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Effects of ovarian hormones on β-adrenergic receptor-mediated relaxation in the female rabbit bladder

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Abstract The effects of ovarian hormones on β-adrenergic receptor-mediated responses in female rabbit detrusor smooth muscles were investigated. Ovariectomized mature female New Zealand white rabbits were untreated or treated with estrogen and/or progesterone for 2 weeks. The contractile responses to carbachol and KCl in the detrusor strips were not significantly different in all groups. As compared with dobutamine and GS-332, isoproterenol and procaterol significantly relaxed the detrusor strips derived from all groups on KCl-induced tonic contractions. Combined with estrogen treatment, isoproterenol, procaterol and GS-332 caused a significant increase in this muscle relaxation. Furthermore, estrogen treatment caused a significant increase in relaxation as a result of forskolin and the cyclic AMP (cAMP) production that was induced by isoproterenol, procaterol and GS-332. However, estrogen treatment did not affect the relaxant response to dibutyryl cyclic AMP. Progesterone treatment did not affect β-adrenergic receptor-mediated responses. These results suggest that estrogen treatment causes the increased relaxant responses mediated by β_2- and β_3-adrenergic receptor subtypes, which may be related to the increased cAMP content in female rabbit detrusor smooth muscles.

Key words Estrogen · Progesterone · β-Adrenergic receptor subtypes · Rabbit bladder

Introduction

The presence of estrogen receptors has been demonstrated in the human lower urinary tract [48]. From a clinical perspective, there have been several reports [6, 10] suggesting the usefulness of estrogen treatment to postmenopausal women with lower urinary tract dysfunction. Although the mechanism of action of estrogen is not fully understood, it has been reported that ovarian hormones influence morphology and function of the lower urinary tract smooth muscles [1, 2, 9, 14, 21, 25, 43]. In bladder function, β-adrenergic receptor activation by noradrenaline may play an important role in the facilitation of urine storage [7, 8, 35]. Although an increase in β-adrenergic receptor responsiveness with estrogen treatment has been reported in several tissues including myometrium [13] and blood vessels [11], there is little information on the effects of ovarian hormones on β-adrenergic receptor-mediated relaxation of the bladder.

β-Adrenergic receptors have now been classified by pharmacological and molecular biological studies into three subtypes: β_1, β_2 and β_3 [27, 42]. Several reports [15, 16, 24, 32, 44, 45] have demonstrated the presence of β-adrenergic receptor subtypes in lower urinary tract smooth muscles. Therefore, the present study was undertaken to determine the effects of ovarian hormones on relaxation of female rabbit detrusor smooth muscles induced by various β-adrenergic receptor selective agonists.

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Materials and methods

Animals and tissues

Forty mature virginal female New Zealand white rabbits weighing 3.3–3.5 kg were used in the present experiment. Bilateral ovariectomy was performed in all rabbits under anesthesia induced by pentobarbital (1 mg/kg) 1 week before any further treatment. The ovariectomized rabbits were divided into four groups: (1) the ovariectomized group (Ox group, $n = 10$) i.e., ovariectomized and untreated, (2) the estrogen group (E group, $n = 10$) i.e., ovariectomized and treated with an intramuscular injection of 0.1 mg/kg/day estradiol, (3) the progesterone group (P group, $n = 10$) i.e., ovariectomized and treated with an intramuscular injection of 1 mg/kg/day progesterone, and (4) the estrogen-progesterone group (EP group, $n = 10$) i.e., ovariectomized and treated with an intramuscular injection of 0.1 mg/kg/day estradiol and 1 mg/kg/day progesterone. The effects of estrogen treatment on the rabbit lower

urinary tract have been studied previously at the dose of about 0.1 mg/kg/day (100–250 pg/ml of serum) for a period of several weeks [1, 23, 25, 40, 43]. These 2- [43] or 3-week estrogen treatment regimens [40] have previously demonstrated marked effects on the rabbit urethra. Furthermore, the effects of progesterone treatment have been studied previously at the dose several times as high as that of estrogen [9, 19, 31]. Thus, for the present experiment we have chosen the doses (0.1 mg/kg per day for estrogen and 1 mg/kg per day for progesterone) over a 2-week treatment period. All rabbits were killed 2 weeks after the start of treatment. A blood sample was taken from each rabbit just prior to ovariectomy, hormonal treatment and sacrifice. To confirm the efficacy of our hormonal manipulations, serum estradiol and progesterone were measured by radioimmunoassay (RIA) as previously described [41].

