Prompt Effect of Progesterone on the Adrenergic Response of Smooth Muscles

Shigeru MORISHITA
Department of Pharmacology, Kawasaki Medical School,
Kurashiki, Okayama 701-01, Japan

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Abstract—1) The contractile effects of epinephrine on the uterus and ductus deferens of the rabbit and the ductus deferens of the monkey were inhibited by the preincubation with progesterone (6.4×10⁻⁵ M) for 1 or 3 min in Locke-Ringer solution. Epinephrine relaxed the guinea pig uterus and taenia caecum. The relaxant effects were enhanced by preincubation with progesterone. Their effects were in a dose-dependent manner. 2) There was no apparent change in the number and affinity of alpha-adrenergic receptors in the uterus of rabbits and the ductus deferens of guinea pigs during the incubation with progesterone. Progesterone has no direct effect on alpha-adrenergic receptors. 3) All smooth muscles yielded reproducible contractile reactions to Ca²⁺ when maintained in depolarizing Tyrode's solution containing K⁺ (40 mmol/l). Their concentration-response curves were inhibited by preincubation with progesterone (6.4×10⁻⁵ M), and they were shifted to the right in a concentration-dependent manner. Established Ca²⁺-induced contractions were rapidly relaxed by the addition of progesterone (6.4×10⁻⁵ M). 4) It suggests that progesterone directly affects the plasma membrane and inhibits the voltage-dependent Ca²⁺ channel and then inhibits smooth muscle contraction.

The hypothesis of a universal mechanism of steroid hormone action has been proposed by Thompson and Lippman (1). Although steroids have a classical receptor-mediated pathway of hormonal action, it is not sufficient to account for all the known effects of steroids. For example, Baulieu and co-workers (2) have demonstrated that progesterone and other steroid molecules can promote the maturation of Xenopus laevis oocytes, although these cells do not contain steroid receptors. Kaya and Saito (3) have recently demonstrated that steroids have a direct, non-genomic effect on the erythrocyte membrane. Therefore, it seems that, in addition to the receptor-mediated mechanism, steroids may also operate through other mechanisms and, in particular, through an effect on the plasma membrane. In the present paper, I examined the direct, non-genomic effects of progesterone on the plasma membrane with the smooth muscle of the guinea pig, rabbit and monkey in relation to the adrenergic mechanism and the Ca²⁺ channel.

Materials and Methods

Materials: The following drugs were used: 4-Pregnene-3,20-dione (progesterone), Sigma Chemical Co.; epinephrine, Daiichi Chemical Co.; phentolamine mesylate (phen tolamine), Ciba Gaigy; dl-propranolol hydrochloride (propranolol), Wako Pure Chemical Ind.; ³H-dihydroergocryptine and ACSII scintillation mixture, Amersham; All other chemicals were purchased from Wako Pure Chemical Ind. Progesterone (1 mg) dissolved in propylene glycol (0.1 ml) was used in the Magnus method. Progesterone dissolved in 2% ethanol was used in the receptor assay. Deionized distilled water was used in making up other solutions. Propylene glycol up to 0.1–0.3 ml had no effect on the excitability of smooth muscles. The 2% ethanol used as a vehicle had no effect in the receptor assay.
Methods: The uterus, ductus deferens and taenia caecum (2–3 cm) were dissected from Hartley guinea pig, New Zealand White rabbit, Flemish giant rabbit and Japanese stump-tailed macaque Macaca fuscata. Organs were cleaned of surrounding connective tissue, vessels and fat tissue and set up in a 50 ml Magnus organ bath containing modified Locke-Ringer solution or K⁺-depolarizing Tyrode’s solution maintained at 37°C and gassed with air. The composition of the modified Locke-Ringer solution (Locke-Ringer solution) was NaCl, 154; KCl, 15.6; CaCl₂, 2.2; MgCl₂, 0.98; NaHCO₃, 11.9; NaH₂PO₄, 0.4; glucose, 5.5 mM; pH 7.1. K⁺-depolarizing Tyrode’s solution (K⁺-Tyrode’s solution) was NaCl, 97; KCl, 40; NaHCO₃, 11.9; NaH₂PO₄, 0.4; glucose, 5.5 mM; pH 7.1. Contractile and relaxant responses were measured under isotonic conditions (1 g) using a Nihon Kohden isotonic transducer connected to an EGB36069 recorder. When maintained in Locke-Ringer solution, cumulative concentration-response curves were obtained to epinephrine (0.1–100 μg/50 ml) by increasing the concentration in logarithmic increments. To observe the effect of progesterone on the Ca²⁺ channel, K⁺-Tyrode’s solution was used. When maintained in K⁺-Tyrode’s solution, cumulative concentration-response curves were obtained to CaCl₂ (0.03–30 mM) by increasing the Ca²⁺ concentration in logarithmic increments. The 100% response was taken as the maximum response (contraction or relaxant) at the higher epinephrine concentration, and each response was taken at 1 or 3 min after the addition of epinephrine to the bath. Progesterone 1 mg/50 ml (6.4×10⁻⁵ M) was added to the bath at 1 min before the application of catecholamine or CaCl₂, and concentration-response curves of catecholamine or CaCl₂ were measured. The time required for relaxation of the Ca²⁺-contracted muscle after the addition of progesterone was compared with the control in which the relaxant time was measured after the wash-out. The bath fluid was exchanged each time for fresh solution, and the organs were then returned passively to their resting tension. Epinephrine was dissolved each time and used immediately.

