Journal of Steroid Biochemistry, 1974, Vol. 5, pp. 173–178. Pergamon Press. Printed in Great Britain

MECHANISM OF ESTROGEN ACTION—INDEPENDENCE OF SEVERAL RESPONSES OF THE RAT UTERUS FROM THE EARLY INCREASE IN ADENOSINE 3′,5′-CYCLIC MONOPHOSPHATE

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(*Received* 24 *September* 1973)

SUMMARY

Pre-treatment or repeated treatment with propranolol does not modify several responses of the rat uterus to estradiol administration. The parameters studied were: hypertrophy of luminal epithelium, increase in RNA and protein content and increased ability for *in vitro* incorporation of [^{14}C]-phenylalanine into proteins in immature or in spayed adult rats. It was confirmed that the early increase in uterine cAMP concentration that follows estradiol injection was abolished by propranolol.

INTRODUCTION

The role of adenosine 3′,5′-cyclic monophosphate (cAMP) in estrogen action on the uterus remains still a matter of controversy. Some evidence supports the view that estrogen action is mediated by increased adenylcyclase activity[1] and increased cAMP level in the rat uterus[2, 3]. The stimulative influence of cAMP on the rat uterus, *in vitro*[4–7], or *in vivo*[8] as well as potentiation of estrogen action by theophylline[9], also constitute partial fulfilment of the criteria[10] for relating the action of a hormone to its effects on the adenylcyclase system. However, the problem in the case of estrogen action on the rat uterus is complicated by the possible involvement of other agents, such as epinephrine[11], histamine[5, 11], serotonine[5, 11] or eosinophils[12], the levels of which are known to be under estrogen control[2, 5, 11–13]. It is neither known how those factors are related to each other, nor their relationship to the different responses which are grouped under the general framework of estrogenicity in this organ and which comprise water accumulation, hypertrophy, hyperplasia and an increase in the number of eosinophils[13] (see e.g., 5, 15, 16 for reviews). An important feature of the available data on this subject is the fact, demonstrated by independent groups, that propranolol, a β-adrenergic blocking drug, is able to suppress the early increase in cAMP concentration[3] and in adenylcyclase activity[1, 17] that follows estrogen stimulation of the uterus. We decided to take advantage of this as a means to investigate the possible modification of several uterine estrogenic responses after the suppression of the initial estrogen induced rise in uterine cAMP. The present report demonstrates that the estrogenic responses studied remained unmodified. The results lead us to conclude that at least some parameters of estrogenicity are not dependent on early changes in cAMP.

EXPERIMENTAL

Rats of the Wistar strain (Institut Pasteur, Brussels) were used. Animals weighing about 150 g were ovariectomized and used 3–4 weeks after the operation. Efficacy of the latter was tested on vaginal smears during four consecutive days. In one experiment immature rats of the same strain were employed. 17β-estradiol benzoate, dissolved in sesame oil was injected subcutaneously at the dose of 10 μg per animal.

Propranolol (Inderal®), when used, was injected I.P. 20 min before estradiol (or vehicle). In some assays, propranolol injections were repeated several hours after estradiol administration.

At the end of the experiment, the rats were killed by neck rupture and exsanguinated. Uteri were removed, freed from any adhering fat and kept on dry ice for further processing. The organs were homogenized with a glass grinder in 1–2 ml of distilled water. An

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equal volume of 10% perchloric acid (PCA) was added. The mixture was kept in an ice-bath for 20 min and then centrifuged at 3–4000 rev./min for 10 min. The sediment was re-suspended in 1–2 ml of 2·5%PCA, centrifuged, extracted with alcohol–ether to remove the lipids and finally dissolved in 2 ml of 0·2N NaOH.

The proteins were measured according to Lowry *et al.*[18]; RNA was measured by the orcinol reagent[19] and DNA by Burton's procedure[20]. Radioactivity measurements were made on aliquots dissolved in Bray's scintillation medium. Counting efficiency with the Nuclear-Chicago-Mark I scintillation counter was assessed by the method of the 2 channels counting ratio and by calibration with an external standard. Morphological observations were made on fixed segments of uterine horns, cut transversally into 5 μm thickness. For measurement of *in vitro* incorporation of [^{14}C]-phenylalanine into proteins, uterine horns were trimmed free of fat, split longitudinally and cut transversally into 4–5 pieces. Uterine fragments were incubated in 2 ml of Krebs–Ringer-bicarbonate medium containing 1 μCi/ml [^{14}C]-phenylalanine (New England Nuclear Corp., Frankfurt a/Main; S.A. 384 mCi/mMol). The medium was bubbled with CO_2/air. After a 1 h incubation period at 37°C, in a shaking water bath, tissue samples were rinsed in cold saline and treated as hereabove described.

