.05 N Tris-HCl, pH 7.0, and treated according to Krishna *et al.* (14) by passage through a column of Dowex AG50W-X8 (0.5 × 3.5 cm). Elution was carried out with distilled water; the first 6.0 ml of eluate, which contained a mixture of ATP, ADP, and cAMP, were evaporated to dryness. The residue was dissolved in 50 μl of distilled H_2O and chromatographed on plastic-backed, thin-layer cellulose sheets, using 95% ethanol–1

M ammonium acetate (75:30, v/v) as developing solvent. The spot corresponding to cAMP, as well as those representing the mixture of ATP and ADP, was cut out and radioactivity was measured in a liquid scintillation apparatus. The radioactivity due to cAMP thus could always be estimated in comparison with the fairly constant total radioactivity associated with ATP and ADP.

Previous experiments showed that [^{14}C]-adenine was incorporated, probably through the action of adenine phosphoribosyltransferase, into intracellular adenine nucleotides of myometrial strips, and particularly into the ATP pool, which provides the labeled substrate for the adenylate cyclase.

About 10% of the added radioactivity was present in the total trichloracetic acid-soluble myometrial extracts. cAMP labeling ranged from 0.25 to 2.5% of the radioactivity incorporated into the ATP-ADP pool. cAMP was separated from these nucleotides by thin-layer chromatography.

The purity of the radioactivity at the cAMP spot was verified by treating the extracts with a cyclic phosphodiesterase preparation purified from bovine heart according to Butcher and Sutherland (15). After this treatment almost no radioactivity could be detected as cAMP on the chromatogram sheet. On the other hand, addition of exogenous [^{3}H]cAMP at the onset of sample homogenization resulted in 60–70% recovery of the tritiated cyclic nucleotide at the final purification step.

Assay of cAMP Levels

cAMP was estimated directly in the trichloracetic acid-soluble extracts according to Gilman (16), using a protein kinase from rabbit skeletal muscle purified through the DEAE-cellulose step (protein fraction eluted with 300 mM phosphate buffer, pH 6.5) and a heat-stable inhibitor prepared from the same muscle extract. All assays were performed in a total volume of 50 µl in 50 mM acetate buffer, pH 4.0, containing 2.2 pmoles of [^{3}H]cAMP and various concentrations of unlabeled cAMP ranging from 0.5 to 15 pmoles. Each sample was analyzed at three different concentrations. When a known quantity of exogeneous cAMP was added to a tissue sample at the onset of homogenization, 95–100% of the cyclic nucleotide, as determined by the above procedure, was recovered in the final extracts.

cAMP levels were expressed as picomoles per milligram of protein or per milligram of tissue, wet weight.

Method for Recording Uterine Responses

Contractile activity of isolated uterine strips was measured with an isometric transducing device. The segments were loaded at a basal tension of 0.2–0.3 g and were perfused at 37° in 30 ml of Krebs bicarbonate buffer (95% O_2–5% CO_2) with the same salt composition as described for the above incubations. The tissue was equilibrated in the organ bath for 30–60 min, with several changes of the bathing fluid. When a steady-state pattern of contractions evolved and the baseline did not change, the different pharmacological agents were added at the indicated concentrations. Sensitivity was adjusted electronically so that the maximum contraction yielded about half a full-scale deflection of the pen. In some experiments the loaded segments, incubated in the absence or presence of the different agents, were used for assay of their cAMP content. In this case, after the mechanical recording, the bath fluid was removed and the segments were immediately extracted with cold 7% trichloracetic acid at 0° as described above for the estimation of cAMP.

RESULTS AND DISCUSSION

cAMP Content of Rat Myometrium

Effects of theophylline, epinephrine, PGE_1, and PGE_2.

The modification of cAMP concentrations in uterine strips under different incubation conditions is shown in Table 1. cAMP content, measured immediately after tissue preparation without further incubation, was 22.0 pmoles/mg of protein (about 2 pmoles/mg of wet tissue). After incubation for 25 min in the absence of theophylline, there was a decrease of about 50% in the level of cAMP. This decrease was attributed to

TABLE 1

cAMP levels in rat myometrium under various incubation conditions

About 50 mg of myometrial strips were extracted directly with 2 ml of cold 7% trichloracetic acid (column A). Other samples were incubated in 1.5 ml of Krebs bicarbonate buffer in the absence or presence of 20 μM adenine for 25 min at 37° (gas phase, 95% O_2–5% CO_2), and the reaction was stopped by extracting the tissue with 7% cold trichloracetic acid. (column B). The tissues in column C were incubated like those in column B (without adenine). Theophylline (10 mM) was then added, and incubation was continued for the indicated time before addition of 7% cold trichloracetic acid. All acid extracts were treated as described under MATERIALS AND METHODS for the estimation of cAMP by the Gilman technique (16). Results are expressed as picomoles of cAMP per milligram of protein or per milligram of wet tissue. Numbers in parentheses designated the number of experiments.

Total cAMP	Without incubation (A)	Without theophylline (B)		With theophylline, 10 mM (C)		
		−Adenine	+Adenine, 20 μM	1 min	5 min	15 min
pmoles/mg protein	22.8 ±2 (3)	12 ±0.49 (9)	10±0.7 (5)	19±1.13 (5)	21.2 ±1.08 (9)	19.1 ±1.02 (13)
pmoles/mg wet tissue	2.02±0.8 (10)	1.02±0.06 (14)			1.93±0.13 (9)	2.12±0.06 (11)

cAMP phosphodiesterase activity in these preparations, since the presence of theophylline in the incubation medium very rapidly restored the initial values. It has also been verified that the presence of adenine at the concentration (20 μM) used in the preliminary labeling experiments with [^{14}C]-adenine did not markedly modify the tissue content of cAMP.

In the absence of theophylline, epinephrine (1 μM) induced a rise in total cAMP levels of rat myometrium (about 50% stimulation) (Table 2). With PGE_2 or PGE_1 (8.8 μM), the stimulation was about 60 and 210%, respectively. Theophylline (10 mM), when added together with epinephrine or PGE, exerted a synergistic effect in increasing cAMP levels, which under these conditions reached 80 pmoles/mg of protein. The cAMP levels of myometrial strips which had been loaded under the conditions used for recording mechanical activity and incubated with theophylline, epinephrine, or PGE_1 were comparable to the cAMP levels found in unloaded myometrial strips (Table 2).