Receptor assay: The assay was done according to the method of Williams and co-workers (4).

Radioligand: ³H-Dihydroergocryptine was chosen as the α-adrenergic radioligand for this study. The reason for using ³H-dihydroergocryptine to identify α-adrenergic receptors was the high potency of dihydroergocryptine as an α-adrenergic antagonist. Its apparent affinity for α-adrenergic receptors as determined from pharmacological studies is greater than that of any other ergot alkaloid (4).

Membrane preparation: Uterus was obtained from rabbit, and ductus deferens was obtained from guinea pig. The tissues were removed and cleaned of surrounding connective tissues in ice cold buffer (0.25 M sucrose, 1 mM MgCl₂, 5 mM Tris-HCl, pH 7.4). The tissues were homogenized in 4–5 volumes of the same buffer using a Polytron PT-10 homogenizer at setting 6 for four 10-s periods at 0°C. After filtration through a single layer of gauze, the homogenate was centrifuged at 400×g for 10 min at 4°C, and the pellet was discarded. The supernatant was centrifuged at 28000×g for 10 min at 4°C. The resulting pellet was suspended in the incubation buffer (10 mM MgCl₂, 50 mM Tris-HCl, pH 7.5) for use in the binding assay.

Binding assay: ³H-Dihydroergocryptin (³H-DHE), 0.015 ml (specific activity 15.1 Ci/mmol) and the membrane preparation, 0.1 ml (3 mg protein/ml) were incubated together for 15 min at 25°C with shaking in a total volume 0.15 ml. Incubations were terminated by diluting the 0.15 ml incubation mixtures with 2.5 ml of incubation buffer (25°C) followed by a rapid filtration through a TOYO GC50 glass fiber filter. Filters were rapidly washed with 20 ml of incubation buffer (25°C). After drying, the filters were removed to vials. Five ml of ACS II (Amersham) scintillation mixture were added to each vial, and tritium was assayed by measuring the radioactivity of each sample for 10 min in a liquid scintillation spectrometer (LSC-900). Counting efficiencies were 40%. Nonspecific binding is defined as binding under 0.01 mM phentolamine.
Specific binding is defined as the total radioactivity bound minus the nonspecific binding. Membrane preparations were assayed with progesterone (1.31 x 10^{-5} M) in 2% ethanol, and then the binding sites and affinity (K_d) compared with the control. Progesterone of more than 1.31 x 10^{-5} M could not be dissolved in 2% ethanol. The protein content of the final suspension was determined by the method of Lowry et al. (5). Student’s t-test was used for comparison of the mean values. The data from each experiment were analyzed by a Scatchard plot. (Fig. 1).