After removal of the bladder, the tissue was immediately immersed in modified Krebs-Henseleit (K-H) solution. The serosal and mucosal layers were dissected and detrusor strips were cut (approximately 4 mm wide and 15 mm long) from the dome region of the bladder.

Functional experiments

The functional experiments were performed as previously described [34, 46, 50]. Five rabbits in each group were used in this experiment. The detrusor strip was suspended in a 20-ml bath filled with K-H solution at 37°C and bubbled with 95% O_2 and 5% CO_2, resulting in a pH 7.4. Each muscle preparation was connected to a force displacement transducer (TB-611 T; Nihon Kohden, Tokyo, Japan) and isometric forces were recorded and monitored on an ink-writing recorder (R-02 A; Rika Denki, Tokyo, Japan). Each strip was stretched to its optimal length and the bath solution was changed every 15 min. The resting tension at the optimal length in all groups was about 1.5 g. This was determined by preliminary experiments, in which muscle strips were stretched stepwise and stimulated with 80 mM KCl solution at each length. When the change in the KCl-induced contraction was within 10% of the previous one, the length was considered to be optimal for maximum isometric force development.

Following equilibration for 90 min, 80 mM KCl solution was administered two or three times at intervals of 60 min until the responses were reproducible, and then contractile responses to carbachol and KCl were obtained. The force elicited by 80 mM KCl solution was biphasic, declining after a rapid initial increase in force and reaching a near-steady-state value (tonic contraction) about 70–80% of peak force by 30 min. Then, cumulative concentration-response curves for isoproterenol, dobutamine, procaterol, GS-332, forskolin or dibutyryl cyclic AMP (DBcAMP) on KCl-induced tonic contractions were determined by stepwise addition of increasing concentrations of each agonist. In the experiments in which various β-adrenergic receptor selective agonists were administered, muscle strips were treated with 10^{-6} M of phentolamine for 30 min. The pretreatment with phentolamine did not affect the contractility induced by 80 mM KCl solution in all groups. At the end of each experiment, the 80 mM KCl solution was replaced with Ca^{2+}-free solution to obtain the maximum relaxation of the strip. We defined 100% relaxation as the difference between the tonic force determined just prior to the administration of drugs and the force measured in Ca^{2+}-free solution.

Measurement of cyclic AMP content

The measurement of cyclic AMP(cAMP) content was performed as previously described [32, 33]. Five rabbits in each group were used in this experiment. The detrusor strip was incubated in K-H solution gassed at 37°C for 30 min, and then with 10^{-5} M of 3-isobutyl-1-methylxanthine (IBMX) for an additional 20 min. After the incubation period, 10^{-5} M of each agonist (isoproterenol, dobutamine, procaterol or GS-332) was added directly to this medium. Before and 1, 3 and 5 min after the administration of each agonist, muscle strips were rapidly frozen in liquid nitrogen and stored at −80°C. Perchloric acid (0.8 ml, 1 N) and 10,000 cpm [^{3}H]cAMP were added to the frozen tissue.The tissue was then homogenized with a motor driven teflon pestle. The homogenates were centrifuged at 7000 *g* for 30 min at 4°C. Pellets were diluted in NaOH, and assayed for protein [28], using bovine serum albumin as standard. The supernatants were neutralized with 6 N KOH, and the resulting precipitate was removed by centrifugation at 7000 *g* for 30 min at 4°C. Aliquots of the supernatant were assayed for cAMP content using a modification of the method of Brown et al. [5] using a cAMP binding protein prepared from rabbit muscle [38]. In 80–90% of samples [^{3}H]cAMP was recovered.With the addition of a known amount of cAMP to samples at the time of the binding assay, complete recovery of added cAMP was achieved.

Solutions and drugs

K-H solution had the following composition (in mM): NaCl 117.70, KCl 4.69, $CaCl_2$ 2.16, $MgSO_4$ 1.20, $NaHCO_3$ 24.39, KH_2PO_4 1.20 and glucose 9.99. By substituting NaCl with equimolar KCl in K-H solution, an 80 mM KCl solution was made. By removing $CaCl_2$ from K-H solution and adding 0.1 mM EGTA, a Ca^{2+}-free solution was made.

