REGULATION BY ESTROGEN AND PROGESTERONE OF PROTEIN KINASES IN RABBIT MYOMETRIUM

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Abstract—We report on hormonal regulation of protein kinases in the rabbit myometrium. The injection of estrogen or progesterone produced 5.2±0.7- or 4.7±0.4-fold increases, respectively, of the total activity when histone was used as the substrate in the cytosol fraction of the tissue. The analysis of the activity by DEAE-cellulose column chromatography revealed that estrogen had no effect on increases in the levels in both types I and II cAMP-dependent protein kinases, on the basis of mg protein, while progesterone produced a 50% decrease in type I cAMP-dependent protein kinase and a 50% increase in type II cAMP-dependent protein kinase. Thus, the activity ratio of types II to type I did not change by treatment with estrogen and increased from 2.8 to 7.9 with progesterone administration. As the activity patterns by treatment with human chorionic gonadotropin closely resembled those seen with progesterone, our findings in case of the latter hormone are probably valid. The results indicate that the levels of protein kinases in the myometrium are altered in response to estrogen and progesterone and may possibly be involved in the physiological functions of the tissue.

The myometrium is one of the target organs for the steroid hormones, and this tissue develops under the influences of estrogen and progesterone. In contrast to the endometrium, a primary increase is observed in the size of the muscle cells, although a small amount of cell division does occur (1). In association with an increase in tissue weight, there are increases in the ratio of RNA per DNA (2, 3), glycogen (4, 5), phospholipid and nitrogen (6). These alterations relate to functions of the myometrium, i.e., the maintenance of pregnancy and parturition.

In previous papers, we reported that multiple protein kinases in the rabbit endometrium are regulated by estrogen and progesterone (7). Levels of types I and II cAMP-dependent protein kinases are separately controlled by these hormones, and levels of protein kinase in human tissues vary with the menstrual cycle (8). The enzyme levels in the rabbit tissue vary with time after injection of human chorionic gonadotropin (CG) by endogenously secreted hormones from ovarian follicles and the corpus luteum (9). In view of these findings, we examined the regulation of protein kinase levels in the rabbit myometrium. The present communication deals with the alteration by estrogen and progesterone of protein kinase levels in this tissue.

Materials and Methods

Materials: cAMP was obtained from the Kohjin Co, DEAE-cellulose from Whatman, calf thymus histone and protamine from Sigma, casein and cycloheximide from Wako Pure Chemical Industries, [32P]orthophosphate (carrier free) from the Japan Radioisotope Association, estradiol benzoate (estra-
1,3,5,(10)-triene-3,17β-diol-3-monobenzozate) and progesterone (4-pregnene-3,20-dione) from the Teikoku Hormone MFG Co. Ltd, sodium pentobarbital (sodium 5-ethyl-5-(1-methylbutyl) barbiturate) from Abbott Laboratories, human CG from the Mochida Pharmaceutical Co. Ltd.

Animals: Female Japanese white rabbits, which were sexually mature and weighing 2.5-3.0 kg, were anesthetized with pentobarbital (25 mg/kg, i.v.) and then bilaterally ovarietectomized via the ventral route. The injection of hormones was begun from the 14th to the 17th day after this surgery. Estradiol (40 μg/kg) was given subcutaneously, daily for six successive days. Progesterone (800 μg/kg) was given daily by the same route for ten successive days after six prime daily injections of estradiol. Control animals (castration alone) were killed on the 17th day after the surgery. Cycloheximide (0.9 mg/kg), when used, was given intraperitoneally, daily for six days concomitant with the administration of estrogen. Human CG (100 I.U. per rabbit) was intravenously administered to an intact rabbit. The specimens of myometrium were obtained on the 7th and 15th days after administration. Ovulation and the hormonal effect after ovulation were confirmed both in the ovary and the endometrium.

