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HORMONAL REGULATION OF UTERINE CONTRACTILITY: THE ROLE OF THE ADENYLATE CYCLASE SYSTEM

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SUMMARY

Oxytocin was effective both in inhibiting isoproterenol-induced uterine relaxation and cAMP generation in uteri from rats which were either in the castrate state or which had received estrogen, progesterone or both steroids after castration. By contrast oxytocin was ineffective in mid-pregnancy. Examination of the consequences of cAMP generation showed that protein kinase activity in the 20,000 *g* supernatant was activated as a consequence of cAMP generation due to isoproterenol. However, total measured kinase activity fell and could be recovered by Triton X-100 extraction. Sucrose density gradient centrifugation of the microsomal pellet resulted in identification of protein kinase activity associated with the ribosomes, the cell membrane and the sarcoplasmic vesicles. Careful examination of the enzyme activity demonstrated great differences from the cytoplasmic enzyme, making it difficult to estimate the role of protein kinase translocation in cAMP action.

INTRODUCTION

A considerable body of data has been accumulated indicating that uterine contractility is regulated through the adenylate cyclase system. Our interest in the relationship between steroid regulated and cell membrane regulated events led us to investigate in the rat uterus the relationship among substances controlling contractility, the adenylate cyclase system and some aspects of steroid influences.

We have shown previously that the β-adrenergic effector isoproterenol, which inhibits contractility, stimulates myometrial adenylate cyclase, a process that is inhibited by oxytocin and prostaglandins of the E & F series [1]. Estradiol did not affect cAMP generation acutely [2].

We now report further studies on uterine contractility and on the responses of the myometrium to cAMP generation.

MATERIALS AND METHODS

Except where otherwise indicated, rats castrated 3–5 days prior to study were employed.

Protein kinase activity and cAMP concentration were measured as previously described [2, 3].

RESULTS

An attempt was made to relate physiological features of myometrial contractility with cAMP concentrations. In Fig. 1 are indicated typical physiological responses. Employing the minimal dose of isoproterenol necessary to hyperpolarize and inhibit contraction in the regularly spontaneously contracting uterus for at least 8 min, we were able to restore contractions with oxytocin in the castrate uterus and uteri from progesterone-treated animals, while in pregnancy, no such stimulation occurred. The mechanical work exerted in contraction in response of oxytocin in the isoproterenol inhibited uterine strips is indicated in Fig. 2. This measurement, accomplished through the use of a compensating planimeter, is the integral of all forces generated in response to the hormone. As is well known, this resultant is due to increased tone, including tetany, increased frequency of contractions and increased amplitude of contractions. The failure of response of the pregnant uterus is highly significant when compared to the castrate, progesterone, and estrogen treated uteri. A substantial increase in contractile response was demonstrated at all concentrations of oxytocin when both hormones were given. These data suggested that

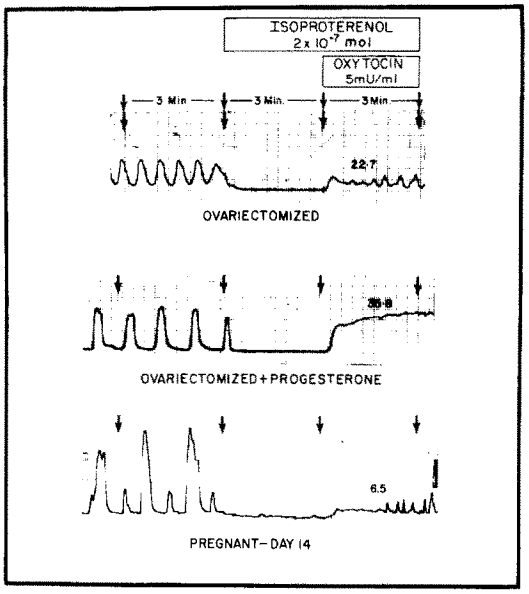

Fig. 1. Influence of oxytocin on isoproterenol-induced uterine relaxation.