Effect of various concentrations of prostaglandins, epinephrine, and oxytocin on [^{14}C]-*cAMP accumulation.* Figure 1a and b shows the dose-response curves for the effects of $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_1, epinephrine, and oxytocin on [^{14}C]cAMP accumulation and total cAMP levels, respectively, in rat myometrial strips incubated in the presence of 10 mM theophylline for 15 min. In both parts of the figure the curves were almost identical for each agent tested. Epinephrine stimulated adenylate cyclase and cAMP accumulation with a maximum effect at 1 μM and an apparent activation constant (concentration giving half-maximal response) of 0.1–0.2 μM. PGE_1 elicited the activation of adenylate cyclase and a rise in total cAMP level with a maximal effective dose of 10 μM and an apparent activation constant of 1–2 μM. Dose-response curves with PGE_2 were superposable on those obtained with PGE_1 (data not shown). The concentration required for PGE_1 were higher than those for epinephrine; however, the maximal responses were similar in magnitude with both agents, whether [^{14}C]cAMP accumulation was measured after prior labeling with [^{14}C]adenine or total cAMP was estimated by the Gilman binding assay. Our previous data (3) dealing with [^{14}C]cAMP accumulation indicated that maximal stimulations by epinephrine and PGE_1 were obtained at concentrations lower than those described in the present study. The reason for this change is unknown, since the experimental protocol does not appear to have been significantly altered except for the origin of the rats. The results re-

TABLE 2

Effects of epinephrine, PGE_1, and PGE_2 on cAMP content of rat myometrium

In experiment A about 50 mg of myometrial strips were incubated for 25 min in Krebs bicarbonate buffer; then theophylline, where indicated, was added, followed by the addition of epinephrine or PGE, and incubations were continued for 5 min. Tissues were extracted and cAMP was determined as described under MATERIALS AND METHODS. Values represent the means ± standard errors of six different experiments. In experiment B myometrial strips were loaded in the bath fluid as under conditions for measuring mechanical activities, incubated for 25 min before the addition of theophylline, epinephrine, or PGE_1, and processed as above.

Addition	Without theophylline	With theophylline, 10 mM
	pmoles cAMP/mg protein	
Experiment A		
Control	11.1 ± 0.9	21.2 ± 1.1
Epinephrine, 1 μM	16.0 ± 1.6	81.3 ± 5.0
PGE_1, 8.8 μM	24.6 ± 2.2	78.4 ± 6.6
PGE_2, 8.8 μM	17.6 ± 1.5	76.0 ± 6.0
Experiment B		
Control	11.1 ± 0.7	18.4 ± 1.3
Epinephrine, 1 μM	17.4 ± 1.4	
PGE_1, 8.8 μM	25.2 ± 2.1	

ported in Fig. 1 are now consistently reproducible.

$PGF_{1\alpha}$, $PGF_{2\alpha}$, and oxytocin did not significantly affect the adenylate cyclase activity or total cAMP level even at concentrations as high as 100 μM, which for the oxytocin preparation corresponds to 100 milliunits/ml. Bhalla *et al.* (5) recently reported similar results for isoproterenol (used as a *beta* adrenergic agent) and PGE_1 in estrogen-treated rat uterus. They also found $PGF_{2\alpha}$ and oxytocin to have no stimulatory effects on cAMP levels.

With the results of Fig. 1a and b and those described in the following experiments, it has been possible to compare the amount of radioisotope which cAMP contains with the absolute levels of the cyclic nucleotide. The results of such a comparison demonstrate in all cases that the change in radioactive cAMP formed reflects the change in the absolute levels of cAMP. It could not be a simple consequence of variability in the specific activity of the ATP pool(s) which serves as the precursor for cAMP.

Time Course of Epinephrine and PGE_1 Effects on Total cAMP Level in the Absence and Presence of Theophylline

Figure 2a shows the kinetics for PGE_1 and epinephrine responses at two different concentrations, when theophylline was absent from the incubation medium. In the case of epinephrine, with the two concentrations used (0.1 μM and 5 μM), there was an increase in cAMP level which appeared to be dose-dependent, with maximal stimulation being obtained in each instance between 30 and 60 sec. The cyclic nucleotide then declined slowly with time and at 15 min remained slightly higher than the control value. The results were quite different with PGE_1: the response was also rapid, but the level of cAMP continued to rise slowly, reaching a maximum at about 5 min for the optimal concentration (25 μM). Within 1–2 min the levels of cAMP, when compared either at submaximal doses of epinephrine and PGE_1 or at their supramaximal doses, were identical for both agents, but because of the decline with time in the case of epinephrine the levels after prolonged incubation (5–10 min) were always higher for PGE_1 than for epinephrine. It was verified, by incubating epinephrine with uterine segments for various periods (30 sec–20 min) and subsequently testing the stimulatory capacity of the incubation medium on a second batch of uterine strips, that this discrepancy was not due to the degradation of epinephrine (results not shown in the present work). Hence two explanations may be advanced for the lack of decline of the PGE_1-induced rise in cAMP as opposed to the decline with epinephrine either an inhibitory effect of PGE_1 on the cyclic phosphodiesterase activity, or inaccessibility of the cAMP formed to the action of the intracellular phosphodiesterase. The latter explanation would of course suggest a sequestered form for the cAMP formed through the stimulation by PGE_1.