The animals were then given pentobarbital (50 mg/kg) and the uterus was quickly removed and dissected free of adhering fat and connective tissue. The myometrium was macroscopically separated from the endometrium using scissors on a cold glass board and placed in physiological saline. No contamination of the endometrium was observed in the myometrium preparation by microscopy. The effect of the hormones on the uterus was confirmed by histologically examining the endometrium according to the method of McPhail (10).

Three to seven experiments were done for each treatment as indicated. Respective experiments were performed using one to three-pooled tissues. The alteration of protein kinase levels with the administration of the hormones was simultaneously examined in the endometrium in order to confirm the effects of the hormones as described previously (7-9).

Preparation of cytosol fraction and DEAE-cellulose column chromatography: All procedures were carried out at 0-4°C. The myometrium was homogenized at high speed for three 20-sec periods interspersed with 30-sec cooling periods with 5 vol. of 0.25 M sucrose, 10 mM Tris-HCl buffer, pH 7.5, and 0.1 mM EGTA in a Hiscotolon. The homogenate was centrifuged at 105,000 x g for 60 min. The cytosol fraction (2.0 ml) containing 7.7 to 17.8 mg of protein was applied to a 1.1 x 3.2 cm column (3 ml of bed volume) of DEAE-cellulose which had been equilibrated with 10 mM Tris-HCl buffer, pH 7.5, 1 mM Mg2+ and 0.05 mM EGTA. The column was washed with about 16 ml of the same buffer. Elution was carried out with a linear gradient of NaCl (0-0.4 M) in the buffer with a total volume of 150 ml. Fractions (3 ml) were collected at a flow rate of about 30 ml/hr.

Assay for protein kinase activity: The standard assay system for protein kinase contained, in a final volume of 0.2 ml, 10 μmoles of sodium acetate buffer, pH 6.0, 100 μg of substrate protein as indicated, 1 nmole of [γ-32P]ATP (2-5 x 10^5 cpm), 2 μmoles of magnesium acetate, 0.01 μmole of EGTA and the indicated amount of protein kinase in the presence or absence of 0.2 nmole of cAMP. Incubation was carried out at 30°C for 5 min in a shaking water bath. The reaction was terminated and the degree of protein phosphorylation measured as described previously (11).

Other methods: [γ-32P]ATP was prepared by the method of Post and Sen (12). Protein
was measured by the method of Lowry et al. (13) with bovine serum albumin as a standard. The other materials and methods used in the present study were essentially as described previously (7-9).

**Results**

Protein kinase activities in the cytosol fraction by hormonal treatment: The protein kinase activities in the cytosol fraction following treatments with estrogen and progesterone were compared to those in the case of castration, with histone as the substrate. The activities were analyzed from three respects, i.e., the total activity per whole tissue, activity per g wet tissue and activity per mg protein (specific activity). The total activity present in the whole tissue increased 5.2±0.7-fold and 4.7±0.4-fold with the administration of estrogen and progesterone, respectively. However, the activity per g wet tissue showed a slight decrease or no change by treatment with these hormones. The activity per mg protein decreased to 60-70% of that seen after castration. These results may be explained by the finding that the rate of increase in protein kinase activity by hormonal treatments was approximately equal to that of the amount of tissue, but less than that of the amount of protein.

Analysis by DEAE-cellulose column chromatography: As protein kinases in the cytosol fraction of the myometrium have multiple forms which may be individually altered by hormonal treatments, the patterns of protein kinase activities were analyzed by DEAE-cellulose column chromatography. For convenience of comparison, the same volume (2.0 ml) of the cytosol fraction extracted from a given amount (0.36 g) of the tissue was applied to the same sized column (1.1×3.2 cm). Each peak of activity was integrated according to the profile on the column, and findings in the case of tissues from castrated rabbits were taken as 100%.

Five experiments were performed for each treatment. This analysis was considered to be a valid statistical assessment and to express activity per certain weight of wet tissue for each treatment.

