EFFECTS OF ESTROGEN AND PROGESTERONE ON ADRENOCEPTORS AND CYCLIC NUCLEOTIDES IN RAT UTERUS

Takashi KANO

Department of Pharmacology, Osaka Medical College, Daigaku-machi,
Takatsuki, Osaka, 569, Japan

Accepted February 17, 1982

Abstract—The effects of estrogen and progesterone on adrenoceptors and cyclic nucleotides were studied in ovariectomized rat uterus. The effects on adrenoceptors were examined by measuring mechanical responses to noradrenaline and by binding site determinations with [^3H]-dihydroergocryptine and [^3H]-dihydroalprenolol. After acute administration of estradiol-17 β , uterine cyclic GMP was progressively and significantly elevated, but cyclic AMP declined through it was not significant. Phentolamine suppressed this cyclic GMP elevation. Both acute and chronic treatments with estrogen increased the number of α -receptors. The increase in uterine cyclic GMP was related to the increase in α -stimulation as a result of increased α -receptors. Chronic treatment with estrogen increased the number of α -receptors. The α -effect induced by noradrenaline was bidirectional in the uterus treated with estrogen chronically; there was contraction in normal Tyrode's solution and relaxation in high K^+ Tyrode's solution. In other hormonal group, either α - or β -effects produced relaxation. Enhancement of the α - and β -effects by estrogen is attributed to an increased number of the receptors. Acute treatment with estrogen decreased the responses of cyclic AMP to isoproterenol and mechanical reactivity. Progesterone also enhanced the β -effect as a result of an increase in the number of β -receptors.

Kuehl et al. (1) reported that administration (i.p.) of estradiol-17 β increased the concentration of uterine cyclic GMP in the rat, and similar studies have been carried out in expectation that cyclic GMP might play some role in the onset of the estrogenic effect in the uterus. As to whether or not the adrenergic system participates in the elevation of cyclic GMP levels by estrogen, Flandroy and Galand (2) denied the participation of the β -receptors *in vivo*, but whether or not the α -receptors in rat uterus participate *in vivo* is still unresolved. In the light of the known close relationship between β -

receptors and cyclic AMP (3), a possible relationship between α -receptors and cyclic GMP is an interesting subject to be studied in rat uterus. It is generally considered that estrogen modulates the adrenergic mechanical activity of the uterus. It has widely been accepted since the time of Ahlquist (4) that the uterus of the rat contains β -receptors which inhibit uterine contractions. The stimulation of α -receptors produces contraction of the uterus in the nonpregnant guinea-pig and rabbit, whereas responses of the rat uterus to α -receptor stimulation has not been thoroughly elucidated because of the

variability in the contractile responses depending on the stage of the sexual cycle. The effect of progesterone on the adrenergic responses has not been sufficiently clarified. So far, no experiments have been performed in which simultaneous determinations of cyclic nucleotides, mechanical reactivity, and binding sites in animals equally treated with estrogen or progesterone.

In order to investigate the effects of estrogen and progesterone on adrenoceptors and cyclic nucleotides in rat uterus, experiments were carried out in the uterus isolated from rats treated with "estrogen-acute", "estrogen-chronic", and "progesterone". The influences of adrenergic agonists and antagonists on the concentration of cyclic nucleotides were also examined. The mechanical reactivity was examined by the measurement of the effect of noradrenaline on the isolated uterus in normal Tyrode's solution and in 25 mM KCl-added Tyrode's solution. In order to determine the number of α - or β -receptors, binding sites for [^3H]-dihydroergocryptine and [^3H]-dihydroalprenolol were measured.

MATERIALS AND METHODS

1) Animals: Female Wistar rats (200–250 g) were used in these studies. The rats at diestrus or metestrus, determined by microscopic observation of vaginal smears, were ovariectomized. Then the rats were maintained under ordinary environmental conditions for 3 weeks and were divided into 4 groups. The animals were killed by a blow on the occipital region followed by bleeding from the carotid artery. The uterus was immediately removed.

2) Drugs and dosages: Estradiol-17 β (Sigma) dissolved in ethanol and diluted with polyethyleneglycol-400 and water (1:4.5:4.5, on a volume basis) was given intraperitoneally (1 $\mu\text{g}/100$ g). The measurement of mechanical reactivity and the deter-

mination of binding sites were carried out 90 min after administration to examine the acute effect of estrogen ("estrogen-acute").

In order to examine the chronic effect of estrogen, 25 $\mu\text{g}/100$ g of estradiol-benzoate (Sigma) dissolved in sesame oil was administered subcutaneously (s.c.) for 4 days ("estrogen-chronic").

Progesterone (Tokyo Kasei) dissolved in sesame oil was administered (s.c.) at a dose of 2 mg/100 g for 4 days ("progesterone").

Atropine (Fuso) was administered (i.p.) at a dose of 250 $\mu\text{g}/100$ g.

($\pm$)-Phentolamine (Regitin[®], Nippon Ciba-Geigy) and ($\pm$)-propranolol (Inderal[®], Sumitomo Chemical) were administered (i.p.) at doses of 50 and 250 $\mu\text{g}/100$ g, respectively, 20 min prior to estradiol-17 β or vehicle injection.