The following pharmacological agents were used: estradiol benzoate and progesterone (Teikoku Zouki, Tokyo, Japan), carbamylcholine chloride (carbachol), isoproterenol hydrochloride, dobutamine hydrochloride, procaterol hydrochloride, forskolin, DBcAMP, phentolamine hydrochloride and IBMX (Sigma, Tokyo, Japan). GS-332 [17, 18] were kindly donated by Tokyo Tanabe, Tokyo, Japan. Other chemicals and materials were of analytical grade and obtained from commercial sources. Concentrations are expressed as final bath concentrations. Isoproterenol was prepared in 5 mM HCl containing 1 mg/ml of ascorbic acid and 0.2-ml aliquots were added to the bath. A stock solution of forskolin (100 mM) was prepared using 100% ethanol with a further dilution in distilled water.

Data analyses

The E_{max} value (the maximum contractile response) was obtained from the maximum stress developed, and the ED_{50} value (the concentration of an agonist producing 50% of the maximum contraction) and the IC_{50} value (the concentration of an agonist producing 50% of the maximum relaxation) were calculated from a semilogarithmic plot of the percentage of the maximum response vs. drug concentration. Statistical analyses between groups and between concentration-response curves were performed using analysis of variance (ANOVA) and the multiple comparison test (Fisher's test). *P* values of 0.05 or less were taken as statistically significant.

Results

Serum estradiol and progesterone levels

The serum estradiol and progesterone levels measured for each group are shown in Table 1. After ovariectomy, both estradiol and progesterone levels in all groups decreased to unmeasurable levels (less than 10 pg/ml estradiol and less than 0.2 ng/ml progesterone). Hormonal treatment significantly increased the estradiol levels in E and EP groups, as compared with the pretreatment level of each group, and the progesterone levels in P and EP groups also increased.

Contractile responses to carbachol and KCl

The contractile responses to carbachol and KCl in female rabbit detrusor smooth muscles were not

Table 1 Serum estradiol and progesterone levels. *n* Number of rabbits obtained in each group, *significantly different from comparable values for pretreatment rabbits ($P < 0.01$)

Groups	*n*	Estradiol (pg/ml)		Progesterone (ng/ml)	
		Pretreatment	Hormonal treatment	Pretreatment	Hormonal treatment
Ox	10	13.62 ± 1.12	Less than 10	0.52 ± 0.11	Less than 0.2
E	10	14.24 ± 1.45	247.60 ± 25.65*	0.64 ± 0.07	Less than 0.2
P	10	13.68 ± 1.33	Less than 10	0.58 ± 0.11	4.98 ± 0.80*
EP	10	13.66 ± 0.96	231.20 ± 34.55*	0.58 ± 0.09	4.60 ± 0.83*

significantly different in all groups. Carbachol (10^{-8}–10^{-4} M) caused concentration-dependent contractions. The E_{max} values for the carbachol-induced contractions in Ox, E, P and EP groups were 6.01 ± 0.68 g, 6.20 ± 0.72 g, 6.24 ± 0.56 g and 6.15 ± 0.37 g, and the ED_{50} values were 0.87 ± 0.06 μM, 0.92 ± 0.09 μM, 0.85 ± 0.05 μM and 0.90 ± 0.07 μM, respectively. The 80 mM KCl-induced phasic contractions in Ox, E, P and EP groups were 4.05 ± 0.15 g, 3.82 ± 0.17 g, 4.03 ± 0.14 g and 3.91 ± 0.16 g, and the tonic contractions were 3.04 ± 0.14 g, 2.81 ± 0.16 g, 3.02 ± 0.17 g and 2.91 ± 0.17 g, respectively.

Effects of ovarian hormones on relaxation induced by various β-adrenergic receptor selective agonists

Isoproterenol (non-selective) (10^{-10}–10^{-6} M) and procaterol (β_2-selective) (10^{-10}–10^{-6} M) caused concentration-dependent relaxation of KCl-induced tonic contractions in female rabbit detrusor smooth muscles from all groups (Fig. 1). The maximum relaxation and the IC_{50} values in the presence of some drugs are shown