A typical result with histone is shown in Fig. 1. Both types I and II cAMP-dependent protein kinases were present in the myometrium from a castrated rabbit (Fig. 1A). The administration of estrogen brought about an increase in both types I and II enzymes to 157±20 and 131±10%, respectively. The peak of type I enzyme was smaller than that of type II enzyme (Fig. 1B). The administration of progesterone resulted in a decrease of type I enzyme to 80±6% and an increase of...
type II enzyme to 235±22% (Fig. 1C). The activity profile with protamine as substrate is shown in Fig. 2. Peaks 1 and 2 which eluted with 0.08 and 0.18 M NaCl, respectively, were observed. The cAMP-independent activity of peak 1 and cAMP-dependent activity of peak 2 increased by treatments with estrogen to 146±7 and 126±11%, respectively, and with progesterone to 259±32 and 126±13%, respectively. Similar experiments with casein as substrate were performed. The administration of estrogen and progesterone resulted in an increase in the peak of cAMP-independent activity eluted with about 0.18 M NaCl to 271±13 and 260±13%, respectively.

The results obtained on the DEAE-cellulose column were further analyzed by calculating the activity per mg protein (Table 1). It was noteworthy that progesterone caused a decrease in type I enzyme and an increase in type II enzyme. The activity ratio of type II cAMP-dependent protein kinase to type I cAMP-dependent protein kinase was unchanged (from 2.8 to 2.4) between the castration and estrogen treatments, but it did increase from 2.8 to 7.9 by treatment with progesterone. With protamine as substrate, peak 1 of the cAMP-independent activity increased by treatment with progesterone. Therefore, the ratio of peak 2 to peak 1 decreased. Estrogen and progesterone showed an increased activity when casein was used as the substrate.

**Effect of cycloheximide on protein kinase levels by treatment with estrogen:** The effects of a protein synthesis inhibitor, cycloheximide, on the treatment with estrogen are shown in Fig. 3. In comparison to the activity profile in Fig. 1B, the administration of cycloheximide plus estrogen decreased both types I and II cAMP-dependent protein kinases to 46±5 and 70±2%, respectively, in comparison to data obtained in the case of castration (Fig. 2).

![Fig. 2. Activity profile with protamine on DEAE-cellulose column of the cytosol fraction. The cytosol fraction (2.0 ml) containing 7.7, 16.1 and 14.8 mg of protein for castration (A), estrogen (B) and progesterone (C) treatments, respectively, was applied to a 1.1 x 3.2 cm column of DEAE-cellulose. Each experiment corresponds to that shown in Fig. 1. Aliquots (0.075 ml) of the fractions were assayed for protein kinase activity with protamine in the presence (■) or absence (□) of 1 μM cAMP.](image)

![Fig. 3. Effect of cycloheximide on protein kinase levels by treatment with estrogen. The cytosol fraction (2.0 ml), containing 13.2 mg of protein and obtained after treatment with estrogen plus cycloheximide, was applied to a 1.1 x 3.2 cm column of DEAE-cellulose. Aliquots (0.075 ml) of the fractions were assayed for protein kinase activity with histone (A) and protamine (B) in the presence (—■—■—) or absence (—□—□—) of 1 μM cAMP.](image)
Table 1. Analysis of the peak of activity on DEAE-cellulose column after various treatments

| Treatment                        | Histone Type I | Histone Type II | Type I/Type II | Protamine Peak 1 | Protamine Peak 2 | Casein Peak 2/Peak 1 |
|----------------------------------|----------------|-----------------|----------------|-------------------|------------------|---------------------|
| Castration (5)                   | 100±4          | 100±6           | 2.8±0.1 (2.3)  | 100±8             | 100±6            | 8.3±0.4             |
| Estrogen (5)                     | 104±14         | 84±6            | 2.4±0.3 (1.3)  | 95±6              | 82±8             | 7.2±0.6             |
| Progesterone (5)                 | 52±4           | 150±16          | 7.9±0.5 (16.1) | 165±21            | 80±8             | 4.1±0.2             |
| Estrogen+cycloheximide (3)       | 32±4           | 47±1            | 4.3±0.5        | 98±8              | 51±4             | 4.4±0.1             |