Similar time courses experiments were performed in the presence of 10 mM theophylline in the incubation medium (Fig. 2b). Again, with epinephrine as well as PGE_1,

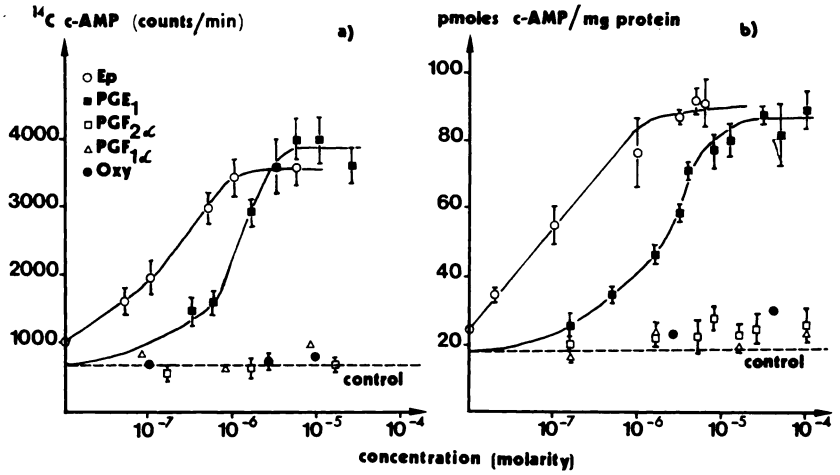

FIG. 1. *Effects of various concentrations of prostaglandins E and F, epinephrine (Ep), and oxytocin (Oxy) on [^{14}C]cAMP accumulation and cAMP levels in rat myometrium*

a. Uterine strips (100 mg) were incubated in 2 ml of Krebs bicarbonate buffer containing 2 μCi of [^{14}C]adenine for 30 min at 37°. At the end of this incubation, strips were transferred into 2 ml of Krebs bicarbonate buffer with 10 mM theophylline and the different agents tested at the concentrations shown. The reaction was stopped after 15 min, and the strips were treated as indicated in the text for assay of [^{14}C]cAMP accumulation. The radioactivity associated with cAMP was estimated as counts per minute per 100 mg of wet tissue, after subtraction of the zero-time control (cAMP value obtained after the first incubation, an average of 500 cpm). b. Uterine strips were incubated in Krebs bicarbonate buffer without adenine for 25 min; then 10 mM theophylline was added, as well as the different agents tested. After 15 min of further incubation, tissue was extracted for total cAMP determination as described in the text. Values represent the means ± standard errors of six different experiments.

the time to reach the plateau was inversely related to the concentration of the agonist. It is also important to note that for a given response, there was no significant difference between the time course of PGE_1-induced cAMP accumulation and that elicited by epinephrine. The responses in both cases were very rapid, being detectable at 30 sec even for the lowest concentrations used in this experiment, i.e., 1.6 μM PGE_1 and 0.1 μM epinephrine. In addition, the presence of theophylline prevented the decline in cAMP levels after epinephrine stimulation.

Effect of Beta and Alpha Adrenergic Blocking Agents on Epinephrine- and PGE_1-Stimulated Adenylate Cyclase Activities

Propranolol (25 μM), a blocking agent of *beta* adrenergic receptors, inhibited by 90% the accumulation of [^{14}C]cAMP as well as the total rise in cAMP level evoked by epinephrine (Table 3). This confirms that in rat myometrium, epinephrine stimulation of adenylate cyclase occurs through *beta* adrenergic receptors (3, 10, 17). On the other hand, propranolol did not modify the response to PGE_1, indicating that there are separate receptor sites for epinephrine and PGE_1.

Phentolamine (25 μM), a blocking agent of *alpha* adrenergic receptors, has no effect on the stimulation by either epinephrine or PGE_1. [^{14}C]cAMP accumulated in the presence of phentolamine and epinephrine was 3480 ± 350 cpm, and with phentolamine plus PGE_1 it was 4040 ± 440 cpm; these values are identical with those obtained in the absence of any adrenergic blocking agent (Table 3). It may be concluded that adrenergic receptors are not involved in the activation of adenylate cyclase by PGE_1 in rat myometrium.

Effect of Combined Epinephrine and PGE_1 on cAMP Accumulation in Rat Myometrium

Table 4 shows the results obtained when PGE_1 and epinephrine were added simultaneously to incubated myometrial strips. At supramaximal concentrations (epinephrine, 5 μM; PGE_1, 25 μM) in the presence of

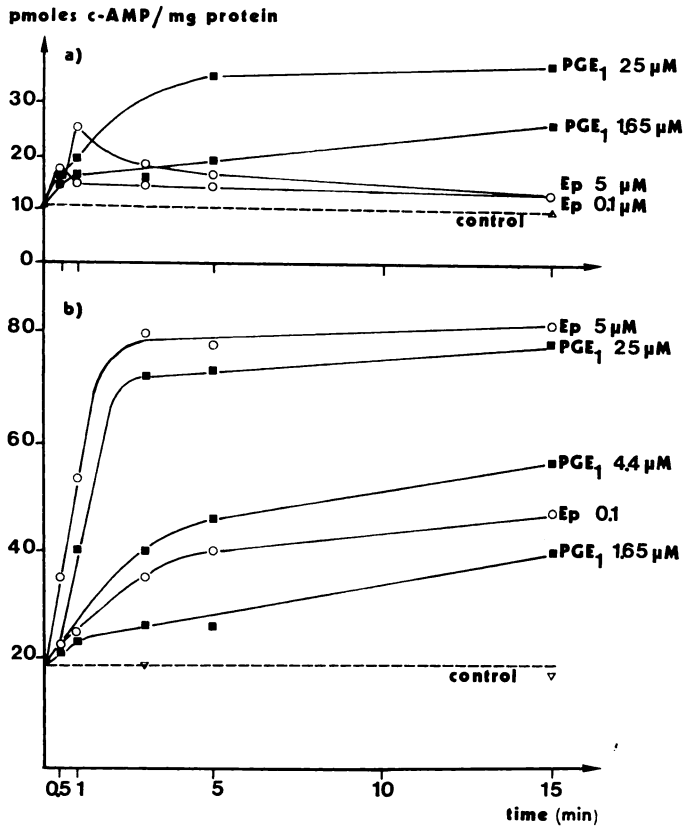

FIG. 2. *Time course for epinephrine (Ep) and PGE_1 effects at various concentrations on total cAMP level of rat myometrium*

Uterine strips were incubated in Krebs bicarbonate buffer for 25 min at 37°. Incubations were then continued in the absence (a) or presence (b) of 10 mM theophylline with the addition of epinephrine (○) or PGE_1 (■) at the indicated concentrations, and reactions were stopped at the times indicated by the addition of cold trichloracetic acid up to 7%. cAMP was assayed as described under MATERIALS AND METHODS. Values represent the means of three different experiments.

theophylline, no additive effects could be demonstrated on [^{14}C]cAMP accumulation or on total cAMP levels. Therefore, although interacting with distinct receptors, PGE_1 and epinephrine seem to activate the same catalytic sites of the adenylate cyclase system: when the enzyme is fully activated by either agent, it can no longer be responsive to the other.