($\pm$)-Phenylephrine (Neo-synesis[®], Kowa) was administered (i.p.) at doses of 50, 100, 250, and 500 $\mu\text{g}/100$ g.

(-)-Isoproterenol (Proterol-L[®], Daiichi) was administered (i.p.) at a dose of 50 $\mu\text{g}/100$ g.

3) Determination of cyclic GMP and cyclic AMP: The isolated whole uterus was frozen in liquid nitrogen and the wet weight was measured with a torsion balance. After addition of 1 ml of 6% trichloroacetic acid (TCA) solution, the uterus was homogenized in a glass homogenizer placed in ice water. The homogenate was centrifuged for 15 min at 4°C at 3,000 rpm, and the pellet was resuspended in 1 ml of 6% TCA solution and centrifuged as above. The supernatants were combined, extracted with three 5 ml aliquots of water-saturated ethylether and stored frozen until assay. For determining protein content by the method of Lowry et al. (5), the pellet was hydrolyzed in 5 ml of 1N NaOH at 70°C for 2 hr.

Cyclic GMP and cyclic AMP were determined by the use of on ^{125}I -RIA kit from Yamasa Co. prepared according to Honma

et al. (6).

4) Determination of noradrenaline: The whole uterus was frozen in liquid nitrogen immediately after isolation and homogenized in a glass homogenizer containing 1 ml of 0.4 N perchloric acid (PCA) solution. The homogenate was centrifuged at 4°C for 20 min at 10,000 rpm. The pellet was resuspended in 1 ml of the PCA solution and centrifuged as above. The supernatants were combined, and noradrenaline was determined by the gas chromatographic-mass fragment-graphic method reported by Kurobe et al. (7). Protein content was measured by the method of Lowry et al. (5).

5) Measurement of mechanical reactivity: The ovarian side (1.5 cm in length) of the longer horn of the excised uterus was suspended in a organ bath where Tyrode's solution had been kept at 37°C and bubbled with a 95% O₂-5% CO₂ mixture gas. The mechanical reactivity of the uterus was measured isometrically by a force displacement transducer, SB 1T-H (Nihon-Kohden), and recorded on a pen-recorder, RJG-4002 (Nihon-Kohden).

After a constant motility of the preparation was confirmed to be maintained for 30 min, noradrenaline (initial dose 10⁻⁹ g/ml) was cumulatively added to the bath to determine alteration of the motility. In experiments for determination of the uterine-relaxing effect of noradrenaline, the tissue preparation was previously contracted by 25 mM KCl-induced depolarization. This concentration of KCl was chosen on the basis of Gabella's report (8). After a constant depolarizing contraction was established, noradrenaline (initial dose 10⁻⁹ g/ml) was cumulatively added to the organ bath. In order to differentiate between the α - and β -effects of noradrenaline, 10⁻⁵ or 2×10⁻⁵ g/ml of phentolamine (Nippon Ciba-Geigy) or propranolol (Sumitomo Chemical) was added in the bath 20 min prior to noradrenaline

addition.

The same experiment was performed with isoproterenol at 2×10⁻⁹ g/ml or greater.

6) Determination of binding sites: The excised uterus was rinsed, with 7 ml of buffer solution (pH 7.4) containing 50 mM Tris-HCl and 4 mM MgSO₄ in an ice water bath, subjected to 3, 10 sec burst by a Polytron P-10 in ice water, transferred to a glass homogenizer, and homogenized. The homogenate was filtered through cheese cloth and centrifuged at 35,000×g for 15 min. After the supernatant was decanted, the pellet was resuspended in 10 ml of the above-mentioned Tris buffer solution, and this suspension (crude membrane fraction) was used for the binding experiments.

In accord with the report by Krall et al. (9), [³H]-dihydroergocryptine (DHE) and (-)-[³H]-dihydroalprenolol (DHA), both prepared by New England Nuclear, were used as radio-ligands for α - and β -receptors, respectively. (±)-Phentolamine (HCl salt, Nippon Ciba-Geigy) and (±)-propranolol (Nakarai Chemicals) were dissolved in 0.02% ascorbic acid solution and used as the unlabeled competitors.

A mixture of 100 μ l of the radio-ligand solution, 100 μ l of 0.02% ascorbic acid solution in the absence or presence of unlabeled competitor, and 1.6 ml of buffer solution (pH 7.4) containing 50 mM Tris-HCl and 4 mM MgSO₄ was preincubated at 30°C for 2 min. Then 200 μ l of the crude membrane fraction that was equivalent to 190–360 μ g of protein was added, and the mixture was incubated for 20 min at 30°C. Binding was terminated by filtration through a Whatman GF/F filter. The filter was washed 3 times each with 5 ml of the Tris buffer solution and placed in a scintillation vial. After addition of 10 ml of scintillation fluid (ACS II®, Amersham), the radioactivity was measured by a Packard Tricarb liquid scintillation spectrometer.

Fig. 1. Time courses of the change in uterine cyclic GMP (left) and change in cyclic AMP (right) after estradiol-17 β administration (i.p.). Points represent means (vertical bars express $\pm$ S.E.). Number of expts. is shown in parentheses. ● estradiol-17 β , 1 μ g/100 g body weight. ○ solvent, 0.2 ml. * $P < 0.05$, ** $P < 0.01$: significantly different from control values.

