Abstract—Properties of [3H]nitrendipine binding, high K+- and Ca++-induced contractions and the inhibition of high K+-induced contractions by verapamil were investigated in the uterine preparations isolated from rats treated with estrogen or progesterone or both. In [3H]nitrendipine binding experiments using crude membrane fractions, treatment with estrogen alone or estrogen + progesterone significantly lowered the K_d; there was very little change in the B_max. In the Ca++-depleted, high K+-containing medium, only the progesterone-, and estrogen + progesterone-treated uteri produced contractions. The estrogen-, estrogen + progesterone-, and estrogen + progesterone-treated uteri showed decreases in concentrations of Ca++ required for the maximal contractions. In the estrogen- and estrogen + progesterone-treated uteri, the dose-response curves by verapamil were shifted to the left in a parallel manner. These findings suggest that estrogen appeared to increase the affinity of calcium channels and increase transmembrane influx of Ca++, leading to enhancement of contractions, whereas progesterone might increase the Ca++ storage in the intracellular sites.
to relax smooth muscles (12, 13).

The binding properties of a calcium channel antagonist, \[^{3}H\]nitrendipine, a derivative of 1,4-dihydropyridine, has been investigated in homogenates of the guinea-pig and rat cerebral cortex, heart and ileum (14, 15), and \[^{3}H\]nitrendipine has been used as a chemical probe for chemical (16, 17) and biological (18, 19) characterization of the calcium channel.

The present experiments using isolated rat uterus preparations were carried out to investigate the changes in the properties of calcium channels and mobilization of Ca\(^{2+}\) during mechanical reaction of the uterus as well as to investigate the effect of estrogen and progesterone on the \[^{3}H\]nitrendipine binding, on the high K\(^{+}\)- and Ca\(^{2+}\)-induced contractions, and on the relaxation by verapamil, an organic calcium channel antagonist.

Materials and Methods

**Animals:** Female Wistar rats at diestrus or metestrus, determined by microscopic observation of vaginal smears, weighing 180–200 g were anesthetized with pentobarbitone (30 mg/kg, i.p.) and then bilaterally ovariec-
tomized through a ventral route. Then the rats were maintained under ordinary environmental conditions for 21 days and were divided into 5 groups. The control group consisted of ovariectomized rats without hormonal treatment. The remaining 4 groups of rats were subcutaneously treated with estradiol benzoate or progesterone dissolved in sesame oil in a dose of 250 \(\mu\)g/kg or 20 mg/kg, respectively. In one of these groups, estradiol benzoate was given daily for 4 days on day 17–20 (Estrogen group). In a second group, progesterone was given daily for 4 days on day 17–20 (Progesterone group). In a third group, estrogen was given daily for 4 days on day 13–16, followed by progesterone which was given daily for another 4 days on day 17–20 (Estrogen→Progest-
erone group, as a model of the luteal phase). In the last group, estrogen and progesterone were simultaneously given daily for 4 days on day 17–20 (Estrogen+Progesterone group, as a model of pregnancy).

The animals were killed on day 21 by a blow on the occipital region followed by bleeding from the carotid artery, and the uteri were quickly removed and dissected free of adhering fat and connective tissue.

**Assay of \[^{3}H\]nitrendipine binding:** The uteri were homogenized in a Polytron P-10 by three 10-sec bursts in 10 vol. of 50 mM Tris-HCl buffer solution (pH 7.4) in ice water, transferred to a glass homogenizer, and further homogenized. The homogenates were filtered through double cheese cloth layers and then centrifuged at 105,000 \(\times g\) for 60 min. After the supernatants were decanted, the pellets (crude membrane fractions) were resuspended in 50 mM Tris-HCl buffer solution (pH 7.4) to obtain a final protein concentration of 150 \(\mu\)g/2 ml and were used for binding assay. The binding assay procedure was as follows: A two-ml aliquot of the thus prepared suspension was incubated with 20 to 800 pM \[^{3}H\]nitrendipine in the presence or absence of 1 \(\mu\)M nifedipine in triplicate for 90 min at 25°C. The mixture was filtered under a vacuum through a Whatman GF/C glass fiber filter and washed with three 3 ml aliquots of 50 mM Tris-HCl buffer solution (pH 7.4). The trapped radioactivity was measured by liquid scintillation spectro-
photometry (Packard, 300C type). The properties of \[^{3}H\]nitrendipine binding were analyzed by the method of Scatchard (20).