Isolated uterine horns were opened longitudinally and the endometrium removed by scraping. Strips 1·5 cm long from the middle portion were removed and anchored to a force displacement transducer (Gross Instruments, Quincy, Mass.). The strips were incubated at 37° in Krebs Ringer Bicarbonate buffer, pH 7·4, containing 1% glucose and a 95% O_2, 5% CO_2 gas mixture bubbled through gently. Contractions were recorded employing a Sanborn D.C. strain gauge and recorder (Model 150–400). When regular contractions were established, the minimal effective dose of isoproterenol, 2×10^{-7} M, was added to the bath. Contractions were completely inhibited for at least 8 min. Three minutes after isoproterenol, oxytocin was added. Upper panel: uterus from rat castrated 5 days previously. Middle panel: subcutaneous injection of progesterone 2 mg/day for 2 days to a 3 day castrate. Lower panel: Pregnancy-day 14.

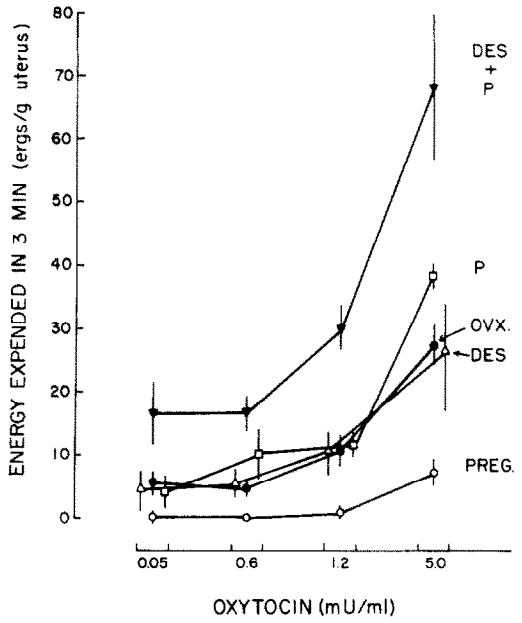

Fig. 2. Influence of endocrine status on the contractile response to oxytocin in the isoproterenol-relaxed uterus.

Uteri from oophorectomized (ovx) or mid-pregnant (PREG) rats or from oophorectomized rats pretreated with either progesterone (P) 2 mg/day for 2 days, diethylstilbesterol (DES) 2·5 mg/day for 3 days or DES 2·5 mg/day for 5 days with P, 2 mg/day for the last 3 days were prepared and incubated as in the legend to Fig. 1. The energy expended in contraction was measured planimetrically and calibrated against a one gram weight. Doses of oxytocin were administered in random order. Between doses the medium was changed and relaxation reestablished with isoproterenol. Each point is the mean of at least 4 determinations.

the pregnant uterus was under at least one additional major influence.

To validate the concept that the level of cAMP was a determining factor in contractility under these circumstances, uteri were treated as above and cAMP levels were measured (Fig. 3). It was found that oxytocin effectively inhibited cAMP generation in castrate, progesterone, and estrogen plus progesterone treated animals but was less effective in the pregnant animals $p < 0{\cdot}1$.

In Fig. 4 is indicated a model of how the cytoplasmic cAMP-dependent protein kinase system has been shown to operate in at least two systems [4, 5]. It appears reasonably well established that the enzyme consists of a regulatory and a catalytic subunit that dissociate on binding of cAMP to the regulatory subunit to release free, active catalytic subunit that selectively catalyzes the phosphorylation of proteins. The physiological role of the heat stable protein inhibitor of cAMP-dependent protein kinase is currently unknown.

If this model were operative in the myometrium, then we should expect to find, consequent to cAMP generation, a decrease in cAMP binding sites and an increase of cAMP independent protein kinase [6, 7]. This concept was confirmed in the data of Table 1. Use of differing doses and times of isoproterenol administration, there was a rough correspondence between the level of cAMP generated and the change of binding sites and cAMP-independent kinase activity. It was noted, however, as illustrated also in Fig. 5 that there was a decline in protein kinase activity found in the presence of cAMP and histone in the treated uteri compared to the controls. This loss of kinase activity also could be demonstrated in the 105,000g supernatant fraction.