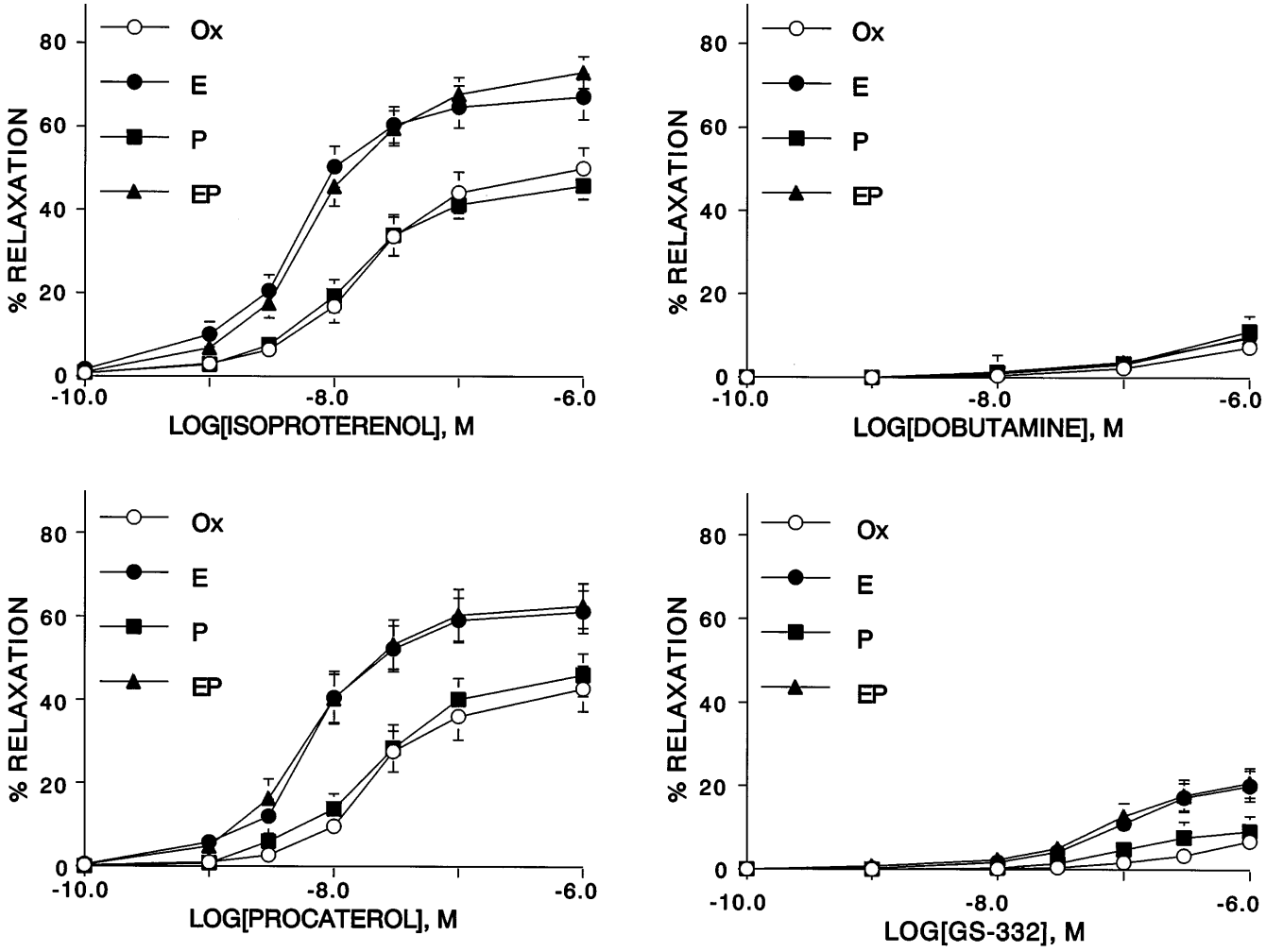

Fig. 1 Effects of ovarian hormones on relaxation induced by various β-adrenergic receptor selective agonists in female rabbit detrusor smooth muscles. For each experiment, relaxant responses are expressed as percentages of the maximum relaxant response obtained in Ca^{2+}-free solution. Each *point* represents the mean ± SEM of the results from five strips from five different rabbits in each group; if not shown, *SE bars* fall within the size of the symbols

Table 2 Maximum relaxation and IC_{50} values of various β-adrenergic receptor selective agonists for KCl (80 mM)-induced tonic contractions in female rabbit detrusor smooth muscles. Values represent means ± SEM of the results from five strips from five different rabbits in each group. *ND*, Not derived. *, Significantly different from comparable values for Ox and P groups ($P < 0.05$)