P value

|       | C-E           | C-P                | E-P                | F-E+cycloheximide |                 |                     |
|-------|---------------|---------------------|--------------------|-------------------|------------------|---------------------|
|       | N.S.*         | P<0.005             | P<0.005            | P<0.005           | P<0.005          | P<0.005            |
|       | P<0.005       | P<0.025             | P<0.005            | P<0.025           | N.S.             | P<0.005            |
|       | P<0.01        | P<0.005             | P<0.005            | P<0.025           | N.S.             | P<0.005            |

Integrated protein kinase activity was calculated according to the peak with each substrate on the DEAE-cellulose column. The activity of each peak with respective substrates used for tissues from the castrated rabbits was taken as 100%, and from this value, the activity of the peak for each treatment was calculated as a percentage. The activity value of each peak in the castration treatment was 1234, 3390, 379, 3153 and 868 pmole/mg protein/min for type I, type II, peak 1, peak 2 and casein, respectively. Numbers in parenthesis represent the number of experiments in "Treatment" and the activity ratio of type II to type I for the endometrium in "Type II/Type I". Values are means±S.E.M. C, E and P in the "P value" represent castration, estrogen and progesterone, respectively. *N.S. means not significant.
Table 2. Analysis of the peak of activity on the DEAE-cellulose column after human CG injection

| Treatment                  | Protein kinase activity per mg protein (\% of control) |
|----------------------------|-------------------------------------------------------|
|                            | Type I | Type II | Type II | Type I |
| No treatment               | 100±7  | 100±9   | 2.1±0.3 |
| Human CG 7th day           | 62±8*  | 159±20* | 5.4±0.2* |
| Human CG 15th day          | 54±4*  | 151±15* | 5.8±0.3* |

Integrated protein kinase activity was calculated according to the peak with histone as the substrate on the DEAE-cellulose column. The activity of the peak for tissues from rabbits given no treatment was taken as 100%, and from this value, the activity of peak for each treatment was calculated as a percentage. The activity value of each peak was 1047 and 2189 pmole/mg protein/min for types I and II, respectively. Numbers in parenthesis in “Treatment” represent the number of experiments. *P<0.005, 1P<0.025, 2P<0.05: P values were calculated in comparison to activities of no treatment. Values are means±S.E.M.

With protamine as substrate, peak 2 decreased to 76±4%, yet there was no decrease in peak 1 (Fig. 3B).

These results were confirmed by an analysis of activity per mg protein as shown in Table 1. The ratio of type II to type I increased from 2.4 to 4.3, indicating that the decrease in type I enzyme was more prominent. These results indicate that cycloheximide primarily represses the stimulatory effect of the hormone on levels of certain protein kinases.

Alteration in protein kinase levels by treatment with human CG: The myometrium was obtained at the 7th and 15th days after administration of human CG. Under these conditions, ovulation occurred 10 to 12 hr after the injection. Therefore, progesterone secreted from the corpus luteum mainly regulates the uterine functions. Even on the 15th day, the progesterone secretion still maintained a plateau (14). These conditions are known as pseudopregnancy since hormones are in the highly dynamic state which occurs during pregnancy. Activity profiles on the DEAE-cellulose column were examined (data not shown). With histone as the substrate, a decrease in type I enzyme and an increase in type II enzyme were seen on the 7th and 15th days after the hormone treatment as compared to cases with no treatment. These activity profiles were similar to those seen with the treatment of progesterone (see Fig. 1C and Table 1). The results were confirmed by analyzing the activity per mg protein from the profile on the column (Table 2).