**Results**

**Contractile effect of epinephrine in Locke-Ringer solution:** The uterus (rabbit) and the ductus deferens (rabbit, monkey and guinea pig) were contracted by epinephrine in Locke-Ringer solution. The uterus and taenia caecum from guinea pig were relaxed. Those concentration-response curves were reproducible and dose-dependent. (Fig. 2) These responses did not change under 0.01 mM propranolol, but were greatly reduced by 0.01 mM phentolamine.

The effects of progesterone on the contractile or relaxant effects of epinephrine:

Progesterone (6.4 x 10^{-6} M) added to the bath 1 or 3 min before the application of epinephrine inhibited the contractile effect of epinephrine on the rabbit uterus and the ductus deferens of rabbit and monkey. The dose-response curves were shifted to the right (Fig. 2 a, b, c). However, progesterone enhanced the relaxant effect of epinephrine on the uterus and taenia caecum of guinea pig. In these cases, the dose-response curves were shifted to the left (Fig. 2 d, e). Progesterone enhanced the contractile effect of epinephrine on the ductus deferens of guinea pig. The dose-response curves were shifted...
Table 1. Effects of progesterone on alpha-adrenergic receptors of smooth muscles

|                    | Rabbit uterus       | Guinea pig ductus deferens |
|--------------------|---------------------|-----------------------------|
|                    | Control             | Progesterone 1.3x10^-5 M    | Control                      | Progesterone 1.3x10^-5 M |
| Binding sites (fmol/mg prot.) | 571.2±40.8          | 583.1±79.3                  | 265.8±39.2                  | 208.0±91.8 |
| K_i (nM)           | 15.5±2.9            | 13.4±3.8                    | 17.4±5.4                    | 17.3±8.9 |

Progesterone (1.3x10^-5 M) did not affect the both binding sites and affinity (K_i) of progesterone receptors in rabbit uterus and guinea pig ductus deferens.

to the left (Fig. 2 f).

Alpha-adrenergic receptor assay: The binding sites (fmol/mg protein) in rabbit uterine membranes were 583.1±79.3 with progesterone and 571.2±40.8 in the control. K_i was 13.4±3.8 with progesterone, and 15.5±2.9 in the control. The binding sites with progesterone in guinea pig ductus deferens were 208.0±91.8, and the control value was 265.8±39.2. K_i was 17.3±8.9 with progesterone, and it was 17.4±5.4 in the control. There was no difference between the incubation with progesterone and the control (Table 1).

Contractile effects of Ca^{2+} in K^+-Tyrode’s solution: All smooth muscles in Ca^{2+}-free, K^+-Tyrode’s solution were contracted by CaCl_2. Their contraction-response curves were reproducible and dose-dependent. (Fig. 3).

The effect of progesterone on the contractile effects of Ca^{2+} in K^+-Tyrode’s solution: Progesterone (6.4x10^-5 M) added to the bath 1 or 3 min before the application of Ca^{2+} caused the inhibition of the contractile effect of Ca^{2+} on all smooth muscles. (Fig. 3 a—f). The dose-response curves were shifted to the right in a concentration-dependent manner by progesterone. (Fig. 4)

Relaxant effects of progesterone: Ca^{2+}-induced contraction of smooth muscles maintained in control relaxation of K^+-Tyrode’s solution was relaxed by the exchange of the bath solution with a Ca^{2+}-free solution. When progesterone was added to the bath during the period when the tissues were being contracted with Ca^{2+}, progesterone relaxed the smooth muscles rapidly. However, these relaxation curves were not significantly different from the relaxation curves after the washout of Ca^{2+} (Fig. 5).