The question of the disposition of the kinase activity was tested, employing Triton X-100, which has been employed widely to solubilize protein kinase activity from particulate fractions. It was found, as illustrated

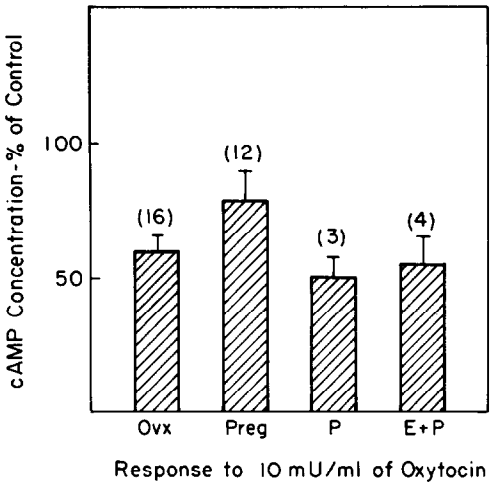

Fig. 3. Uteri from rats treated as indicated in the legend to Fig. 2 were incubated in Eagles Minimal Essential Medium (for Spinner Culture) for 20 min at 37° to achieve baseline status. They were then incubated for 10 min in the presence of isoproterenol (10^{-5} M) or isoproterenol plus oxytocin (10 mU/ml) and cAMP was measured as previously indicated. All incubations were carried out in the presence of 10^{-3} M theophylline. The numbers indicate the number of uteri assessed.

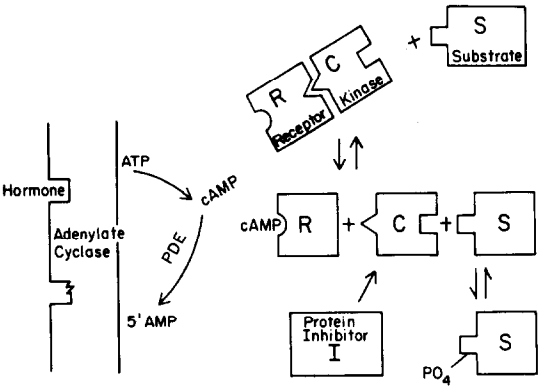

Fig. 4. A model of the responses to increased cAMP concentration.

in Fig. 6, for three experiments, that use of Triton X-100 would increase slightly protein kinase activity in the 20,000*g* supernatant and eliminate the difference between values in control and treated uteri.

These data suggested that the protein kinase had been translocated to a component of the microsomal pellet.

Employing an 8–45% linear sucrose density gradient the components of the microsomal pellet were separated. It was shown that Ca uptake and binding were located principally at 34% sucrose, the membrane fragments migrated somewhat more slowly, peaking at 30% sucrose, and the ribosomal elements even more slowly. The distribution of protein kinase and Ca^{45} uptake and binding are presented in Fig. 7. When 5′ nucleotidase and Na/K ATPase activity were measured, they were found to correspond quite closely with the conventionally measured protein kinase activity.

We were unable to identify a definite increment in protein kinase activity in the particulate fractions from isoproterenol-treated animals.

We then initiated studies of the characteristics of protein kinase activity in the microsomal pellet. In Fig. 8 the time course of phosphorylation in the cytosol is given. The reaction is linear for about 5 min, and cAMP stimulated. It requires histone and (not shown) is essentially unaffected by Triton X-100. When isoproterenol-treated uteri are compared with controls, the initial rate in the presence of histone is increased in

Table 1. After incubation of uteri from 15 castrated rats per point, as described in the legend to Fig. 3, castrated rat uteri were rinsed, frozen and then homogenized in 2 volumes of protein kinase assay buffer (0·05 M) acetate buffer pH 6·5 containing 0·005 M $MgCl_2$, 0·02 M NaF ·005 M theophylline. The homogenates were centrifuged first at 1500 *g* for 10 min and then at 20,000 *g* for 15 min. The 20,000 *g* supernatant was employed for protein kinase assay in the presence or absence of 2×10^{-6} M cAMP, in the presence of 0·24 mg F_2 histone (Sigma) and 10^{-4} M ATP in a vol. of 0·120 ml. cAMP binding sites were measured by the method of Chambaut[8].