On the other hand, in the presence of PGE_1 and epinephrine at concentrations near their half-maximal effective doses, cAMP levels were always higher than those obtained with each agent when present alone, and averaged approximately the calculated values for epinephrine- plus PGE_1-induced effects at these nonsaturating concentrations. These results would indicate that as long as the adenylate cyclase is not fully stimulated, the presence of PGE_1 at the concentration used does not modify the response to epinephrine, and vice versa. The latter observations differ to some extent from those published by Bhalla *et al.* (5), who described the stimulation of adenylate cyclase in estrogen-treated rat uteri by PGE_1 in a dose-dependent manner (0.5–50 μM); however, PGE_1 was always less effective than the *beta* adrenergic agonist isoproterenol. Moreover, at low concentrations (0.5–5 μM), PGE_1 inhibited isoproterenol-induced cAMP accumulation. According to these

TABLE 3

Effect of propranolol on epinephrine- and PGE_1-induced rise in cAMP levels of rat myometrium

Uterine strips were treated as described under MATERIALS AND METHODS for [^{14}C]cAMP accumulation (column A) and for total cAMP determination (column B) (see also legend to Fig. 1). After the addition of theophylline to the incubation medium, tissue strips were incubated for 5 min in the absence or presence of 25 μM propranolol before the subsequent addition of epinephrine or PGE_1, and incubation was continued for 15 min at 37°. Means ± standard errors of six different experiments are shown.

Addition	Propranolol, 25 μM	[^{14}C]cAMP accumulated (A)	Total cAMP (B)
		cpm	*pmoles/mg protein*
Basal levels	−	700±150	22.5±0.75
	+	800±146	24.6±1.0
Epinephrine 5 μM	−	3400±285	82.5±4.0
	+	1050±100	35.2±3.0
PGE_1, 8.8 μM	−	3960±330	85.0±5.0
	+	3580±310	81.0±5.1

TABLE 4

Effects of combination of epinephrine and PGE_1 on [^{14}C]cAMP accumulation and total cAMP levels in rat myometrium

Uterine strips were treated as described under MATERIALS AND METHODS for [^{14}C]cAMP accumulation (column A) and for total cAMP determination (column B) (see legend to Fig. 1). Epinephrine or PGE_1, combined or individually, were added at the indicated concentrations, and incubation was continued at 37° for 15 min in the presence of 10 mM theophylline. Means ± standard errors of six different experiments are shown.

Conditions	[^{14}C]cAMP (A)		Total cAMP (B)	
	Experimental	Calculated if additive	Experimental	Calculated if additive
	cpm		*pmoles/mg protein*	
Control	980±150		19±2.0	
Epinephrine, 5 μM	3800±260		84±5.5	
PGE_1, 25 μM	3780±250		90±7.2	
Epinephrine + PGE_1	4000±650	6520	105±8.5	155
Epinephrine, 0.1 μM	2500±350		45±4.4	
PGE_1, 1.6 μM	2200±300		52.5±4.9	
Epinephrine + PGE_1	3170±320	3720	72±4.25	78

results, PGE_1 at 3 μM, used in the experiment of Table 4, should have inhibited the epinephrine response 40%). Under the present experimental conditions, as far as cAMP accumulation is concerned, rat myometrium appears to be more responsive to PGE_1 than in the experiments described by Bhalla *et al.* (5). It is therefore possible that under our experimental conditions much lower doses of PGE_1 might produce an inhibitory effect on the *beta* adrenergic stimulation, but such doses were not tested in the present study.

Comparison of Effects of Epinephrine and PGE_1 on cAMP Levels of Scraped and Stripped Myometrial Preparations

All the experiments described above were performed with myometrial preparations obtained by scraping off the endometrial cells. As described under MATERIALS AND METHODS, histological observations showed these samples to be contaminated with 30–40% of the original endometrium. In order to evaluate the contribution of the endometrium to the responses of the uterine preparations to epinephrine and PGE_1, a series of experiments were performed with stripped myometrial preparations, which were shown to be virtually free of endometrium and also of circular muscle fibers. Some of the results are shown in Table 5. The protein content of the stripped myometrium averaged 66% of the total protein found in the intact uterus, while in the scraped preparations about 80% of the protein was present. Hence, for the comparisons to be meaningful, results were expressed as picomoles of cAMP per milligram of total protein of the intact tissue as well as cAMP per milligram of protein in the indicated preparation. The stripped myometrium possessed an adenylate cyclase which could be stimulated equally by epinephrine and PGE_1 (Table 5). The values for cAMP levels after both stimulations are comparable to those described above. This shows that endometrial cells contaminating the scraped tissue preparation were not involved to any significant extent in the sti-

TABLE 5

Comparison of effects of epinephrine and PGE_1 on cAMP levels of scraped and stripped myometrial preparations

Scraped or stripped myometrium was prepared as described under MATERIALS AND METHODS. About 50 mg of either preparation were incubated under conditions specified in Table 2, experiment A. Results are expressed as picomoles of cAMP per milligram of total protein of the intact uterus or as per milligram of protein of the tissue preparation. Results represent the means ± standard errors of three different experiments.