**Measurement of mechanical reactivity:** The ovarian side (1.5 cm in length, 20 mg in weight) of the horn of the excised uterus was suspended in an organ bath (50 ml) where Tyrode's solution (composition of the nutrient solution (mM): NaCl, 137; KCl, 2.7; CaCl\(_2\), 1.8; MgCl\(_2\), 1.1; NaH\(_2\)PO\(_4\), 0.4; NaHCO\(_3\), 12; and dextrose, 5.6) was kept at 37±0.5°C and bubbled with a 95% O\(_2\)-5% CO\(_2\) mixture gas. The response of preparations was recorded isometrically on an ink-writing oscillograph (RJG-4002, Nihon Kohden Kogyo Co., Japan). The initial tension applied to the preparation was adjusted to 0.3 g.

**Studies on high K\(^{+}\)-induced contractions:** High K\(^{+}\)-induced contractions were elicited by exposure of the uterine strip to 25 mM KCI-added Tyrode's solution or Ca\(^{2+}\)-depleted Tyrode's solution for 5 min after the uterine strip was suspended in Ca\(^{2+}\)-depleted, 1 mM EGTA-containing Tyrode's solution for 10
min. The concentration of KCI was decided on the basis of Gabella’s report (21).

Studies on Ca**+-induced contractions: Ca**+-induced contractions were elicited by cumulative addition of CaCl₂ (1x10⁻⁵ to 3x10⁻² M) to Ca**+-depleted, 27.7 mM K⁺-containing Tyrode’s solution, for 5 min after the uterine strip was suspended in Ca**+-depleted, 1 mM EGTA-containing Tyrode’s solution for 10 min. CaCl₂ was added directly to the bathing fluid (12, 22). Maximum contractions of the individual preparations were taken as 100%.

Studies on relaxation by verapamil: High K⁺-induced contraction was elicited by addition of 25 mM KCl to normal Tyrode’s solution. When the contraction reached its steady state, verapamil (1x10⁻⁸ to 1x10⁻⁵ M) was added cumulatively to the bathing fluid, and then 1x10⁻⁴ M papaverine was added directly to the bathing fluid to fully relax the preparation (21). When the K⁺-contracted preparation was relaxed by varapamil to this lowest level of tension, the degree of inhibition of contraction was expressed as 100% (23).

Drugs: Verapamil was supplied by Eisai Co., Ltd., Osaka, Japan; nifedipine by Bayer Yakuhin Co., Ltd., Osaka, Japan; [³H]nitrendipine by Japan Radioisotope Association (78.4 Ci/mmol, New England Nuclear), Tokyo; ethyleneglycol-bis (β-aminoethyl ether)-N,N',N''-tetraacetic acid (EGTA), papaverine hydrochloride, estradiol benzoate and progesterone by Sigma Chemical Co., U.S.A.; and pentobarbitone sodium by Abbott Laboratories, U.S.A. All other chemicals were of reagent grade.

Other methods: Protein was measured by the method of Lowry et al. (24), using bovine serum albumin as a standard. The results were expressed as the mean±standard error for each group. Significance of the difference was examined by Student’s t-test.