Cyclic AMP concentrations were measured in whole uterine horns that were put in water immediately after removal and kept for 5 min in a boiling water bath. The tissue was homogenized in 1 ml bidistilled water, using a glass-to-glass grinder. The homogenate was centrifuged 30 min at 20,000 ***g*** and the supernatant was lyophilized and redissolved in 100–200 μl of bidistilled water (Van Sande *et al.*, unpublished). The method of Gilman[21] was then used for measuring cAMP concentration. This method is based on competition with [^{3}H]-cAMP for binding with bovine muscle protein (Gilman's simplified preparation for routine use—ref.[21], presumed to be cAMP-dependent protein kinase. [^{3}H]-cAMP used in these assays had a specific activity of 25 Ci/mMol (New England Nuclear Corp.).

RESULTS

Table 1 shows a twofold increase in cAMP concentration in the uterus of spayed rats, 5 min after the injection of 17β-estradiol. This increase is prevented by a pre-treatment of the animals with 50 μg propranolol. These results confirm previous findings by Szego and Davis[2, 3]. No increase in cAMP was observed in the vagina after the administration of estradiol.

Table 1. Cyclic AMP content of the uterus and the vagina of spayed rats under estradiol stimulation. Effect of pre-treatment with propranolol

	Cyclic AMP concentration (pmol/mg protein)[a] ± S.D.	
	Uterine horns	Vagina
Control (vehicle alone)	6·9 ± 0·9	12·6 ± 2·7
17β-estradiol benzoate[b]	14·4 ± 2·8[c]	13·3 ± 2·3
Propranolol + 17β-estradiol benzoate[b]	7·4 ± 0·6	10·5 ± 2·8

[a] The mean for groups of 4 animals, killed 5 min after injection of estradiol benzoate (or vehicle).

[b] 10γ of 17β-estradiol benzoate/animal administrated subcutaneously. Propranolol (Inderal) dissolved in 0·9% NaCl: 50 μg/100 g body weight I.P., 20 min before estradiol injection.

[c] Significantly different (*t* test) from control and propranolol groups at *p* level equal to 0·02 and 0·05 respectively.

Propranolol was unable to modify the luminal hypertrophy induced 18 h after estradiol administration to spayed rats: uterine sections from animals treated by estradiol alone or by any combination of propranolol and estradiol treatments tested, were completely undistinguishable from each other. Several schedules of treatment with propranolol were tested (consisting of repeated injections every 30 min during 4, 6, 8 or 10 h) with identical results. Repetition of injections at short intervals was aimed to test a possible objection of short action of propranolol as compared with the slow turnover of the estrogen. The duration of propranolol action was tested directly by measuring the epinephrine-induced increase in cAMP in the uterus of rats that were treated with 50 μg propranolol/100 g body weight 20 min, 2, 4 and 6 h prior to the administration of epinephrine. The results of this test are illustrated in Table 3. They clearly show that the inhibitory action of propranolol persists at least up to 6 h after its administration.

Table 4 shows in immature animals sacrificed 24 h after estradiol injection that the hormone-induced increase in protein and ribonucleic acid contents was not abolished by propranolol treatment, nor was the luminal epithelium hypertrophy which is characteristic of estrogen stimulation.

Table 5 shows the *in vitro* incorporation of [^{14}C]-phenylalanine into proteins by uterine horns taken from rats that were sacrificed 2 h after estradiol treatment, as compared with uterine horns from control animals. The incorporation of the labelled amino-acid by estrogen-stimulated horns is approximately 130%

Table 2. Estradiol-induced hypertrophy in the uterine luminal epithelium of spayed rats treated with propranolol

Treatment	Number of animals	Hypertrophy of the uterine luminal epithelium (18 h after estradiol)[c]
Estradiol 10 μg (=E)[a]	8	+
Propranolol 50 μg/injection (=P)[b]	5	–
E + P with P given 20 min before E and injected every other 30 min during:		
2 h	5	+
4 h	5	+
6 h	5	+
8 h	5	+
10 h	5	+
Control (vehicle)	8	–

[a] 17β-estradiol benzoate in oil (injected I.P.).

[b] Propranolol (Inderal®) 50 μg/100 g body weight in each injection (I.P.).

[c] + Normal positive response; i.e., presence of the classical signs of estrogen-induced luminal hypertrophy (very high epithelium, lining up of the nuclei in basal position, presence of a dense nucleolus). Uterine sections of all the animals within the positive groups (indicated by +) were absolutely identical in this respect from sections obtained from animals receiving estradiol alone.