Incubation conditions	cAMP in stripped myometrium		cAMP in scraped preparations	
	pmoles/mg total protein	*pmoles/mg protein in tissue preparation*	*pmoles/mg total protein*	*pmoles/mg protein in tissue preparation*
Without theophylline	10.3 ± 1.1	15.5 ± 1.64	8.8 ± 0.75	11.0 ± 0.9
With theophylline	20.0 ± 1.65	30.0 ± 2.5	17.0 ± 1.05	21.2 ± 1.3
+ Epinephrine, 5 μM	92.0 ± 7.5	140.0 ± 11.5	65.0 ± 4.25	81.3 ± 5.3
+ PGE_1, 8.8 μM	89.2 ± 6	135.0 ± 9	65.0 ± 5.5	81.0 ± 6.5

mulation of cyclic AMP formation observed through the actions of epinephrine and PGE_1.

Correlation with Uterine Motility

Correlations between the effects on adenylate cyclase activity of the different pharmacological agents and their capacity to alter uterine motility were systematically tested. Contractile activity of the isolated uterine strips was measured in the same physiological buffer and with identical dose ranges of active substances as those used for the preceding experiments. As shown in Fig. 3a and b, $PGF_{2\alpha}$ elicited the same type of tetanic contractions as oxytocin, but neither agent modified total cAMP level or its accumulation in uterine strips (results of Fig. 1). When epinephrine (0.3 μM) was added to $PGF_{2\alpha}$- or oxytocin-stimulated strips, immediate relaxation was observed in both cases. $PGF_{1\alpha}$, another agent which did not alter cAMP levels, also induced contractions (Fig. 3c).

PGE_1 (Fig. 3d) stimulated uterine contractions, even though it elevated cAMP levels. On the other hand, epinephrine, whose effect is identical with that of PGE_1 on adenylate cyclase, antagonized the PGE_1-induced contraction of uterine strips. Identical results were obtained with PGE_2 or with PGE_1. Finally, PGE_1, at a concentration known to elevate intracellular cAMP levels, was unable to provoke relaxation of $PGF_{2\alpha}$-stimulated uterine contractions, whereas subsequent addition of epinephrine to $PGF_{2\alpha}$ plus PGE_1 immediately counteracted the induced contractions (Fig. 3e). Likewise PGE_1 failed to inhibit oxytocin-induced contractions, while epinephrine added to oxytocin plus PGE_1 abolished the contractions (results not shown).

In these experiments the contractile agents were used at their maximal effective concentrations. It is noteworthy that for PGE_1 the maximal response for contraction could be measured at 1.5 μM, which was not the maximal dose for adenylate cyclase stimulation. Furthermore, PGE_1 at 25 μM, which maximally stimulated adenylate cyclase, still provoked full contractility responses and did not antagonize contractions elicited by $PGF_{2\alpha}$ or oxytocin, in spite of the high intracellular cAMP levels under these conditions. It has also been verified that the presence of either $PGF_{2\alpha}$ or oxytocin does not modify the response of myometrial adenylate cyclase to PGE_1.[2]

The ability of dibutyryl-cAMP to prevent the contractions induced by $PGF_{2\alpha}$ and PGE_1 is shown in Fig. 4. A 1–2 mM concentration of the substituted cyclic nucleotide had to be used, and a preliminary incubation time of 10 min was necessary (this might reflect the time required for its intracellular penetration). These results are in close agreement with previously published observations (8, 10, 17) in which DB-cAMP was shown to mimic epinephrine or isoproterenol

[2] M. F. Vesin and S. Harbon, unpublished observations.

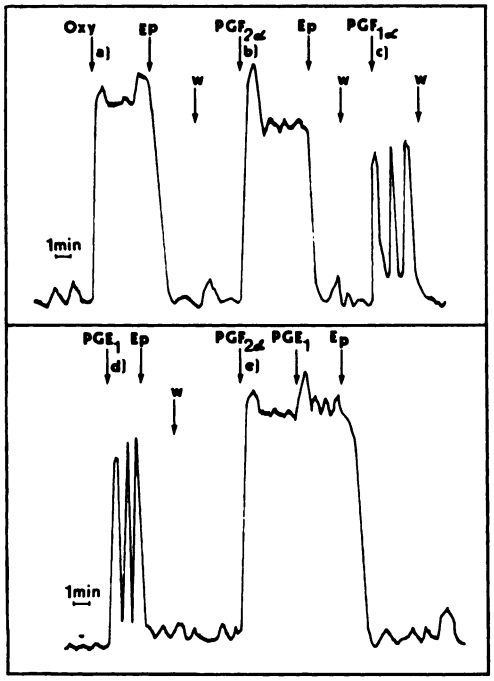

FIG. 3. *Tracing of isometric contractions of isolated rat uterus in the presence of oxytocin and various prostaglandins: effect of epinephrine*

The concentration of oxytocin (Oxy) was 0.4 milliunit/ml; epinephrine (Ep), 0.3 μM; $PGF_{2\alpha}$, 0.5 μM; $PGF_{1\alpha}$, 1.5 μM; PGE_1, 1.5 μM. w = washing with buffer solution (recording stopped during the washing period).

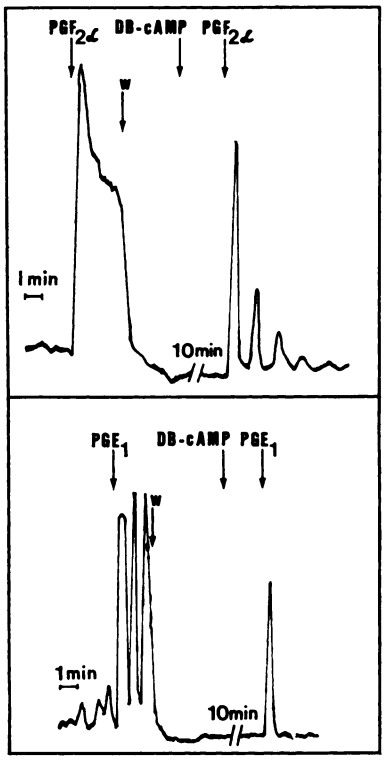

FIG. 4. *Tracing of isometric contractions of isolated rat uterus: effect of DB-cAMP on $PGF_{2\alpha}$- and PGE_1-induced contractions*

The concentration of PGE_1 was 1.5 μM; $PGF_{2\alpha}$, 0.5 μM; DB-cAMP, 2 mM. w = washing with buffer solution (recording stopped during the washing period).

in antagonizing the contractions elicited by oxytocin in isolated rat uterus.