7) Statistical analysis: The results were expressed as the mean $\pm$ standard error for each group. Significance of the difference was examined by the Student's *t*-test.

RESULTS

1) Changes in uterine cyclic nucleotides:

Time courses of the changes in uterine cyclic GMP and cyclic AMP concentrations after estradiol-17 β administration (i.p.) are shown in Fig. 1. The cyclic GMP concentration of the control was 236.2 ± 24.8 fmoles/mg protein. Cyclic GMP increase after estradiol-17 β was significant at 60 and 90 min ($P < 0.05$ and 0.01 , respectively). The cyclic GMP level reached its highest level (464.9 ± 31.0 fmoles/mg protein) at 120 min after estradiol-17 β administration, and then it decreased to the control level at 240 min. The concentration of cyclic AMP was 15.6 ± 4.4 pmoles/mg protein in the controls. After estradiol-17 β , cyclic AMP tended to decrease gradually, but the differences from the corresponding control levels were not significant.

The concentration of cyclic GMP at 60 min after treatment with estradiol-17 β alone or estradiol-17 β in combination with atropine are shown in Fig. 2. Cyclic GMP levels

Fig. 2. Effect of atropine on response of cyclic GMP to estradiol-17 β . Points represent means (vertical bars express $\pm$ S.E.). Number of expts. is shown in parentheses. * $P < 0.05$: significantly different from control values.

after estradiol-17 β with atropine were significantly higher ($P < 0.05$) than those of the control.

Changes in the concentration of cyclic

GMP after estradiol-17 β in the rats pretreated with phentolamine or propranolol are shown in Fig. 3. In animals pretreated with 50 or 250 μ g/100 g of phentolamine, the increase in cyclic GMP by estradiol-17 β treatment was suppressed at 60 and 90 min. The suppression was significant ($P < 0.01$) at 60 min in the 50 μ g group and at 90 min in the 250 μ g group. Pretreatment with propranolol caused no significant changes in the concentration of cyclic GMP.

Pretreatment with either phentolamine or propranolol caused no significant alteration in the concentration of cyclic AMP after estradiol-17 β (results are not shown in figures).

Sixty minutes after 500 μ g/100 g of phenylephrine, uterine cyclic GMP was significantly ($P < 0.05$) increased to 341.0 ± 52.1 fmoles/mg protein ($n=6$) from its control level.

Effects of estradiol-17 β or 500 μ g of phenylephrine on uterine cyclic GMP and cyclic AMP were measured in the rats pretreated with progesterone for 4 days. Cyclic GMP was determined at 90 min after estradiol-17 β or at 60 min after phenylephrine administration, and the results are shown in Fig. 4. No significant changes were observed in the concentration of uterine cyclic GMP or cyclic AMP in the animals treated with progesterone alone (data for cyclic AMP are not shown in the figure). Estradiol-17 β significantly ($P < 0.01$) increased uterine cyclic GMP whether it was used alone or in combination with progesterone, while phenylephrine produced a significant ($P < 0.05$) increase in uterine cyclic GMP only when it used alone. Animals treated with phenylephrine combined with progesterone were associated with no significant increase in uterine cyclic GMP.

Fig. 3. Effect of phentolamine (left) and propranolol (right) on time course of the change in uterine cyclic GMP after estradiol-17 β administration (i.p.). Points represent means (vertical bars express $\pm$ S.E.). Number of expts. is shown in parentheses. $\circ$ Effect of pretreatment with phentolamine or propranolol on time course of changes in uterine cyclic GMP after solvent (0.2 ml) administration (i.p.). ** $P < 0.01$: significantly different from the time course of values after estradiol-17 β administration (i.p.) described screen tone.

Fig. 4. Effect of progesterone on response of uterine cyclic GMP to estradiol-17 β (left) and to phenylephrine (right). Points represent means (vertical bars express $\pm$ S.E.). Number of expts. is shown in parentheses. * $P < 0.05$ ** $P < 0.01$: significantly different from control values.

The concentration of uterine cyclic AMP in the rats treated with isoproterenol alone or isoproterenol combined with estradiol-17 β 60 min before, are demonstrated in Fig. 5. In the animals treated with isoproterenol alone, cyclic AMP increased significantly ($P < 0.01$), while no significant changes were observed in animals treated with isoproterenol combined with estradiol-17 β .

Time courses of the changes in whole uterine wet weight and protein content after estradiol-17 β administration (i.p.) are demonstrated in Fig. 6. The wet weight and protein content of the control uterus are 101.1 ± 13.4 mg, and 10.0 ± 1.0 mg, respectively. Both values remained constant until 120 min after the treatment. At 240 min after the treatment, although no increase was observed in the protein content, the wet weight was significantly ($P < 0.05$) increased. In the animals pretreated with 250 μ g/100 g of phentolamine, no increase in these

parameters was observed.

2) Concentration of uterine noradrenaline:

The concentration of uterine noradrenaline was determined at 60 and 120 min after estradiol-17 β administration (i.p.). The results are shown in Fig. 7. The control contained 65.4 ± 3.8 ng/mg protein of noradrenaline. Decreased noradrenaline levels were detected at 60 and 120 min, but the differences were insignificant.