Discussion

Uterine smooth muscle has been known to be profoundly influenced by ovarian steroids
Fig. 4. Effects of progesterone on cumulative concentration response curves of smooth muscle with Ca²⁺ in K⁺ (40 mmol/l)-Tyrode’s solution. The contraction of smooth muscle with Ca²⁺ was inhibited in a concentration-dependent manner with progesterone.

Fig. 5. The relaxant effects of progesterone on established Ca²⁺-induced contractions of K⁺ depolarized smooth muscles. The control relaxation (□) was obtained by exchange the Ca²⁺ solution with Ca²⁺-free solution during established Ca²⁺-induced contractions of smooth muscles maintained in K⁺ (40 mmol/l)-Tyrode’s solution. Progesterone (6.4×10⁻⁵ M) (▲) immediately relaxed the established Ca²⁺-induced contractions of smooth muscles maintained in K⁺-Tyrode’s solution. This relaxation was not significantly different from the control relaxation, and significantly different from the established Ca²⁺-induced contraction (■).

It is known that not only uterine smooth muscle but also the smooth muscle of blood vessels from rats (8). Smooth muscle of the guinea pig ileum (9) and airway smooth muscle of the guinea pig (10) exhibit changes in contractile activity in response to steroids. The mechanism responsible for the effects of these steroids, however, remains largely unknown. Among the diverse effects of steroids, the great majority correspond to receptor-mediated events. It appears, however, that some of these effects of steroids may represent direct non-genomic effects (2, 3).

In the present paper, we studied the non-genomic effects of steroids, in particular those of progesterone, on smooth muscles. In Locke-Ringer solution, rabbit uterus, rabbit ductus deferens, monkey ductus deferens and guinea pig ductus deferens were contracted by epinephrine added to the organ bath. This mechanism is thought to be through the alpha-adrenergic receptor. These effects are dose-dependent. However, at 1 or 3 min after the addition of progesterone to the organ bath, epinephrine depressed the contractile-response in a competitive manner. Because of the promptness of this effect, it should not be caused by genomic effects through a receptor-mediated mechanism (Fig. 2). On the other hand, the contraction of guinea pig ductus deferens was enhanced by progesterone. It seems, however, that this is also evidence for non-genomic effects because this effect was produced in a very short time, and it disappeared promptly by the washout with steroid-free medium. However, progesterone has antipodal effects on smooth muscle, enhanced or depressed contraction. Therefore, it is difficult to explain that the depressing effect is merely through competitive antagonism.

Although Brink and co-workers (10) showed that glucocorticoids were able to enhance the contraction of guinea pig airway smooth muscle by histamine and barium in
vitro, they did not explain the mechanism for this effect. In an in vitro experiment with guinea pig uterus and taenia caecum, we demonstrated that progesterone rapidly enhanced the relaxant activity of epinephrine. This is also evidence for the non-genomic effect of progesterone because of the prompt effect. There was no apparent change in the number and affinity of alpha-adrenergic receptors for $^3$H-DHE on rabbit uterus and guinea pig ductus deferens during the incubation with progesterone. Progesterone has no direct effect on alpha-adrenergic receptors (Table 1).

Ca$^{2+}$-free, Mg$^{2+}$-free, bicarbonate-buffered K$^+$-Tyrode's solution made the taenia preparations more sensitive to Ca$^{2+}$ than the regular Tyrode's solution (Durbin and Jenkinson (11), Ferrarin and Capendo (12), Spedding and Weetman (13)). In this paper, all smooth muscles yielded reproducible contractile-response curves to Ca$^{2+}$ when maintained in depolarizing Tyrode's solution containing K$^+$ (Fig. 3). The contractile mechanism of smooth muscles was profoundly influenced by Ca$^{2+}$. Ca$^{2+}$ enters into the cell through Ca$^{2+}$ channels. Two Ca$^{2+}$ channels are proposed for muscle contraction: One is the voltage dependent channel that is activated by electric stimulations, and the other is the receptor-operated channel that is activated by chemical stimulations (14-17). There are two types of receptor-operated channel: One is the depolarizing receptor-operated channel, and the other is the non-depolarizing receptor operated channel (15, 16). Non-depolarizing receptor-operated channels are classified as phospholipid methylations (18), which are mediated by phosphatidylinositol response (19, 20) and others (21, 22). It seems that all smooth muscles have at least a voltage dependent channel because their responses were dependent on the Ca$^{2+}$ concentration in K$^+$-Tyrode's solution.