Propranolol inhibits the relaxing effect of epinephrine (10, 17). When the *beta* adrenergic blocking agent (10 μM) was added to a $PGF_{2\alpha}$-stimulated uterine horn prior to the addition of epinephrine, no relaxation was induced by the latter (Fig. 5). Also, if propranolol was added after epinephrine had inhibited the stimulating effect of $PGF_{2\alpha}$, normal contractions in response to $PGF_{2\alpha}$ were restored, as epinephrine could no longer exert its relaxing effect.

The results obtained in this comparative study deserve a few comments.

1. It seems clear that the contractile effects of oxytocin and $PGF_{2\alpha}$ on rat uterus did not involve a prior interaction with the adenylate cyclase system and that the site of their antagonism to epinephrine or DB-cAMP is located at a step beyond cAMP formation.

2. In the case of epinephrine, there seems to be a good correlation between its effect on adenylate cyclase stimulation, with a subsequent rise in cAMP levels, and its relaxing effect on rat myometrium. Both epinephrine activities are potentiated by theophylline (9, 10), inhibited by a *beta* adrenergic blocking agent, propranolol, and appear to be closely related in time. In addition, DB-cAMP, with some restrictions for the high concentrations needed, is able to mimic epinephrine in inducing relaxation.

3. The results obtained with PGE are more complex. During stimulation of rat myometrium by two unrelated agents, epinephrine and PGE_1, enhanced formation of both total and radioactive cAMP was shown to

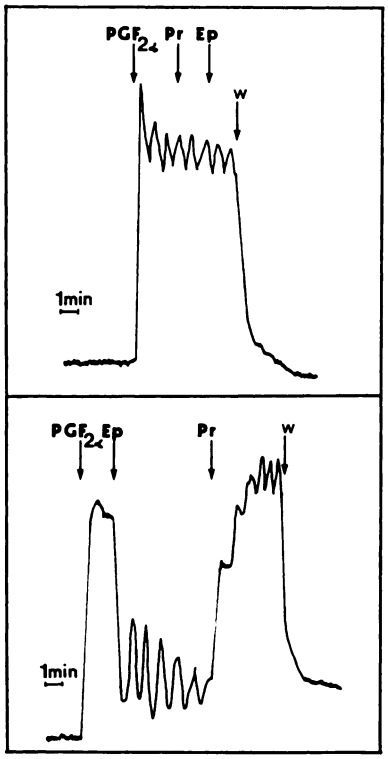

FIG. 5. *Tracing of effect of propranolol on epinephrine-induced relaxation of isolated rat uterus, stimulated by* $PGF_{2\alpha}$

The concentration of $PGF_{2\alpha}$, was 0.5 μM; propranolol (Pr), 10 μM; epinephrine (Ep), 0.3 μM. w = washing with buffer solution (recording stopped during the washing period).

occur to a similar extent and to follow similar time courses. However, the ultimate physiological responses were opposite, i.e., relaxation in the former case and contraction in the latter. Our results indicate that a single adenylate cyclase is involved in both epinephrine and PGE stimulation, since no additive effects could be observed when both agents were used at supramaximal concentrations in the presence of theophylline (Table 4). In addition, when the effect of combined epinephrine and PGE_1 was examined in the absence of theophylline, it was found that incubation of myometrial strips with 25 μM PGE_1 resulted in contractions with a rise in cAMP level (after 3 min) to a value of 29.5 ± 2.3 pmoles/mg of protein. Subsequent addition of 5 μM epinephrine resulted in relaxation with no detectable extra increase in cAMP after an additional 1-min incubation.

At first sight these results may cast some doubt on or even invalidate, the hypothesis that cAMP is the effective intracellular mediator of smooth muscle relaxation. As an alternative explanation, the hypothesis could be advanced that compartmentalization and consequent "unreactivity" of the cyclic nucleotide formed on PGE stimulation occur. These interpretations will be further discussed under CONCLUSIONS.

Similar rises in cAMP level during prolonged isometric contractions of rabbit colon by carbamylcholine or potassium ions have recently been observed (18). Consequently it appeared important to determine the significance of the rise in cAMP level under the influence of the contractile PGE. The following experiments were designed first in order to verify whether both PGE effects were causally related.

Effects of Polyphloretin Phosphate and 7-Oxa-13-prostynoic Acid on Prostaglandin-Induced Contractions and Stimulation of Adenylate Cyclase

PPP, a polymeric phosphorylated polyanionic derivative of phlorizin, has been found to antagonize some stimulating actions of both PGE and PGF on certain isolated smooth muscle preparations (19–21). In addition, Fried *et al.* (22) have synthesized compounds structurally related to prostaglandins, among them the 7-oxa-13-prostynoic acid derivative, which actively antagonized the stimulating effect of PGE and PGF on a smooth muscle preparation of gerbil colon.

In the present comparative study it seemed important to investigate the effects of these prostaglandin antagonists on both the prostaglandin-induced contractions and stimulation of adenylate cyclase. Figure 6a represents a typical experiment in which the response of an isolated uterine horn to PGE_1 (1.5 μM) was examined in the absence and presence of increasing concentrations of PPP. The organ was incubated for 10 min with PPP before the addition of PGE_1, and after the response had been recorded it was washed three or four times with the buffer before the addition of a subsequent dose of