Treatment		Protein kinase activity pMols ^{32}P/mg protein				cAMP Binding sites		cAMP pMols/mg protein	
		Control		Treated					
		−cAMP	+cAMP	−cAMP	+cAMP	Control	Treated	Control	Treated
10^{-4} M	Isoproterenol	307	2662	861	1452	3·48	1·73	30·0	490·0
	10 min	248	1916	411	646	2·71	0·96	17·5	70·0
		105	514	318	435	3·56	0·86	9·0	250·0
10^{-5} M	Isoproterenol	437	1187	607	770	11·40	3·40	3·5	39·6
	30 min	308	1476	501	551	8·00	6·70	6·4	34·4
5×10^{-6} M	Isoproterenol	243	1317	283	813	14·50	11·90	5·4	15·2
	20 min								
5×10^{-6} M	Isoproterenol	303	1303	503	1144	12·60	10·20	1·3	1·7
	30 min	347	769	663	770	20·00	12·80	9·4	28·3
		277	686	357	408	5·40	4·40	5·7	22·4

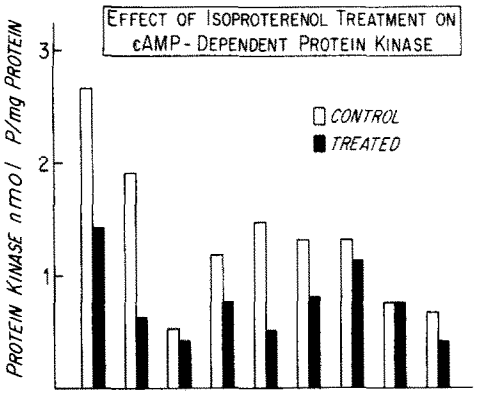

Fig. 5. Protein kinase activity in the presence of cAMP and Histone in control and isoproterenol treated uteri. See legend to Table 1.

the absence of cAMP and decreased in the presence of cAMP, confirming the previous observations.

When the microsomal pellet was examined in the absence of Triton X-100 (Fig. 9), the rate of phosphorylation was very slow for 8–10 min. In the presence of histone it never rose, but in the absence of histone very substantial phosphorylation occurred. The phosphorylation process was essentially independent of cAMP and was grossly increased in the isproterenol-treated

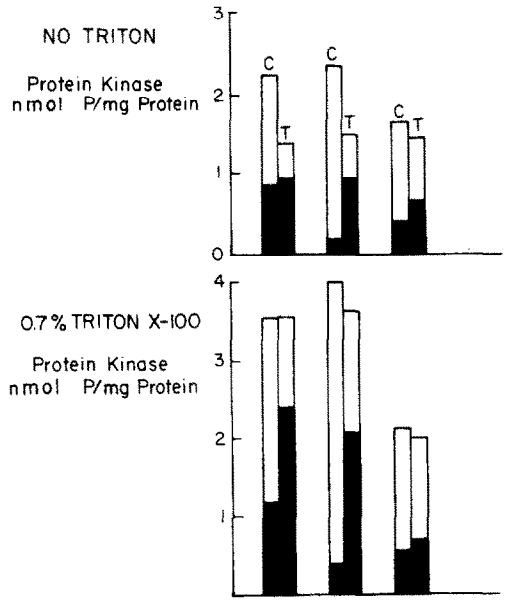

Fig. 6. Effect of incubation in the presence of Triton X 100 on protein kinase activity.

Uteri were incubated as described in the legend to Fig. 3 and the 20,000g supernatant fraction prepared. Portions were assayed for cAMP-dependent and independent protein kinase activity in the presence of absence of Triton X 100.