Drugs	Maximum relaxation (%)				IC_{50} (nM)			
	Ox	E	P	EP	Ox	E	P	EP
Isoproterenol (non-selective)	49.99 ± 4.96	67.17 ± 5.43*	45.82 ± 3.25	72.99 ± 3.80*	18.74 ± 3.12	5.22 ± 0.50*	16.05 ± 5.20	7.13 ± 0.77*
Dobutamine (β_1-selective)	7.32 ± 1.10	9.89 ± 2.20	11.10 ± 3.63	9.61 ± 2.54	ND	ND	ND	ND
Procaterol (β_2-selective)	42.68 ± 5.47	61.13 ± 5.13*	45.98 ± 5.13	62.55 ± 5.37*	21.75 ± 2.06	6.95 ± 0.80*	23.24 ± 4.57	6.96 ± 0.89*
GS-332 (β_3-selective)	6.79 ± 1.00	20.05 ± 3.66*	9.20 ± 3.66	20.80 ± 3.47*	ND	91.62 ± 9.41	ND	92.15 ± 28.31

in Table 2. The maximum relaxation in the presence of isoproterenol and pocaterol was significantly larger, 2nd the IC_{50} values for isoproterenol and procaterol were significantly smaller in E and EP groups than in Ox and P groups. There were no significant differences in the maximum relaxation and the IC_{50} values in the presence of isoproterenol and procaterol between E and EP groups and between Ox and P groups.

The relaxant response to dobutamine (β_1-selective) (10^{-10}–10^{-6} M) was extremely small in all groups (Fig. 1). The relaxant response to GS-332 (β_3-selective) (10^{-10}–10^{-6} M) was small in Ox and P groups. On the other hand, in E and EP groups, GS-332 caused a marked concentration-dependent relaxation on KCl-induced tonic contractions (Fig. 1). In E and EP groups, the maximum relaxation to GS-332 was about 30% of that detected for isoproterenol or procaterol.

Effects of ovarian hormones on relaxation induced by forskolin and DBcAMP

Forskolin (10^{-7}–3×10^{-5} M) and DBcAMP (10^{-4}–3×10^{-3} M) caused concentration-dependent relaxation of KCl-induced tonic contractions in female rabbit detrusor smooth muscles from all groups (Fig. 2). The maximum relaxation to forskolin and DBcAMP for different samples is shown in Table 3. The maximum relaxation to forskolin was significantly larger in E and EP groups than in Ox and P groups. There were no significant differences between E and EP groups and between Ox and P groups in maximum relaxation determined in the presence of forskolin. On the other hand, DBcAMP equally relaxed the detrusor strips from all groups.

Effects of ovarian hormones on the cAMP content

After the administration of 10^{-5} M of isoproterenol and procaterol, the cAMP content increased in female rabbit detrusor smooth muscles from all groups (Fig. 3). The increments in cAMP content 3 min after the administration of isoproterenol and procaterol in E and EP groups were significantly larger than those in Ox and P groups, and there were no significant differences in these values between E and EP groups and between Ox and P groups. Dobutamine did not significantly increase the cAMP content in all groups (Fig. 3). The cAMP content in Ox and P groups was not increased by GS-332 (10^{-5} M). On the other hand, in E and EP groups, the cAMP content significantly increased 3 min after the administration, and the values were about 30% of those where isoproterenol or procaterol (Fig. 3) were involved.

Discussion

In the present experiment, KCl-induced contractile responses were not significantly different after hormonal