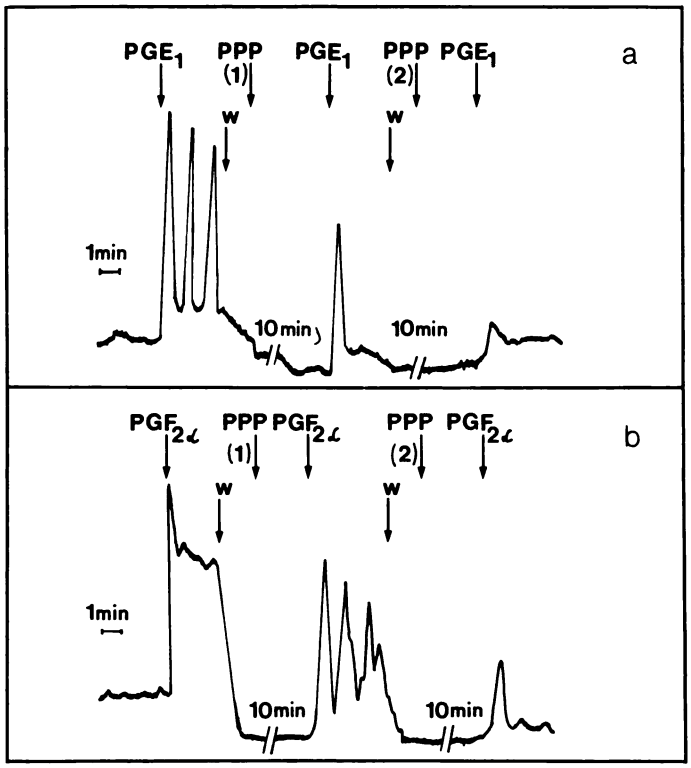

FIG. 6. *Tracing of effects of different concentrations of PPP on PGE_1- and $PGF_{2\alpha}$-induced contractions of isolated rat uterus*

The concentration of PGE_1 was 1.5 μM; $PGF_{2\alpha}$, 0.5 μM; PPP, 100 μg/ml (*1*), 260 μg/ml (*2*) w = washing with buffer solution (recording stopped during the washing period).

inhibitor and PGE_1. PPP at 260 μg/ml almost completely inhibited the stimulation response to 1.5 μM PGE_1 (under these conditions PPP, 120 μg/ml, gave 50% inhibition). Contractions induced by 0.5 μM $PGF_{2\alpha}$ were also completely abolished in the presence of PPP at 260 μg/ml (Fig. 6b). Under the same conditions contractions elicited by oxytocin (0.3 milliunit/ml) were not significantly affected. The results are in agreement with those of Eakins *et al.* (19, 20), who found that PPP antagonized the actions of both PGE and PGF compounds on isolated gerbil colon and rabbit uterus, while the contractions produced by other agonists, such as acetylcholine in the latter organ and acetylcholine, bradykinin, or 5-hydroxytryptamine in the former, were not reduced by concentrations of PPP which markedly antagonized the responses to PG.

The same type of experiments, using PY_1 as a prostaglandin antagonist, is shown in Fig. 7. Complete inhibition of PGE_1 (1.5 μM)-induced contraction could be obtained with 30 μM PY_1, and 50% inhibition with 10 μM PY_1. With $PGF_{2\alpha}$, inhibition was also complete for an antagonist to agonist ratio of 60:1. Under identical conditions contractions induced in myometrial strips either by oxytocin (0.3 milliunit/ml) or by carbamylcholine (3 μM) were not antagonized by PY_1 (45 μM) (data not shown). The inhibition by PY_1 of PGE- and PGF-induced contractions was reversible in both cases, with three successive washings with buffer for 2–5 min restoring a normal response to PGE_1 or $PGF_{2\alpha}$. On the other hand, with PPP, it was found that the inhibition of the PG effects lasted for a long time (30–60 min), in spite of repeated washings with fresh solutions. During this period of inhibition myometrial responses to oxytocin were virtually unaffected.

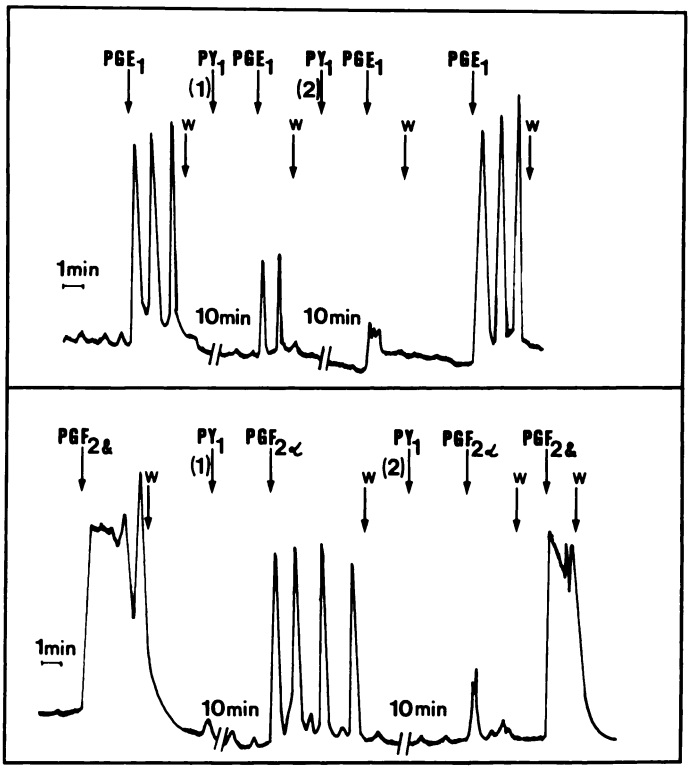

FIG. 7. *Tracing of effects of different concentrations of PY_1 on PGE_1- and $PGF_{2\alpha}$-induced contractions of isolated rat uterus*

The concentration of PGE_1 was 1.5 μM; $PGF_{2\alpha}$, 0.5 μM; PY_1, 8 μM (*1*), 30 μM (*2*). W = washing with buffer solution (recording stopped during the washing period).

The action of the two prostaglandin antagonists was also tested on adenylate cyclase stimulation, and the results are summarized in Table 6. In this case, as in the experiments above, PPP or PY_1, when used, was added 10 min prior to PGE_1. It is evident that the rise in total cAMP, as well as in [^{14}C]cAMP accumulation induced by various concentrations of PGE_1, was not modified by the presence of PPP or PY_1, even though at the concentrations used both antagonists completely inhibited PGE_1-induced contractions of the myometrium. PPP and PY_1 alone had no effect on basal cAMP levels. In addition (data not reported), they did not affect the normal activation of adenylate cyclase by epinephrine. Hence the dual effects of PGE_1 in rat myometrium, i.e., contraction and stimulation of the adenylate cyclase, may be completely dissociated under these conditions.