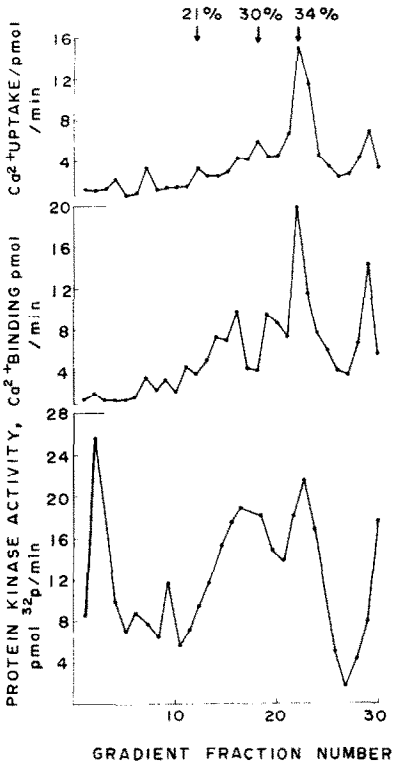

Fig. 7. Separation of microsomal components of myometrium by sucrose density gradient centrifugation.

The 105,000g microsomal pellet was resuspended in 0·01 M Tris buffer pH 7·4 containing 4% sucrose and layered on an 8–47% sucrose gradient. Centrifugation was carried out in the Spinco SW41 rotor for 2·5 h at 153,000g max. 0·4 ml samples were collected.

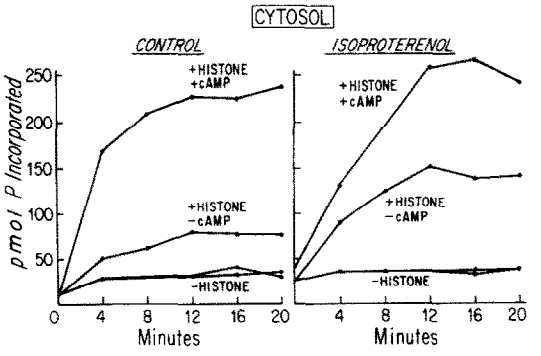

Fig. 8. Time course of cytoplasmic protein phosphorylation of control and treated castrate rat uterus.

Phosphoprotein formation was measured in the presence and absence of histone and cAMP over time. Ten control and treated uteri were incubated *in vitro* as in the legend to Fig. 3. Each point is the mean of duplicates and this experiment was representative of several.

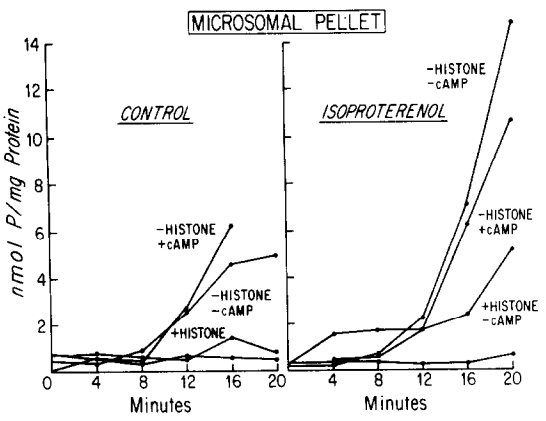

Fig. 9. Microsomal protein phosphorylation.
Time course of microsomal protein phosphorylation of control and treated castrate rat uterus prepared as in the legend to Fig. 8. The microsomal pellet was taken up in kinase assay buffer.

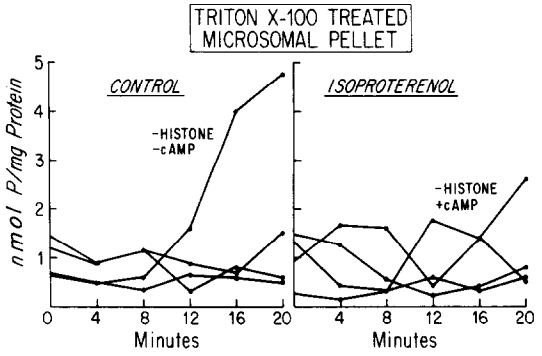

Fig. 10. Microsomal protein phosphorylation in the presence of Triton X 100. See legend to Fig. 9.

uteri. In the presence of Triton X-100, there was a variable and inconsistent low level of phosphorylation (Fig. 10).