[c] – No response; i.e., histological aspect of the luminal epithelium exactly the same as that observed in control, ovariectomized animals (flat epithelium, no visible nucleolus).

of the control value. This early parameter of estrogen stimulation is also insensitive to the propranolol treatment.

DISCUSSION

Increase in uterine cAMP concentration under estrogen stimulation

Our results confirm previously reported findings by others[2, 3] showing that estrogens induce an early increase in uterine cAMP concentration in the rat uterus, and that this is abolished by pre-treatment with propranolol. Consistent with these observations is the fact that adenylcyclase activity increases under estrogen stimulation of the rat uterus and that propranolol is also able to suppress this response[1]. A recent report describes opposite findings, but only at 4 h post-hormone injection[22] and using as test the slight (49%) increase in "the ability of uteri to biotransform adenosine-^{3}H into cAMP-^{3}H during a 60 min incubation at 37°C. It is possible therefore that loss of propranolol *in vitro* allows for some action of bound estradiol on the parameter studied. Contrary to our results and similar findings by others[1, 2, 3], Sanborn *et al.*[23] did not observe any increase of cAMP in castrated rat uteri under treatment with estradiol-17β. They concluded that "acute elevation of cAMP is not a regular feature of estrogen action". The authors also noted that there was substantial variability within a given experiment and between experiments *in vivo*. Admittedly, in some of our experiments the changes in cAMP concentration were low or not significant as compared to control values. It may be significant that the control values for the cAMP level were particularly high in the *in vivo* experiments of Sanborn *et al.*[23]. We can offer no explanation for the lack of constancy of the cAMP measurements in this material: in our laboratory positive and negative findings were obtained whether estradiol was injected subcutaneously or intraperitoneally, and independently of the fact that the animals were or were not anaesthetized, or of the fact that theophylline was, or was not, administered. But since the positive evidences were highly significant in our experiments, as well as in experiments done by others, we are inclined to believe that the negative results are due to the poorly controlled labilisation of the estrogen-induced rise in cAMP by some unknown factor. It may also be suggested from preliminary (unpublished) findings that the increase in uterine cAMP is located *in part* of the organ only so that in some cases it may be masked by the basal level of *total* cAMP content in the uterus. The fact that we did not observe any increase in the vaginal cAMP concentration after estrogen treatment is reminiscent of similar findings by Rosenfeld and O'Malley[1] in the case of the chick oviduct. If confirmed, this would indicate that cAMP is not involved

Table 3. Uterine increase in cAMP concentrations, 5 min after epinephrine injection: effect of pretreatment with propranolol[a]

Epinephrine (20 μg/I.P.)	Propranolol (50 μg/100 g body weight/I.P.)	pmol cAMP/mg protein (±S.D.)
–	–	11·6 ± 1·4 (11)[a]
+	–	20·9 ± 1·7 (8)[b]
+	+ (20 min before epi.)	8·5 ± 1·2 (7)
+	+ (2 h before epi.)	10·9 ± 1·2 (9)
+	(4 h before epi.)	12·6 ± 0·9 (11)
+	(6 h before epi.)	11·8 ± 1·1 (10)[c]

[a] Numbers between brackets refer to number of animals in each group.

[b] Significantly different from control value at P level < 0·001 (Student's "t" test).

[c] Significantly different from "epinephrine alone", P level < 0·001.

Table 4. Effect of propranolol on the 24 h uterine response to estradiol in immature rats

Treatment	Protein content (μg/μg DNA) ± S.D.	RNA content (μg/μg DNA) ± S.D.	Luminal epithelium hypertrophy
1. Control (saline)	17·0 ± 0·7	0·31 ± 0·02	–
2. Propranolol[a]	20·0 ± 1·0 (n.s.)[c]	0·34 ± 0·03 (n.s.)[c]	–
3. 17β-estradiol[b]	39·4 ± 3·0 (s)	0·69 ± 0·05 (s)[c]	+
4. Propranolol[c] + 17β-estradiol	33·4 ± 2·9 (s)	0·65 ± 0·04 (s)[c]	+

[a] Animals (4–5 in each group) were sacrificed 24 h after I.P. injection of 50 μg propranolol (Inderal), dissolved in 0·9% NaCl.

[b] 17β-estradiol benzoate was injected s.c. (1 μg/animal) and the rats were sacrificed 24 h thereafter.

[c] Propranolol (50 μg) was injected 20 min before, and 5 h after estradiol injection; animals were sacrificed 24 h after estradiol administration.

[d] + = Increase in cell height and dense nucleoli (see footnote to Table 2).

[e] n.s. = Not significantly different from control values.

s. = Significantly different from control values at P level $\leqslant 0{\cdot}001$.

in the response of all the tissues that are target organs for estrogens. Finally, the contradictory uterine cAMP levels in the estrogen-stimulated uterus may evidently indicate that an increase in cAMP really is a non-constant side-effect of the hormone and that it is not a prerequisite for the development of estrogenic responses.