Spedding (23) and Godfraind (24) have tried to clarify the characters of Ca$^{2+}$ entry blockers using depolarized smooth muscles. According to them, the inhibitory effects of progesterone on Ca$^{2+}$-induced contraction are considered to be through the inhibition of the voltage-dependent channel, as is the case with the Ca$^{2+}$ entry blockers. To clarify the mechanism of Ca$^{2+}$ entry blockers, Spedding (23, 25) compared the relaxing times of Ca$^{2+}$-induced contracted muscles with various Ca$^{2+}$ entry blockers, and nifedipine, verapamil and diltiazem were found to relax them at once. Diphenylalkylamine, which resembles the calmodulin inhibitors, relaxed them after more than one hour. As progesterone behaves similarly to nifedipine, verapamil and diltiazem (Fig. 5), its effect on the plasma membrane may be similar to those of nifedipine and others. Progesterone has a prompt effect on the erythrocyte membrane (3).

The effects of catecholamine through alpha-adrenergic receptor are various. Guinea pig taenia caecum has constant tension in Locke-Ringer solution. In this case, it can explain how progesterone inhibits the voltage dependent channel. However, the contraction of guinea pig ductus deferens caused by epinephrine is enhanced by progesterone, and this can not be explained by inhibition of the voltage dependent channel. Rat ductus deferens that has a phosphatidylinositol response (26, 27) yielded a good concentration response curve to epinephrine. The concentration-response curve is shifted to the left by progesterone. The receptor operated channel of the phosphatidylinositol response is not confirmed on smooth muscles of which the contractions are inhibited. From these facts, the stimulating effect of progesterone may be due to a difference in the characteristics of the Ca$^{2+}$ channel. Either way, it is considered that the direct effect of progesterone on smooth muscles differ from species to species and from organs to organs. Although human erythrocyte membrane was influenced the most by progesterone (3) among several other steroids, the central nervous cell membrane of the mouse was affected most effectively by corticosterone (H. Kaya et al., unpublished data). This might be due to a difference in the constituents of the cell membrane.

It is conceivable that the effect of progesterone on the contractility of smooth muscles in various species and organs extended by catecholamine is not through the
progesterone receptors, but due to direct effects on the plasma membrane. Among them, the inhibition of the voltage dependent Ca^{2+} channel might be suspected.