3) Determination of mechanical reactivity:

No difference was observed in the uterine wet weight and protein content between the control and the "estrogen-acute" group. The uterus of the "estrogen-chronic" group was essentially comparable to those of rats at the estrus (data not shown) in terms of the wet weight and protein content. The uterus of the "progesterone" group was similar to that of the controls except that the protein content was higher in the former (data not shown).

Fig. 5. Effect of estradiol-17 β on response of cyclic AMP to isoproterenol. Points represent means (vertical bars express $\pm$ S.E.). Number of expts. is shown in parentheses. * $P < 0.05$; significantly different from control values.

Fig. 7. Changes in uterine noradrenaline levels after estradiol-17 β administration (i.p.). Points represent means (vertical bars express $\pm$ S.E.). Number of expts. is shown in parentheses.

Fig. 6. Time course of the change in uterine wet weight and protein content after estradiol-17 β administration (i.p.). Points represent means (vertical bars express $\pm$ S.E.). Number of expts. is shown in parentheses. * $P < 0.05$; significantly different from control values.

In the control, "estrogen-acute" and "progesterone" groups, a spontaneous motility of the uterus was observed when the uterine preparation was suspended in the bath. In the "estrogen-acute" group, spontaneous motility was somewhat irregular and infrequent. In the "estrogen-chronic" group, some uterine preparations showed irregular spontaneous motility, but it disappeared after about 20 min.

Effects of noradrenaline *in vitro* on the uterine motility are demonstrated in Fig. 8. Disappearance of uterine spontaneous

motility took place at 10^{-5} , 10^{-6} , and 10^{-5} g/ml of noradrenaline in the control, "estrogen-acute", and "progesterone" groups, respectively. Neither phentolamine nor propranolol pretreatment at the concentration of 10^{-5} g/ml modified these effects of noradrenaline. In the "estrogen-chronic" group, noradrenaline at 10^{-8} , 10^{-7} , and 10^{-6} g/ml caused a contraction of about 500 mg of tension, but at 10^{-5} g/ml, it produced relaxation. While pretreatment with propranolol did not change the uterine response to noradrenaline, pretreatment with

Fig. 8. Effect of noradrenaline (g/ml) on isolated rat uterus (left) and effect of noradrenaline and isoproterenol (g/ml) on K^+ -induced contracture of isolated rat uterus (right). A: noradrenaline, B: noradrenaline after propranolol, C: noradrenaline after phentolamine, D: isoproterenol.

phentolamine abolished contraction produced by noradrenaline. These results suggest that α - and β -stimulations by noradrenaline produced relaxation except for the "estrogen-chronic" group in which α -stimulation caused contraction. In order to separate the α - and β -effects of noradrenaline, the relaxing effect of noradrenaline on K^+ -induced contracture was studied in the presence and absence of phentolamine or propranolol. The results obtained in the control uterus are demonstrated in Fig. 8. According to this experiment, relaxation produced by noradrenaline after pretreatment with propranolol can be called a "rapid relaxation", whereas relaxation produced by noradrenaline in the phentolamine pretreated uterus is a "slow relaxation" which is quite similar to the relaxation produced by isoproterenol.

A stable contraction of 1266.7 ± 16.7 mg ($n=3$) of tension was produced at 25 mM

KCl in the control uterus. Similar contraction was produced in the uterus from the "estrogen-acute" and "progesterone" groups. The tension of the KCl-induced contraction in the "estrogen-chronic" group was 2900 ± 81.6 mg ($n=3$) and significantly ($P < 0.01$) greater than those of the other 3 groups.

The relaxations mediated by α - and β -receptors are demonstrated in Fig. 9. Relaxation induced by α -stimulation occurred at 10^{-5} g/ml of noradrenaline in the uterus from the control group. In the "estrogen-acute" group, 10^{-6} g/ml of noradrenaline relaxed the uterus by $13.0 \pm 4.7\%$. The relaxation at 10^{-5} g/ml is significantly ($P < 0.01$) greater than that determined in the control uterus. The difference was not significant at 5×10^{-5} g/ml. In the uterus from the "estrogen-chronic" group, 10^{-8} g/ml and higher concentrations of noradrenaline

Fig. 9. Dose-response curves of noradrenaline in K^+ -contracted, isolated rat uterus. left: Effect of noradrenaline after pretreatment with propranolol. right: Effect of noradrenaline after pretreatment with phentolamine.

caused a dose-dependent relaxation. The relaxation at 5×10^{-5} g/ml was $92.3 \pm 4.0\%$ and significantly ($P < 0.05$) greater than those in the control and "estrogen-acute" groups. No difference was observed between the "progesterone" and control groups. As shown in the right panel of Fig. 9, β -mediated relaxation was $4.3 \pm 2.5\%$ at 10^{-6} g/ml of noradrenaline in the control uterus. This relaxation was also dose-dependent. In the uterus from the "estrogen-acute" group, no relaxation was induced by 10^{-6} g/ml of noradrenaline. In the uterus from the "estrogen-chronic" group, 10^{-8} g/ml of noradrenaline produced relaxation by $18.7 \pm 2.0\%$. The relaxation was linearly increased with elevating noradrenaline concentration, and at 10^{-5} g/ml, the relaxation was significantly ($P < 0.05$) greater than those in the control and "estrogen-acute" groups. The uterus was maximally relaxed, 100% ($n=3$), by 5×10^{-5} g/ml. In the "progesterone" group, the relaxation was $3.5 \pm 1.1\%$ at 10^{-7} g/ml and reached its maximum at 5×10^{-5} g/ml. Noradrenaline at 10^{-7} , 10^{-6} , and 10^{-5} g/ml, however, produced significantly ($P < 0.01$, 0.01, and 0.05, respectively) smaller relaxations than those observed in the "estrogen-chronic" group.