Discussion

The present investigation indicates that steroid hormones such as estrogen and progesterone alter the levels of multiple protein kinases in the myometrium of rabbits. The mechanism may be based on a \textit{de novo} synthesis of enzyme proteins (see Fig. 3) as in the case of the endometrium (7) and Chinese hamster ovary cells (15). Cycloheximide counteracted the stimulatory effects of estrogen and progesterone in the endometrium (7). In view of the similarity of the estrogen effect between the endometrium and myometrium, it is suggested that cycloheximide has the identical effect on progesterone action for the myometrium as it has with that for the endometrium. To our knowledge, this is the first report to demonstrate hormonal regulation of protein kinases in the myometrium. When making a comparison of the results obtained in the case of the endometrium (7–9) and the myometrium (the present study), the differences are as follows: 1) The increase in total activity of
cAMP-dependent protein kinase in the myometrium (about 5-fold) was less than that seen in the endometrium (9 to 16-fold) by treatment with the hormones. 2) The activities of cAMP-dependent protein kinase in the myometrium were 4.6, 2.9 and 3.3 nmole/mg protein/min for castration, estrogen and progesterone treatments, respectively. These values were higher than 0.7, 0.4 and 0.8 nmole/mg protein/min for the respective treatments in the endometrium (7), indicating that the specific activity of the enzyme is higher in the myometrium than in the endometrium. 3) The effect of estrogen on the increase of type I cAMP-dependent protein kinase was less in the myometrium (see Fig. 1 and Table 1). 4) Type II cAMP-dependent protein kinase was slightly decreased by treatment with estrogen (see Table 1), although the activity per wet tissue weight increased (see Fig. 1). Therefore, the activity ratio of type II to type I was greater (2.4) than that seen in the endometrium (1.3). 5) With protamine as a substrate, peak 1 of cAMP-independent activity that was eluted at 0.08 M NaCl on the DEAE-cellulose column was less than that of the endometrium under conditions of treatment with progesterone (see Fig. 2 and Table 1). 6) Peak 2 of cAMP-dependent activity with protamine was found to be at a similar level to that of castration with the hormone treatment (see Fig. 2 and Table 1). 7) In general, the hormone effects on the increase in protein kinase activities were less in the myometrium than in the endometrium. On the basis of cell specificity, the differences may reflect the effects of the hormones on both tissues.

The alteration in protein kinase levels by these hormones may have a functional significance. Uterine contractility is regulated in response to certain hormones such as oxytocin and epinephrine (16-18). A possible involvement of cAMP in the contractility has been demonstrated by the findings that stimulation of β-adrenergic receptors relaxes the myometrium with a concomitant increase in cAMP (19, 20). The stimulation of myometrial adenylate cyclase is inhibited by prostaglandins and oxytocin which are known to cause contraction. Furthermore, dibutyryl cAMP and phosphodiesterase inhibitors mimic β-adrenergic stimulants (21-24). Recent findings strongly support the involvement of cAMP-dependent protein kinase in the relaxation of the myometrium: 1) The phosphorylation of specific proteins increases Ca++-uptake into uterine microsomes through cAMP-dependent endogenous phosphorylation of the 48,000-dalton protein (25). Thus, intracellular Ca++ is sequestered in the sarcoplasmic reticulum. 2) β-Adrenergic stimulation relaxes smooth muscle (26-28). The mechanism was suggested to be based on the stimulation of cAMP-dependent phosphorylation of proteins which enhances Na+/K+ transport (26) or ATP-dependent Ca++ transport through the cellular membrane (27, 28). 3) Myosin light chain kinase which is considered to be involved in the contraction and relaxation cycle in smooth muscles is reportedly phosphorylated by cAMP-dependent protein kinase (29-32). The phosphorylated enzyme has a lower activity in the phosphorylation of myosin light chain, and therefore, there is a decrease in muscle contraction.

In addition, protein kinases are considered to be involved in many other metabolic processes (33, 34). In view of the active metabolism of a variety of compounds such as glycogen, proteins and nucleic acid in the myometrium, the change in protein kinase levels, as induced by estrogen and progesterone, may reflect regulation of physiological processes.

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