Results

Effects on [³H]nitrendipine binding: The specific [³H]nitrendipine binding to crude membranes prepared from the control uteri was saturable monophasically (Fig. 1A). An apparent dissociation constant (K₀) of 0.270±0.0333 nM (n=8) was obtained with 20 to 800 pM [³H]nitrendipine. Scatchard analysis revealed a single population with a Hill coefficient of 0.98 and gave the value of 53±3.8 fmol/mg protein for the maximal number of binding sites (Bmax) (Fig. 1B).

The K₀ values in the Estrogen (n=7) and the Estrogen+Progesterone (n=4) groups were 0.166±0.0254 and 0.156±0.0195 nM, respectively. These values were significantly (P<0.05, in both groups) lower than that in the control group. The K₀ value in the Estrogen-Progesterone group (n=4) was 0.183±0.0183 nM, and this was lower than that in the control group, though insignificantly. The membranes of the Progesterone group (n=6) showed a K₀ value of 0.282±0.0312 nM without any great difference from that of the control group. The Bmax values of the treated groups were 52 to 60 fmol/mg protein and were comparable to that of the control group (Fig. 2). Hill coefficients calculated for the individual groups were 1.00 to 1.05.

Effects on high K⁺-induced contractions: When the uteri were treated with 25 mM KCl-containing Tyrode’s solution, high K⁺-induced contractions were observed in each group (Fig. 3). In the control group (n=5), the mean tension which developed was 1370±144 mg. In the Estrogen (n=5), the Estrogen→Progesterone (n=4) and the
Estrogen+Progesterone (n=4) groups, the tensions which developed were 3125±476, 2700±240 and 3967±303 mg, respectively, and showed significant (P<0.01, P<0.001 and P<0.001, respectively) increases of 128, 97 and 190%, respectively, from that in the control group. The contractions in the Progesterone group (n=5, 1440±97 mg) and the control group differed very little from each other. In the Estrogen group, spontaneous rhythmic contractions were observed. When the uteri were treated with Ca++-depleted, 1 mM EGTA-containing Tyrode’s solution and then with 25 mM KCl-containing Ca++-depleted Tyrode’s solution, high K+ induced contractions developed as periodical contractions only in the Progesterone (n=6) and Estrogen→Progesterone (n=5) groups. No contractions developed in the control (n=8), the Estrogen (n=6) and the Estrogen+ Progesterone (n=5) groups (Fig. 4).

Effects on Ca++-induced contractions:
When the uteri were treated with 1×10^{-5}–3×10^{-2} M CaCl2, which was cumulatively added to the Ca++-depleted, high K+ containing Tyrode’s solution, the Ca++-induced contractions were observed in the control group (n=6) in a dose-dependent manner in the CaCl2 range from 1×10^{-4} M to 3×10^{-2} M. The maximum contraction was attained at 3×10^{-2} M CaCl2 (Fig. 5). The Ca++-induced contractions in the Estrogen group (n=4) were slight at 3×10^{-5} M CaCl2 and maximum at 3×10^{-3} M CaCl2. When CaCl2 was added up to 1×10^{-2} M to 3×10^{-2} M, the preparation was fully relaxed. In the Estrogen→Progesterone (n=4) and the Estrogen+ Progesterone (n=4) groups, maximum contractions were observed at 3×10^{-3} M and 1×10^{-2} M CaCl2, respectively, and relaxations of 13.9 and 95.2%, respec-
tively, were observed at $3 \times 10^{-2}$ M CaCl$_2$. The Ca$^{2+}$-induced contractions in the Progesterone group (n=4) were significantly (P<0.05) larger than those in the control group only at $3 \times 10^{-3}$ M CaCl$_2$. Maximal contractions of the individual groups were taken as 100% tension. Points and vertical bars express the mean±S.E. of values. The number of experiments were four to six. *: P<0.05, **: P<0.01 and ***: P<0.001; Significantly different from control values, respectively.