4) Determination of binding sites: DHE and DHA binding sites were assayed by incubating uterine preparation with DHE and DHA present at concentrations between 10^{-9} and 10^{-8} M. As shown in Fig. 10, approximately 50% of the bound DHE and 60% of the bound DHA were displaced by 10^{-6} M phentolamine and 10^{-6} M propranolol, respectively. Those sites for which binding could be inhibited were defined as specific sites. Specific binding of DHE and DHA reached equilibrium after 20 min at 30°C .

Animals were divided into 4 groups as done in the experiment for the mechanical reactivity and subjected to the binding

Fig. 10. Competitive inhibition of ^3H -ligand binding by the unlabeled competitor (control uterus).

experiment. The Scatchard analysis of the binding of DHE and DHA is shown in Fig. 11. The K_D values of DHE estimated from the figure were almost the same among the 4 groups. B_{max} determined with the preparation obtained from animals of the control and "progesterone" groups were 3.00 and 3.20 pmoles/mg protein, respectively, and these two values were almost the same. In the "estrogen-acute" group, the B_{max} was 6.30 pmoles/mg protein and was higher than the control value by 110%. Chronic treatment with estradiol-benzoate gave the highest B_{max} of 6.85 pmoles/mg protein which was higher than the control value by 128%. The contents of the specific binding sites at 10^{-8} M DHE were 2.51 ± 0.23 , 4.71 ± 0.12 , 5.14 ± 0.20 , and 2.35 ± 0.23 pmoles/mg protein ($n=5$) for the control, "estrogen-acute", "estrogen-chronic", and "progesterone" groups, respectively. The contents for the "estrogen-acute" and "estrogen-chronic" groups were significantly

Fig. 11. Scatchard analysis of the data from the DHE (left) and DHA (right) binding experiments. Lines were determined by linear regression analysis. K_D (DHE): control, 1.60×10^{-9} M; estrogen-acute, 1.63×10^{-9} M; estrogen-chronic, 1.94×10^{-9} M; progesterone, 1.77×10^{-9} M. K_D (DHA): control, 2.28×10^{-9} M; estrogen-acute, 2.20×10^{-9} M; estrogen-chronic, 2.43×10^{-9} M; progesterone, 2.25×10^{-9} M.

($P < 0.01$) higher than that for the control. K_D values of DHA were almost the same among the groups as shown in Fig. 11. The B_{max} was 6.10 and 6.20 pmoles/mg protein for the control and "estrogen-acute" groups, respectively. These values were almost the same. Chronic treatment with estradiol-benzoate increased B_{max} by 103% to 12.4 pmoles/mg protein. Treatment with progesterone increased B_{max} by 107% and produced the highest value of 12.6 pmoles/mg protein. The contents of the specific binding sites at 10^{-8} M DHA were 4.81 ± 0.51 and 5.00 ± 0.33 pmoles/mg protein ($n=5$) in the control and "estrogen-acute" groups, respectively, and there was no significant difference between these groups. Treatment with estradiol-benzoate and progesterone

increased the binding sites to 9.24 ± 0.92 and 8.68 ± 0.47 pmoles/mg protein ($n=5$), respectively. These values were significantly ($P < 0.01$) higher than the control.

DISCUSSION

Szego et al. (10) reported that estrogen caused an increase in the concentration of uterine cyclic AMP in rats.

In the present investigation, uterine cyclic AMP was shown to have a tendency to decrease throughout the observation period until 240 min after treatment with estradiol- 17β (i.p.). Accordingly, it seems unlikely that cyclic AMP acts as a messenger for estrogen.

Kuehl et al. (1) reported for the first time that estrogen increased the concentration of

uterine cyclic GMP *in vivo* in rats. Flandroy and Galand (2, 11) and Nicol et al. (12) also reported that estrogen elevated uterine cyclic GMP in animals including immature rats. In the present experiment, uterine cyclic GMP in ovariectomized rats was progressively and significantly elevated after administration of estradiol-17 β . The concentration reached its peak after 120 min and returned to the control level within 240 min after treatment with estradiol-17 β . This increase in cyclic GMP preceded the increase in wet weight of the uterus. Uterine wet weight was found to be increased at 240 min after the hormonal treatment. This increasing effect of estradiol-17 β on uterine wet weight may be attributed to water imbibition caused by estrogen at its initial stage of action (13) because there was no concomitant increase in uterine protein. Flandroy and Galand (2) demonstrated a biphasic cyclic GMP response; a first peak near 2–4 hr, a drop around 6 hr, and again a high cyclic GMP content associated with [3 H]-thymidine uptake in the uterus at 18–24 hr and beyond. It seems reasonable to expect that the effect of estrogen on uterine tissue might involve an increase in uterine cyclic GMP.