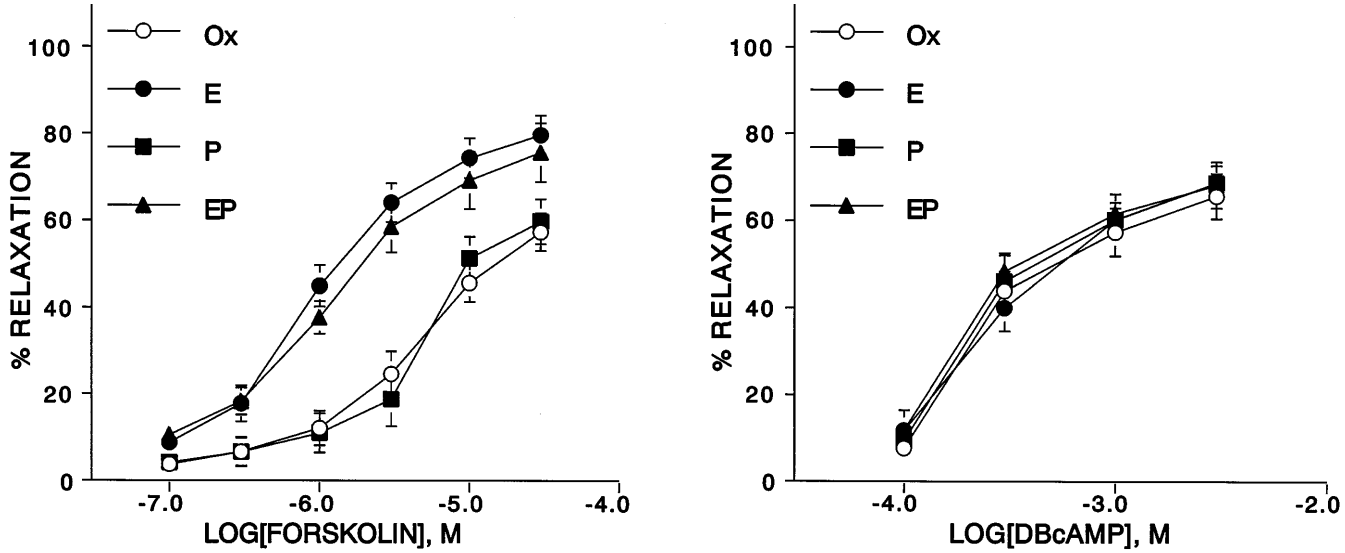

Fig. 2 Effects of ovarian hormones on relaxation induced by forskolin and DBcAMP in female rabbit detrusor smooth muscles. For each experiment, relaxant responses are expressed as percentages of the maximum relaxant response obtained in Ca^{2+}-free solution. Each *point* represents the mean ± SEM of the results from five strips from five different rabbits in each group; if not shown, *SE bars* fall within the size of the symbols

treatment in rabbit detrusor smooth muscles. This result implies that the contractility of rabbit detrusor smooth muscles to KCl were not affected by estrogen and/or progesterone replacement. This is consistent with the data reported by Levin et al. [23, 25]. Batra and Andersson [1] also reported that the contractile response to KCl did not change 1 week after the estrogen replacement in ovariectomized rabbits. Thus, in the present experiments, it is possible to compare the relaxation induced by various β-adrenergic agonists in rabbit detrusor smooth muscles precontracted with KCl among groups.

There is considerable evidence suggesting that estrogen modulates β-adrenergic receptor function in various animal tissues, including brain [29], uterus [13, 30], lung [31], heart [19], liver [49] and blood vessels [11]. However, there is little information regarding the effect of estrogen on β-adrenergic receptor function in bladder. Levin et al. [23, 25] reported that, in immature female rabbits injected with estrogen for 4 days, there is not a marked increase in isoproterenol-induced relaxant response and β-adrenergic receptor density in rabbit detrusor smooth muscles. In contrast, the present study demonstrated that estrogen replacement for 2 weeks caused a significant increase in β-adrenergic receptor responsiveness in ovariectomized rabbit detrusor smooth muscles. This discrepancy may be due to the different design, the different period of estrogen treatment and the difference in age of the animals.

β-Adrenergic receptors were initially classified into β_1- and β_2-adrenergic receptor subtypes by Lands et al. [20]. They have now been classified in pharmacological and molecular biological studies into three subtypes: β_1, β_2 and β_3 [27, 42]. The three subtypes have markedly different tissue distributions: (1) β_1-adrenergic receptor subtypes are expressed mainly in the heart, where they are the target for β-adrenergic receptor antagonists that reduce heart rate, (2) β_2-adrenergic receptor subtypes are expressed in the uterus, skeletal muscle and in the lungs where they are the target for β-adrenergic receptor agonists that induce bronchorelaxation, and (3) β_3-adrenergic receptor subtypes are expressed primarily in adipose tissue, where they are likely to regulate noradrenaline-induced changes in energy metabolism and thermogenesis [27, 42]. Furthermore, it has been known that there are species differences in β-adrenergic receptor subtypes involved in the relaxation of the bladder [15, 16, 24, 32, 44, 45]. Pharmacological studies have indicated the presence of β_2-adrenergic receptor subtypes in the rabbit bladder and demonstrated that the relaxation

Table 3 Maximum relaxation in the presence of forskolin and DBcAMP for KCl (80 mM)-induced tonic contractions in female rabbit detrusor smooth muscles. Values represent means ± SEM of the results from five strips from five different rabbits in each group. *, Significantly different from comparable values for Ox and P groups ($P < 0.05$)

Drugs	Maximum relaxation (%)			
	Ox	E	P	EP
Forskolin	57.30 ± 4.35	79.61 ± 4.49*	59.71 ± 5.16	75.61 ± 6.76*
DBcAMP	65.68 ± 5.19	68.75 ± 2.97	68.68 ± 3.99	68.24 ± 5.30