In the control, the Estrogen, the Progesterone, the Estrogen→Progesterone and the Estrogen+Progesterone groups, the ED50 values of Ca$^{2+}$ which induced 50% of maximal contraction were 2.34±0.789 x 10$^{-3}$, 6.50±0.567 x 10$^{-4}$, 1.61±0.216 x 10$^{-3}$, 9.29±1.182 x 10$^{-4}$ and 5.12±0.877 x 10$^{-4}$ M, respectively. These values of the 4 hormonally treated groups were not significantly different from that in the control group. The maximum contractions were 1640±67, 2028±77, 2078±268, 3638±270 and 3750±401 mg tension, respectively.

**Effects on the action of verapamil:** When the uteri were cumulatively treated with $1 \times 10^{-8}$ to $1 \times 10^{-5}$ M verapamil in the high K$^+$-containing Tyrode’s solution, the tissue preparations the control group (n=5) were dose-dependently relaxed in the range of verapamil concentrations from $1 \times 10^{-8}$ M to $1 \times 10^{-5}$ M (Fig. 6). In the Estrogen (n=4) and Estrogen+Progesterone (n=4) groups, the relaxations by verapamil were significantly (P<0.05 and P<0.01, respectively) greater than that in the control group at $3 \times 10^{-8}$ to $1 \times 10^{-5}$ M verapamil, the dose-response curves of verapamil being shifted to the left in a parallel manner. In the Progesterone (n=5) and the Estrogen→Progesterone (n=4) groups, the relaxations by verapamil were not significantly greater than that in the control group, but the dose-response curve in the Estrogen+Progesterone group was slightly shifted to the left.

In the control, the Estrogen, the Progesterone, the Estrogen→Progesterone and the Estrogen+Progesterone groups, the IC50 values of verapamil which produced 50% inhibition of 25 mM K$^+$-induced contractions were 1.98±0.472 x 10$^{-7}$, 3.58±0.971 x 10$^{-8}$, 1.72±0.325 x 10$^{-7}$, 1.24±0.424 x 10$^{-7}$ and 3.13±0.675 x 10$^{-8}$ M, respectively. These IC50 values of the Estrogen and the
Estrogen+Progesterone groups were significantly (P<0.05 and P<0.05, respectively) lower than that in the control group. The differences in tension between the 25 mM K+-induced maximum contractions and maximum relaxations by 1×10^{-4} M papaverine were 1434±91, 3191±479, 1552±103, 2815±244 and 4113±281 mg tension, respectively.

**Discussion**

We found that the specific binding of [3H]-nitrendipine to crude uterine membranes prepared from ovariectomized rats was saturable with high affinity. The specific binding of [3H]nitrendipine observed in the present study had a K_D value of 0.270 nM (Fig. 1). The K_D values of [3H]nitrendipine binding in crude uterine membranes were significantly lowered by treating the rats with estrogen or estrogen+ progesterone and insignificantly lowered by treatment with progesterone or estrogen→progesterone. The B_max values in the treated groups differed very little from that in the control group (Fig. 2). These data suggest that calcium channels of the Estrogen and the Estrogen+ Progesterone groups were at a high affinity state, with unchanged numbers of calcium channels, because the [3H]nitrendipine binding has been shown to indicate the properties of calcium channels in the cerebral cortex, heart, ileum and skeletal muscle (25, 26). In the membranes of the rats treated with estradiol benzoate in a dose of 25 µg/kg (one tenth of the presently used dose), K_D and B_max values for the Estrogen group and the control group differed very little from each other (data not shown). Therefore, estrogen seems to increase the affinity of the calcium channel, and progesterone may have no effect on the calcium channel.