Possible involvement of a cholinergic mechanism in estrogen-induced elevation of cyclic GMP was suspected because an increase in cyclic GMP by acetylcholine was demonstrated in myocardium by George et al. (14) and in rat uterus, *in vitro*, by Nicol et al. (15). The present result obtained from the experiments using atropine, however, did not support the idea that cholinergic muscarinic receptors might be involved in the estrogen-induced increase in cyclic GMP.

Ham et al. (16) denied the participation of the prostaglandin F series in elevation of cyclic GMP following estrogen.

As stated previously (2, 17), propranolol did not alter the increased uterine cyclic GMP or physiological responses of the uterus to

estrogen, suggesting that the estrogen-induced increase in cyclic GMP is independent of adrenergic β -receptors. As for the uterus of the rat, few experiments have been carried out *in vivo* in the connection with the α -receptors. Phentolamine significantly suppressed an initial increase in cyclic GMP following estradiol-17 β , and phenylephrine significantly increased cyclic GMP when used at a relatively large dose. This fact suggests that α -receptors participate in the estradiol-17 β -induced elevation of cyclic GMP. It was also studied here whether adrenergic transmitters or receptors are more greatly affected by estrogen. Regarding the effect of estrogen on adrenergic transmitter, Rudzik et al. (18) reported that administration of diethylstilbestrol for 4 days decreased noradrenaline concentration in rat uterus. Bengtsson (19) demonstrated that chronic treatment of estrogen inhibited K $^{+}$ -induced noradrenaline release in the uterus. According to the present observations, the concentration of uterine noradrenaline was not significantly changed in the "estrogen-acute" group where estrogen increased cyclic GMP. It suggests that estrogen does not increase noradrenaline in rat uterus under physiological conditions.

Since it has thus been indicated that estrogen exerts its effect not on the noradrenaline content but on α -receptors, investigation was extended to observing the effects of estrogen and progesterone on α - and β -receptors by means of determination of mechanical reactivity and binding sites.

Since Ahlquist's report (4), it has widely been accepted that β -receptors are present in the uterus, and they mediate uterine relaxation in the rat. However, there is yet a controversy on the presence and significance of α -receptors in rat uterus. Some have suggested the presence of excitatory α -receptors (20–22), but others have a negative opinion on this (23–25). Similarly, there

are positive (21, 26, 27) and negative opinions on the presence of inhibitory α -receptors.

According to the present observations, α -stimulation induced inhibitory responses except for the "estrogen-chronic" group in which α -stimulation produced bidirectional changes: ordinary uterine preparations from this group were contracted, while preparations producing K^+ -induced contracture were relaxed. The α -inhibitory effect on the K^+ -contracted preparation was most marked in the "estrogen-chronic" group followed by the "estrogen-acute" group. The response in the "progesterone" group was comparable to that in the control.

K^+ -induced contracture was significantly greater in the "estrogen-chronic" group than in the other groups. It suggests that estrogen stimulates the mechanism for increasing Ca^{2+} influx.

Adrenergic β -response was greatest in the "estrogen-chronic" group followed by the "progesterone" group, although Milenov et al. (25) reported that estrogen inhibited β -effects. Dose- β -response curves in these groups were apparently shifted toward the left. Compared with the control, uterine responses in the "estrogen-acute" group were slightly suppressed. As to the inhibitory effect of noradrenaline, α -receptors played a predominant role in the "estrogen-acute" group, while β -receptors were predominant in the "estrogen-chronic" and "progesterone" group.

Estrogen was stated to increase DHE binding sites of rabbit uterus (28, 29) and to increase DHA binding sites of rat uterus (9). In the present experiments, no changes were observed in the affinity of DHE binding sites. The number of binding sites of this type was increased in the uterus of the "estrogen-chronic" and "estrogen-acute" groups, whereas no change was observed in the "progesterone" group. DHA binding

sites were increased in the "progesterone" and "estrogen-chronic" group. The population of DHA binding sites was unchanged in the "estrogen-acute" group.

Mechanical reactivity of the uterus was heightened in parallel with an increase in binding sites as observed in the α -effect in the "estrogen-acute" group, in the α - and β -effects in the "estrogen-chronic" group, and in the β -effect in the "progesterone" group. It suggests that these increased reactivities are attributed to the increased number of receptors. In the light of the present data for mechanical reactivity and binding sites in the "estrogen-chronic" group, α -mediated contraction could neither be explained by an increased number of receptors nor by an estrogen-induced qualitative change in receptors.

As observed in the "estrogen-acute" group, the increase in binding sites for DHE occurred earlier than the increase in DHA binding sites and it occurred simultaneously with the increase in cyclic GMP content. This increase in binding sites, however, seems to precede the enhancement of mechanical reactivity.