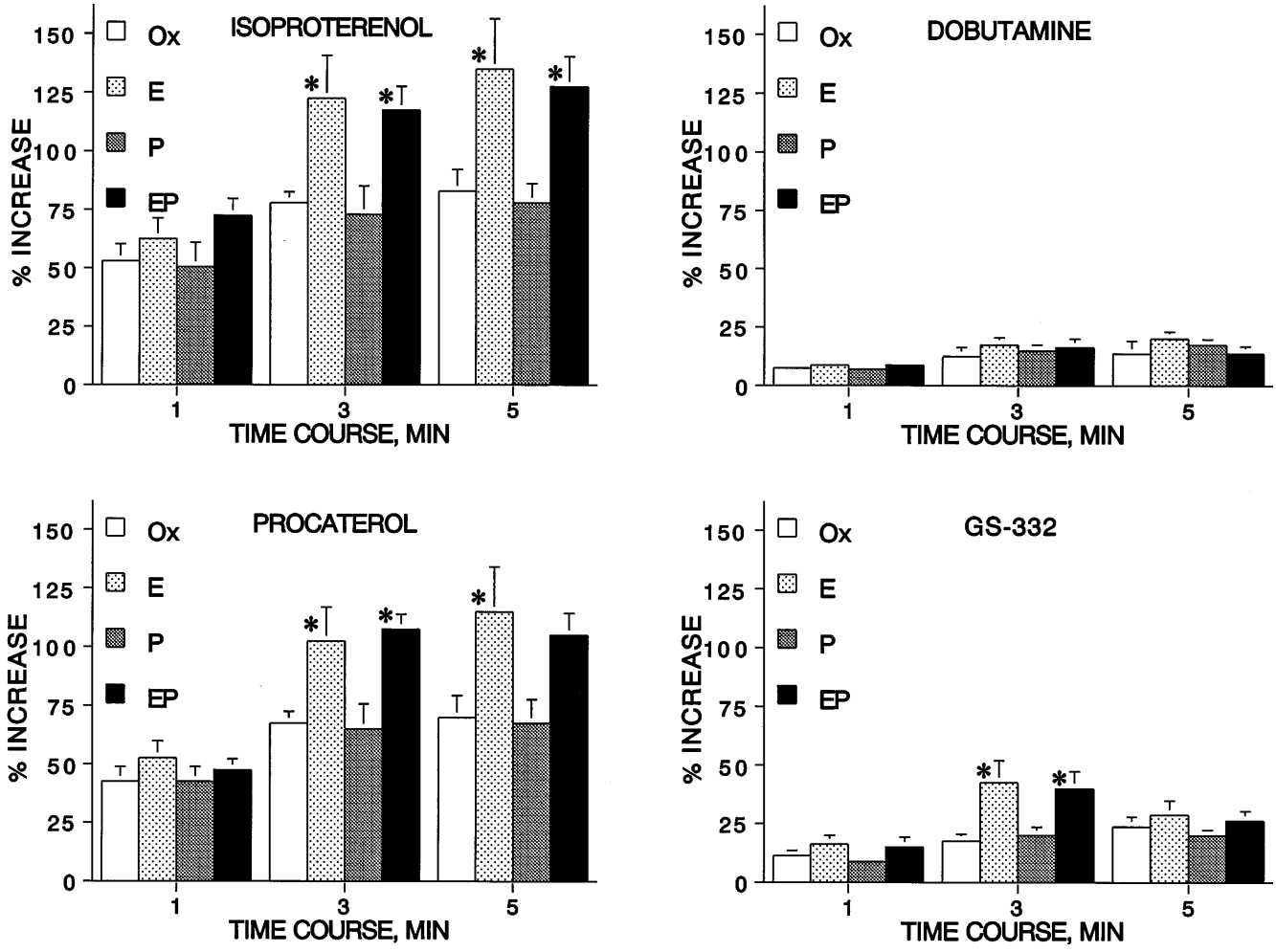

Fig. 3 Effects of ovarian hormones on the cAMP content increased by various β-adrenergic receptor selective agonists in female rabbit detrusor smooth muscles. The cAMP content was measured before and 1, 3 and 5 min after the administration of each agonist in all groups. For each experiment, the increased content is expressed as a percentage of the content measured before the administration of each agonist. Each *bar* represents the mean ± SEM of the results from five strips from five different rabbits in each group; if not shown, *SE bars* fall within the size of the symbols. *Significantly different from comparable values for Ox and P groups ($P < 0.05$)