DISCUSSION

Evidence from many sources including the data depicted in Figs. 1 and 2 have indicated that uterine contractility differs substantially during pregnancy from the castrate state. Steroid treatment, as given, failed to duplicate the oxytocin unresponsiveness noted during pregnancy and in fact, the combination of DES and P in sufficient dosage to exceed mid-pregnancy values of P, resulted in a significantly more responsive uterus. In this extremely complex situation where muscle stretch, intracellular calcium content and endogenous catecholamine and prostaglandin content vary so extensively, it is difficult to characterize the effects of steroid hormone vs the other factors. It was of importance to note, however, that unresponsiveness in mid-pregnancy was related to ineffectiveness of oxytocin in inhibiting isoproterenol-induced cAMP generation.

Examination of the biochemical consequences of cAMP generation indicated that the expected activation of phosphorylating activity occurred. However, there was a loss of total kinase activity that was Triton X-100-recoverable, suggesting that there was translocation of the protein kinase enzyme to a particulate fraction. Although excellent separation of the principal components of the microsomal pellet could be obtained by density gradient centrifugation in sucrose, examination of protein kinase activity under standard conditions for the assay failed to demonstrate a consistent change. However, when the time course of phosphorylation was examined, it was found that the microsomal kinases were hysteretic enzymes that were histone inhibited. Under these circumstances, it was impossible to detect selective transfer of enzyme activity. Furthermore, although Triton X-100 was effective in eluting catalytic activity from the pellet fraction, it inhibited late onset phosphorylation almost completely.

We are led to conclude that the loss of protein kinase activity associated with cAMP generation is more than a simple translocation and that its elucidation is intimately dependent on characterization of the protein kinase enzymes of the microsomal particles.

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DISCUSSION

Cooke:

Corbin, Soderling and Park (*J. biol. Chem.* **248** (1973) 1813) have published evidence to show that the activity of isolated protein kinases from adipose tissue is affected by dilution of the enzyme which is prevented by the addition of 0·5 M NaCl. Have you looked at the effect of NaCl?

Korenman:

No. I'm not sure that it is relevant to the difference between the hormone-treated and the untreated uteri under the same condition.

Crabbé:

Do you get the same kind of physiological response using vasopressin instead of oxytocin?

Korenman:

We really have not tried it because we expected it to be exactly the same. There is a whole literature in which it is shown that the two act very similarly.

Crabbé:

If so, I would appreciate your comment on the fact that on one of the target tissues for vasopressin, namely the distal part of the nephron, there obviously seems to be a generation of *c*-AMP triggered by the hormone whereas in this tissue it would be the opposite taking place.

Korenman:

Well, I think the reason why a tissue is a tissue is that it has a specific receptor and a specific response. We have no idea how oxytocin inhibits isoproterenol effects. We don't know whether it is competitive or non-competitive. It is not strictly dose-related. This is another loose end that we have not tried to solve.

Lindner:

When working with intact uterus, one gets a striking increase in *c*-AMP production with prostaglandin E2 or F2α (*J. Endocr.* **58** (1973) 525). I understand from your work that this is different when you work with isolated myometrium. Is that so?

Korenman:

We get the exact same effect with prostaglandin F2 with myometrial strips or whole uteri. We get identical results. With PGE2 we get a bi-phasic result. I did not go into this. We get a substantial inhibition isoproterenol effect with doses from 10^{-9} M to 5×10^{-7} M. Above 10^{-7} M we get a stimulation. PGE2 seems to stimulate another adenylate cyclase activity in the uterus. We think that it is vascular rather than myometrial. There is a parallel in the literature with the fat cell and fat cell blood vessels.