Ball et al. (30) suggested that cyclic GMP is a second messenger for the α -response. Furthermore, Schultz et al. (31, 32) reported that α -adrenergic stimulation of the ductus deference caused a Ca^{2+} -mediated increase in cyclic GMP in rats. In the present study, a large dose of phenylephrine increased cyclic GMP in rat uterus. In conclusion, it seems reasonable to expect that enhanced α -stimulation as a result of estrogen-induced increase in α -receptors elevates cyclic GMP in rat uterus. Estrogen may also enhance the Ca^{2+} -dependency of rat uterus.

According to Pedroza et al. (33), noradrenaline increased the uptake of estradiol by rat uterus *in vivo* and *in vitro*. Flandroy and Galand (34) hypothesized that an estrogen-receptor complex acts directly on

the enzymatic metabolism of cyclic GMP. Kuehl et al. (1) demonstrated that estrogen increased uterine cyclic GMP in parallel with decreased hydrolysis of cyclic GMP without change in guanylate cyclase activity and suggested that estrogen inhibits the activity of phosphodiesterase. Therefore it does not eliminate the possibility that enhancement of α -stimulation accelerates the entrance of estrogen into rat uterus and that an estrogen-receptor complex acts on an enzyme(s) involved in cyclic GMP metabolism, probably phosphodiesterase. Cyclic GMP may consequently be increased by estrogen.

Regarding the effect of progesterone on cyclic GMP level, Beatty et al. (35) suggested that progesterone decreased guanylate cyclase and phosphodiesterase activities. In the present experiments, progesterone alone produced no change in cyclic GMP levels, and the suppressive effect of progesterone against phenylephrine-induced increase in cyclic GMP seems to be due mainly to the relatively high inhibitory effect of progesterone on guanylate cyclase. On the other hand, the increase in cyclic GMP by estradiol-17 β after pretreatment with progesterone seems to be due to synergistic or summative effect of estrogen and progesterone on phosphodiesterase.

In the "estrogen-acute" group, the β -effect on mechanical reactivity was slightly suppressed. Furthermore, estradiol-17 β inhibited the responses of cyclic AMP to isoproterenol as reported by Rinard and Chew (36). Therefore, the suppression of β -receptors by acute treatment with estrogen is suggested. However, chronic treatment of estrogen increased the number of receptors which was accompanied with elevation of mechanical function.

Acknowledgements: I wish to express my gratitude to Professor Dr. Joichi Ando of the Osaka Medical College for his critical

review of this manuscript and helpful discussions. I am also grateful to Assistant Mr. Kenji Ishii of the Osaka Medical College for his helpful suggestions. I also appreciate the kindness of the members of the Department of Pharmacology, Osaka Medical College.

REFERENCES

- 1) Kuehl, F.A., Jr., Ham, E.A., Sanford, C.H., Nicol, S.E. and Goldberg, N.D.: Estrogen-related increase in uterine guanosine 3',5'-cyclic monophosphate level. *Proc. natn. Acad. Sci. U.S.A.* **71**, 1866-1870 (1974)
- 2) Flandroy, F. and Galand, P.: Changes in cGMP and cAMP content in the estrogen-stimulated rat uterus: temporal relationship with other parameters of hormonal stimulation. *J. Cyclic. Nucleotide. Res.* **4** (1978)
- 3) Robinson, G.A., Butcher, R.W. and Sutherland, E.W.: *Cyclic AMP*, p. 31. Academic Press, New York and London (1971)
- 4) Ahlquist, R.P.: A study of adrenotropic receptor. *Am. J. Physiol.* **153**, 583-600 (1948)
- 5) Lowry, O.H., Rosebrough, N.J. and Farr, A.L.: Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193**, 265-275 (1951)
- 6) Honma, M., Satoh, T., Takezawa, J. and Ui, M.: An ultrasensitive method for simultaneous determination of cyclic AMP and cyclic GMP in small-volume samples from blood and tissue. *Biochem. Med.* **18**, 257-273 (1977)
- 7) Kurobe, Y., Shimada, M. and Hashimoto, Y.: Massfragmentographic determination of plasma catecholamines. *Japan. J. Pharmacol.* **28**, Supp. 72P (1978)
- 8) Gabella, G.: Effect of potassium on the mechanical activity of taenia coli, uterus and portal vein of the guinea pig. *Q. Jl exp. Physiol.* **63**, 125-146 (1978)
- 9) Krall, J.F., Mori, H., Fuck, M.L., LeShon, S.L. and Koreman, S.G.: Determination of adrenergic catecholamine receptor in rat myometrium and their regulation by sex steroid hormones. *Life Sci.* **23**, 1073-1082 (1978)
- 10) Szego, C.M. and Davis, J.S.: Adenosine 3',5'-monophosphate in rat uterus: acute elevation by estrogen. *Proc. natn. Acad. Sci. U.S.A.* **58**, 1711-1718 (1968)
- 11) Flandroy, F. and Galand, P.: Estrogen-related changes in uterine and vaginal cAMP and cGMP. *Archs int. Physiol. Biochim.* **85**, 965-967 (1977)
- 12) Nicol, S.E. and Goldberg, N.D.: Inhibition of