of the rabbit bladder as a result of adrenergic stimulation is mediated mainly by β_2-adrenergic receptor subtypes [24, 32]. In contrast, β_3-adrenergic receptor subtypes have been found mainly in the human bladder [15, 16, 44, 45]. In the present study, isoproterenol (non-selective agonist) and procaterol (β_2-adrenergic receptor selective agonist) significantly relaxed the detrusor strips from all groups as compared with dobutamine (β_1-adrenergic receptor selective agonist) and GS-332 (β_3-adrenergic receptor selective agonist). Although dobutamine did not cause any relaxant response in rabbit detrusor smooth muscles precontracted with KCl in all groups, GS-332 produced relaxant responses approaching 30% of the values determined for isoproterenol or procaterol in E and EP groups. These results suggest that β_2- and β_3- adrenergic receptor subtypes exist in rabbit detrusor smooth muscles, and that the relaxant responses induced by β-adrenergic agonists are mediated mainly by β_2-adrenergic receptor subtypes. Furthermore, after estrogen replacement, β_3-adrenergic receptor subtypes may have a partial contribution to the relaxation. In the present experiment, the fact that estrogen replacement increased equally isoproterenol-, procaterol- or GS-332- induced responses suggest that the mechanism contributing to the increased relaxant response may be the same for each β-adrenergic agonist.

In the present experiment, estrogen replacement caused significant increases in cAMP content induced by isoproterenol, procaterol or GS-332. The binding of β-adrenergic agonist with β-adrenergic receptors results in the binding of GTP to G_s which, in turn, activates the catalytic component of adenylyl cyclase and results in the conversion of ATP to cAMP [39]. To determine which components of the β-adrenergic receptor-adenylyl cyclase system were altered with ovarian hormones, we studied the effects of ovarian hormones on (1) the relaxant response to forskolin, which increases cAMP content by interaction at the catalytic unit of adenylyl cyclase, and (2) the relaxant response to DBcAMP, which is a cell permeable cAMP analogue. The relaxant

response to DBcAMP in KCl-induced tonic contractions were similar in all groups. Thus, the increased effects of the response to isoproterenol, procaterol or GS-332 in E and EP groups do not appear to involve events distal to cAMP synthesis in rabbit detrusor smooth muscles. The effects of estrogen on β-adrenergic agonist-induced relaxation of rabbit detrusor smooth muscles may be related also to differences in cAMP synthesis, which may be modified by changes in G_s or in the catalytic unit of adenylyl cyclase. The relaxant response to forskolin, which has been shown to relax rabbit detrusor smooth muscles through activation of the catalytic unit of adenylyl cyclase [33], significantly increased in E and EP groups. On the basis of above findings, it is suggested that the cAMP content increased by estrogen replacement is in part related to change in the biochemical property of the catalytic unit of adenylyl cyclase in rabbit detrusor smooth muscles.

In the present experiment, progesterone did not have significant effects on β-adrenergic receptor responsiveness in rabbit detrusor smooth muscles. Progesterone produces a relaxation of the uterus smooth muscles by inducing β-adrenergic receptor formation [6]. Raz et al. [36, 37] have also shown that progesterone facilitates β-adrenergic receptor responsiveness in the female rat ureter and pregnant dog urethra. In contrast, the antagonistic effect of progesterone in estrogen-induced responses has been demonstrated in several reports, in which progesterone treatment caused a reduction in estrogen receptors [3, 12, 22], while Batra and Iosif [4] have shown that the number of estrogen receptors in the female rabbit bladder and urethra is not affected by progesterone treatment. With regard to cholinergic effects of progesterone, Levin et al. [26] suggested that progesterone may have an anticholinergic effect on the pregnant rabbit bladder. In the present experiment, carbachol-induced contractions were not significantly different in all groups, suggesting that the anticholinergic effects of progesterone may be ruled out. The absence of a response to progesterone in this experiment may be due to the experimental animal or design. However, we could not explain this fully. Thus, further studies are needed to elucidate the effects of progesterone on the lower urinary tract. Since progesterone reduces estrogen-induced endometrial proliferation [47], a combination of progesterone with estrogen would seem to be the recommended therapy.

In conclusion, the present study has demonstrated that estrogen treatment caused increased relaxant responses mediated by β_2- and β_3-adrenergic receptor subtypes, which might be related to the increased cAMP content induced by change in the biochemical property of the catalytic unit of adenylyl cyclase in female rabbit detrusor smooth muscles. On the other hand, progesterone treatment did not affect β-adrenergic receptor responsiveness in female rabbit detrusor smooth muscles. These results may support the usefulness of estrogen for the therapy of urinary incontinence in postmenopausal women.

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