It remained to establish the possibility that PGE_1 might become a relaxing agent after blockade of its contractile effect by PY_1, while still stimulating cAMP accumulation. In the presence of PY_1 (45 μM), which completely inhibits contractions evoked by PGE_1 (3 μM) (incubation conditions as in Fig. 7), the prostaglandin failed to inhibit contractions induced by oxytocin. This result is in contrast to that found with epinephrine or DB-cAMP (Fig. 4), which strongly blocked the contractile effect of oxytocin. Also, when PGE_1 was inhibited by PY_1, it could not antagonize the response to carbamylcholine, another contractile agent of the rat uterus. Therefore, in spite of the fact that the contractile effect of PGE_1 was blocked, cAMP formed through the action of this prostaglandin could not act as an intracellular mediator for relaxation.

TABLE 6

Effect of polyphloretin phosphate and 7-oxa-13-prostynoic acid on PGE_1 activation of myometrial adenylate cyclase

Uterine strips were treated as described under MATERIALS AND METHODS for [^{14}C]cAMP accumulation (column A) and for total cAMP determination (column B) (see also legend to Fig. 1). After the addition of theophylline to the incubation medium, tissue strips were incubated for 10 min in the absence or presence of PPP or PY_1, as indicated. PGE_1, at the concentrations shown, was then added, and incubation was continued for 15 min at 37°.

Inhibitor	[^{14}C]cAMP (A)			Total cAMP (B)			
	No PGE_1	1.65 μM	3.3 μM	No PGE_1	0.65 μM	1.55 μM	3.3 μM
	cpm			*pmoles/mg protein*			
None	870±62	2930±305	3860±330	20.0±1.02	25.2±5.3	45.3±3.0	61.6±6
PPP, 150 μg/ml	760±23	2770±260		21.6±1.2	29.8±4.3	44.5±4.5	65.0±1.8
PPP, 300 μg/ml	970±120	2570±392	3760±350	20.5±3.0	29.5±3.3	43.0±6.4	57.2±1.2
PY_1, 30 μM	878±92	2560±240			26.4±2.15	45.8±3.5	
PY_1, 60 μM	988±75	2670±290	3900±345	20.6±1.2		45.1±3.96	61.7±2.41

CONCLUSIONS

The results reported here agree with the previously published observations (7–10) supporting the concept that cAMP is a mediator for relaxation of uterine smooth muscle induced by *beta* adrenergic agonists, since a good correlation has been demonstrated between epinephrine activation of adenylate cyclase and its relaxing effect. In addition, protein kinases which are stimulated by cAMP have recently been described in both endometrium and myometrium of bovine uteri (23) and in rat myometrium.[3]

In the case of PGE_1 our previous observations (3) have been confirmed: it stimulates adenylate cyclase and induces contractions of rat myometrium. According to Bhalla *et al.* (5), bovine endometrium possesses an adenylate cyclase system which is stimulated by PGE_1 but not by the *beta* adrenergic agonist isoproterenol. It has been demonstrated that the effects of epinephrine as well as PGE_1 on adenylate cyclase stimulation, described in the present work, are definitely localized in the myometrium. Paradoxical results were thus obtained with epinephrine and PGE_1, which exert opposite effects on rat uterine motility despite the fact that both agents stimulate cAMP accumulation in the myometrium. At least for the moment, these data are difficult to interpret unless one speculates either that the rise in intracellular cAMP levels might not be the exclusive mechanism involved in the relaxation of the myometrium, or that the "unreactivity" of cAMP formed under PGE stimulation is due to its intracellular compartmentalization. The latter hypothesis would imply that PGE_1 and epinephrine stimulate cAMP formation in one and the same compartment of the myometrium, which may represent an inactive pool of cAMP, and that the cyclic nucleotide has to become available to its intracellular binding protein in order to be able to act as the mediator of relaxation. The latter step could be achieved in the case of epinephrine, and even with DB-cAMP or theophylline, while in the case of PGE_1 cAMP would remain as an "inactive pool."

Identical results would obtain if epinephrine, but not PGE_1, stimulates cAMP accumulation in an additional intracellular compartment, which contained the actual active pool of cAMP for the relaxing effect. The absolute increase of cAMP in this additional compartment then would have to be very small as compared to the increase in the inactive pool, so that any rise in cAMP induced by epinephrine, after maximal simulation by PGE_1, could escape detection. Possible compartmentalization of cAMP has

[3] L. Borstad and S. Harbon, manuscript in preparation.

also been suggested by Andersson (18) in various smooth muscles.

Moreover, the results described in this paper do not eliminate the hypothesis that the relaxing action of epinephrine cannot be attributed, at the level of membrane interaction, to the single mechanism of adenylate cyclase stimulation. An additional membrane mechanism may of course operate, with the combination of both mechanisms resulting in relaxation. In the case of PGE_1, activation of adenylate cyclase would be the only one to operate. Both hypotheses need further experimental exploration.

Finally, experiments performed with the two prostaglandin antagonists, polyphloretin phosphate and 7-oxa-13-prostynoic acid, have clearly distinguished two PGE activities. These antagonists do inhibit prostaglandin (E and F)-induced contractions but do not modify the response of myometrial adenylate cyclase to PGE_1. It is noteworthy that in a variety of tissues other than smooth muscle some of the prostaglandin effects appear to be mediated via cAMP (24). In such tissues PPP and PY_1 antagonize the prostaglandin activation of adenylate cyclase as well as the specific metabolic response evoked by the prostaglandins in the particular tissue (25, 26). All these findings, added to the observation that $PGF_{1\alpha}$ and $PGF_{2\alpha}$ do not alter cAMP levels in rat myometrium, provide evidence that the stimulation of uterine contractions by prostaglandins may occur independently of their action on adenylate cyclase. However, the possibility of a role for cAMP in modulating the prostaglandin response has not been excluded. Interaction of the prostaglandins with other membrane enzymatic systems, particularly ion carriers, has been described (4, 24); such mechanisms could easily be involved in the regulation of the prostaglandin activities exhibited in uterine smooth muscle cells.

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