- estrogen induced increase in uterine guanosine 3',5'-cyclic monophosphate levels by inhibitor of protein and RNA syntheses. *Biochemistry Res.* **15**, 5490-5496 (1976)
- 13) Kalman, S.M.: Some studies on estrogen and uterine permeability. *J. Pharmacol. exp. Ther.* **115**, 442-448 (1955)
 - 14) George, W.E., Polson, J.B., O'Toole, A.G. and Goldberg, N.D.: Elevation of guanosine 3',5'-cyclic phosphate in rat heart perfusion with acetylcholine. *Proc. natn. Acad. Sci. U.S.A.* **66**, 398-403 (1970)
 - 15) Nicol, S.E., Sanford, C.E., Kuehl, F.A., Jr., Ham, E.A. and Goldberg, N.D.: Estrogen-induced elevation of uterine cyclic GMP concentration. *Fedn. Proc.* **33**, 284p (1974)
 - 16) Ham, E.A., Cirillo, V.J., Zanetti, M.E. and Kuehl, F.A., Jr.: Estrogen-directed synthesis of specific prostaglandin in uterus. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1420-1424 (1975)
 - 17) Tchernitchin, A., Tchernichin, X., Rodriguez, A., Mena, M.A., Unda, C., Mairesse, N. and Galand, P.: Effect of propranolol on various parameter of estrogen stimulation in rat uterus. *Experientia* **33**, 1536-1537 (1977)
 - 18) Rudzik, A. and Miller, J.A.: The effect of altering the catecholamine content of the uterus on the rate of contraction and the sensitivity of the myometrium to relaxin. *J. Pharmacol.* **138**, 88-95 (1962)
 - 19) Bengtsson, B.: Estrogen induced inhibition of ³H-noradrenaline release in the uterus and portal vein of rat. *Acta physiol. scand.* **104**, 287-298 (1978)
 - 20) Mann, M.: Sympathin and the rat uterus. *J. Physiol.* **110**, 11p (1949)
 - 21) Butterworth, K.R. and Randall, M.J.: The effect of α - and β -adrenoceptor blocking agents on the response of the rat uterus to catecholamines throughout the oestrous cycle. *Brit. J. Pharmacol.* **40**, 160-161 (1970)
 - 22) Butterworth, K.R. and Jarman, D.A.: Effect of temperature on changes induced by oestrous, in α - and β -adrenoceptor activity of rat uterus. *Brit. J. Pharmacol.* **51**, 462-464 (1974)
 - 23) Rudzik, A.D. and Miller, J.W.: The mechanism of uterine inhibitory action of relaxin-containing ovarian extract. *J. Pharmacol. exp. Ther.* **138**, 82-87 (1962)
 - 24) Levy, B. and Tozzi, S.: The adrenergic receptive mechanism of rat uterus. *J. Pharmacol. exp. Ther.* **142**, 178-184 (1963)
 - 25) Milenov, K. and Chernaeva, L.: Effect of ovarian hormones on the myometrium adrenergic reactivity. *Agressologie* **16**, 107-112 (1974)
 - 26) Ahlquis, R.P.: The adrenergic receptor. *J. pharmacol. Sci.* **55**, 359-367 (1966)
 - 27) Miller, J.W.: Adrenergic receptors in the myometrium. *Ann. N.Y. Acad. Sci.* **139**, 788-798 (1967)
 - 28) Roberts, J.M., Insel, P.A., Goldfien, R.D. and Goldfien, A.: α -Adrenoceptor but not β -adrenoreceptor increase in rabbit uterus with oestrogen. *Nature* **270**, 624-625 (1977)
 - 29) Williams, L.T. and Lefkowitz, R.J.: Regulation of rabbit myometrial alpha adrenergic receptors by estrogen and progesterone. *J. clin. Invest.* **60**, 815-818 (1977)
 - 30) Ball, H.H., Kaminsky, N.I., Hardman, J.G., Broabus, A.E., Sutherland, E.W. and Liddle, G.W.: Effect of catecholamine and urinary cyclic nucleotides in man. *J. clin. Invest.* **51**, 2124-2129 (1972)
 - 31) Schulz, G., Hardman, J.G., Schultz, K., Baird, C.E. and Sutherland, E.W.: A new enzymatic assay for guanosine 3',5'-cyclic monophosphate and its application to the ductus deferens of the rat. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1721-1725 (1973)
 - 32) Schultz, G., Schultz, K. and Hardman, J.G.: Effect of norepinephrine on cyclic nucleotide levels in the ductus deferens of the rat. *Metabolism* **24**, 429-437 (1975)
 - 33) Pedroza, E., Garcia-Biénere, W. and Rosner, J.M.: Interaction of 17 β -estradiol and L-norepinephrine on the rat uterus. *Hormon Res.* **6**, 193-198 (1975)
 - 34) Flandroy, F. and Galand, P.: Estrogen-induced increase in uterine cGMP content: A true hormonal action? *Mol. Cell. Endocrinol.* **13**, 281-290 (1979)
 - 35) Beatty, C.H., Bocek, R.M., Herrington, P.T., Young, M.K. and Brenner, R.M.: Effect of estradiol-17 β and progesterone on cyclic nucleotide metabolism in myometrium of macaques. *Biol. Reprod.* **21**, 309-318 (1979)
 - 36) Rinard, G.A. and Chew, C.S.: Uterine cyclic AMP formation: Biphasic effect of estrogen on catecholamine sensitivity. *Life Sci.* **22**, 2043-2050 (1978)