Independence of some estrogenic responses from an increase in cAMP

It was the aim of the present investigation to eliminate with certainty any early increase in uterine cAMP after estrogen injection and to see what were the consequences at the level of several parameters of estrogenic stimulation. This was achieved by using several treatments with the β-adrenergic blocking drug propranolol. The results in Table 3 exclude the possibility that the inhibitory action of the drug was short-acting. It is therefore safe to conclude that any response to estradiol treatment which remains unmodified under propranolol is independent of an increase in the cAMP concentration during the first 6 hours of estrogen action. This conclusion thus applies to estrogen-induced luminal hypertrophy in spayed and in immature animals, as well as to increase in protein and RNA content after 24 h of hormone treatment (Table 2 and Table 4).

An earlier parameter of estrogen stimulation, namely the increased ability for *in vitro* incorporation of [^{14}C]-phenylalanine into proteins by uterine horns taken 2 h after estradiol injection was also insensitive to blockage by propranolol. The fact that the morphologic expression of the estradiol-induced hypertrophic

Table 5. *In vitro* incorporation of [^{14}C]-phenylalanine in the uterus of spayed rats 2 h after *in vivo* administration of 17β-estradiol, alone or together with propranolol

Treatment	Incorporation of [^{14}C]-phenylalanine into protein fraction (c.p.m./μg protein) (± S.D.)
Control (vehicle alone)	195·6 ± 7·9 (5)[a]
Estradiol	249·0 ± 6·5 (4)[b]
Propranolol	207·0 ± 24·8 (3)
Propranolol + estradiol	267·0 ± 15·7 (5)

[a] Numbers between brackets refer to number of animals in the group.

[b] Significantly different from control value at P level between 0·005 and 0·001. 17β-estradiol benzoate (10 μg/animal) was injected s.c. Controls received vehicle alone. Propranolol (50 μg Inderal) was injected 20 min before estradiol or vehicle. Treated animals were sacrificed 2 h after estradiol (or vehicle) administration. Uterine horns were dissected, opened longitudinally and incubated for 1 h in Krebs–Ringer bicarbonate, in the presence of 1 μCi/ml [^{14}C]-phenylalanine.

response is maintained despite propranolol treatment suggests that several other parameters of growth and anabolic responses may also escape inhibition by the β-receptor blocking drug.

Besides the presently reported increase in RNA and protein content, Singhal *et al.*[24] have also shown that propranolol fails to modify the estradiol-induced stimulation of uterine glycogen synthesis as well as increased activity of several key glycolytic enzymes. However, we do not agree with the paradoxical conclusion reached by these authors[24] that these observations imply mediation of estrogen action by cAMP. On the contrary, our findings, as well as their own results, tend to disprove this assumption (which rests mainly upon experiments demonstrating some stimulative action of cAMP on uterine horns, *in vitro*[4–7]).

Among the intricate features that constitute the general framework of estrogen action, there are several steps which may be bound to the adenylcyclase system, either as a cause or an effect. It has been suggested, for example, that uterine cAMP elevation occurs as a secondary consequence to site-specific liberation of amines by estrogen[2]. Release of histamine and serotonine under estrogen stimulation[5, 11] and discharge of epinephrine[11] are well documented facts. It is known that serotonine and histamine show some estrogenmimetic properties in the rat uterus[25]. On the other hand, data relating epinephrine action on the uterus of spayed rats to changes in cAMP level suggest that changes in the cAMP concentration are due to an indirect action of estradiol through epinephrine release.

Since the uterus constitutes a mixture of target-cell populations it may be that the action of estrogen nevertheless implies a cAMP mediation for some of these target tissues. Our present data, together with available data of the same nature, do not exclude such a hypothesis. A distinction may be made, perhaps, between the true genomic response, and early responses (such as water imbition, histamine releasing and estrogen priming effects) which, as suggested by Tchernitchin[13] may be related to two different binding systems for estrogen, namely the 5S–8S system[26–29] and the eosinophil binding system[12]. Work is in progress to investigate the participation of both types of estrogenic responses on the basis of their sensitivity to propranolol treatment.

Acknowledgements—The work was supported by a grant from the Caisse Générale d'Epargne et de Retraite (Fonds Cancer). The authors are grateful to Mr. L. Szabo for skilful technical assistance and to Mrs. D. Legrand and Miss Ch. Borrey for preparation of the manuscript. They wish to thank Dr. A. Tchernitchin for helpful criticisms and advice and Mrs. X. Tchernitchin who helped so much in improving the style and language of the manuscript.

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