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References
1 Thompson, E.B. and Lipman, M.E.: Progress in endocrinology and metabolism mechanism of action of glucocorticoid. Metabolism 23, 159–202 (1974)
2 Baulieu, E.E., Godeau, F., Schorderet, M. and Slatkine, S.S.: Steroid-induced meiotic division in Xenopus laevis oocytes: surface and calcium. Nature 275, 583–588 (1978)
3 Kaya, H. and Saito, T.: Effect of progesterone and its 17-alpha-hydroxyl derivative on human erythrocyte membrane. Japan. J. Pharmacol. 39, 299–306 (1985)
4 Williams, L.T., Mullikin, D. and Lefkowitz, R.J.: Identification of alpha-adrenergic receptor in uterine smooth muscle membranes by 3H-dihydroergocryptine binding. J. Biol. Chem. 251, 6915–6923 (1976)
5 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275 (1951)
6 Batra, S. and Bengtsson, B.: Effects of diethylstilbestrol and ovarian steroids on the contractile responses and calcium movements in rat uterine smooth muscle. J. Physiol. (Lond.) 276, 329–342 (1978)
7 Kubli-Garfias, C., Medrano-Conde, L., Beyer, C. and Bondani, A.: In vitro inhibitor of rat uterine contractility induced by 5-alpha and 5-beta progesterins. Steroids 34, 609–619 (1980)
8 McCalden, T.A.: The inhibitory action of oestradiol-17-beta and progesterone on venous smooth muscle. Br. J. Pharmacol. 53, 183–192 (1975)
9 Seaman, I., Fontaine, J., Famaey, J.P. and Reuse, J.: The inhibitory effect of steroidal sex hormone on the responses of the guinea pig isolated ileum to nicotine and serotonin. Arch. Int. Pharmacodyn. Ther. 230, 340–343 (1977)
10 Brink, C., Ridgway, P. and Douglas, J.: Modification of airway smooth muscle responses in the guinea pig by hydrocortisone, in vitro and in vivo. J. Pharmacol, Exp. Ther. 203, 1–11 (1977)
11 Durbin, R.P. and Jenkinson, D.H.: The calcium dependence of tension development in depolarized smooth muscle. J. Physiol. (Lond.) 157, 80–96 (1961)
12 Ferrari, M. and Carpenido, F.: On the mechanism of action of some myolytic agents on depolarized guinea pig taenia coli. Arch. Int. Pharmacodyn. Ther. 174, 223–232 (1968)
13 Spedding, M. and Weetman, D.F.: The mechanism of the relaxant effect of 2-3 pyridylisogen on the isolated taenia of the guinea pig caecum. Br. J. Pharmacol. 63, 659–664 (1978)
14 Nonomura, Y.: Regulation of contraction in smooth muscle. Protein, Nucleic Acid and Enzyme 28, 340–350 (1983)
15 Okada, Y., Yada, T., Ueda, S. and Oiki, S.: Role of intracellular Ca^{2+} in cellular functions. Metabolism and Disease 20, 439–447 (1983) (in Japanese)
16 Kitamura, K. and Kuriyama, H.: Calcium channels: its properties and functions. Metabolism and Disease 22, 307–316 (1985) (in Japanese)
17 Yamamoto, C.: Voltage-dependent calcium channel. Metabolism and Disease 22, 317–326 (1985) (in Japanese)
18 IWATA, M. and HIRATA, F.: Biochemical mechanism of signal transduction. Protein, Nucleic Acid and Enzyme 26, 858–867 (1981)
19 Kajikawa, N., Nishiyama, K. and Kishimoto, A.: Transmembrane control and protein phosphorylation. Metabolism and Disease 18, 1117–1125 (1981) (in Japanese)
20 Takenawa, T.: Hormone receptor and stimulated turnover of inositol phospholipid: role of PI response in the enhancement of Ca^{2+} influx. Protein, Nucleic Acid and Enzyme 27, 1291–1306 (1982)
21 Suematu, E., Kanmura, Y., Itoh, T. and Kuriyama, H.: Alpha-adrenoceptor. Protein, Nucleic Acid and Enzyme 29, 1338–1352 (1984)
22 Fukui, H. and Wada, H.: Receptors for calcium mobilizing ligands and receptor operated calcium channel. Metabolism and Disease 22, 327–335 (1985) (in Japanese)
23 Spedding, M.: Assessment of "Ca^{2+} antagonist" effect of drug in K+ depolarized smooth muscle. Arch. Pharmacol. 318, 234–240 (1982)
24 Godfraind, T.: Mechanisms of action of calcium entry blockers. Fed. Proc. 40, 2866–2871 (1981)
25 Spedding, M.: Calcium antagonist subgroups. TIPS 6, 109–114 (1986)
26 de Scarnati, O.C. and Lapetina, E.G.: Adrenergic stimulation of phosphatidylinositol labelling in rat vas deferens. Biochim. Biophys. Acta 360, 288–305 (1974)
27 Jones, L.M., Cockerot, S and Michell R.H
Stimulation of phosphatidylinositol turnover in various tissues by cholinergic and adrenergic agonists, by histamine and by caerulein.
Biochem. J. 182, 869–876 (1979)