Potentiations of the high K+-induced contractions in normal Tyrode's solution were observed in the Estrogen, the Estrogen→Progesterone and the Estrogen+Progesterone groups (Fig. 3). The potentiations by estrogen seem to be due to an increase in the intracellular Ca^{++} concentration because the high K+-induced contraction is known to be elicited by increased Ca^{++}-influx through the voltage-dependent calcium channels (6, 7). Ca^{++}-influx is important for increasing the intracellular levels of Ca^{++} under this experimental condition. These results are in contrast to findings from the uterus of estrogenized rats reported by Batra and Sjogrew (27); They reported that the uptake of 45Ca^{++} was increased by more than two-fold in the isolated depolarized uterus from the rats continuously treated with estrogen. Although we could not obtain any direct evidence, it is thought that Ca^{++}-influx is the most important cellular process for increasing the intracellular level of Ca^{++} during high K+-induced contraction of uterine smooth muscle cells. The potentiations of contractile force in the Estrogen→Progesterone and the Estrogen+Progesterone groups may be related to the physiological events in preparing for labor. The potentiation in the Progesterone group was very little. The mechanism of the spontaneous rhythmic contractions observed in the Estrogen group is likely due to the use of a sub-maximal concentration of K^+ (25 mM).

When the uteri were treated with high K^+-containing, Ca^{++}-depleted medium after treatment with EGTA, the high K^+-induced contraction developed only in the Progesterone and the Estrogen→Progesterone groups (Fig. 4). In these conditions, the high K^+-induced contractions may release Ca^{++} from intracellular Ca^{++} storage because extracellular Ca^{++} was removed with EGTA and because Ca^{++}-depleted medium was used (28). These observations indicate that progesterone, but not estrogen has an action of increasing intracellular Ca^{++} storage. Progesterone does not require priming by estrogen for effecting this action. No contraction was induced by high K^+ in the Estrogen+Progesterone group. The mechanism is not known. Furthermore, in the Estrogen group, the high K^+-induced contraction did develop in normal Tyrode's solution (Fig. 3), but did not develop in EGTA-treated tissues in Ca^{++}-depleted Tyrode's solution (Fig. 4). Accordingly, most of the ability of high K^+ to elicit contractile responses was thought to be due to influx of Ca^{++} through the voltage-dependent calcium channel from the medium into the uterine smooth muscle cells. The potentiation of high
K+-induced contraction by treatment with estrogen suggests that the function of the calcium channel was accelerated by estrogen and that the potentiation of the contraction was a result of increased influx of Ca++ from the medium into the muscle cells. Progesterone may increase Ca++ storage of intracellular site(s). However, no effects of estrogen and progesterone on Ca++-efflux from the muscle cells into the medium were found.

As indicated by the present study and by other investigators (12, 22), isolated uterine preparations which were suspended in Ca++-depleted, high K+-containing solution were contracted by cumulative addition of CaCl₂. It suggests that Ca++ ions added to the external medium induced contractions of uterine smooth muscle cells after they get into the intracellular spaces (9, 29). The Estrogen, the Estrogen→Progesterone, and the Estrogen + Progesterone groups showed decreases in the concentrations of CaCl₂ which were required for inducing the maximal contractions (Fig. 5). It is likely that these hormonal treatments enhanced the influx of Ca++ into uterine smooth muscle cells at a low concentration of Ca++. Ca++-induced contractions in the Progesterone group differed significantly or not significantly from that in the control group. The enhancement of Ca++-influx appeared to be related to lowered Kₚ values in the [³H]nitrendipine binding (Fig. 2).

In the Estrogen and the Estrogen+Progesterone groups, the dose-response curves for the relaxation of high K+-induced contractions by calcium-antagonistic verapamil were shifted to the left in a parallel manner, and IC₅₀ values of verapamil were significantly decreased (Fig. 6). Verapamil is reported to inhibit voltage-dependent calcium channels in smooth muscles (10, 13) and to diminish contractile responses of uterine muscles isolated from rats and mice (30). In the light of these findings, estrogen seems to stimulate the function of calcium channels. The relaxing effect of verapamil seen in the Progesterone group differed very little from that seen in the control group.

Estrogen appeared to increase the affinity of calcium channels (and extracellular Ca++ dependency) and to increase transmembrane influx of Ca++, leading to enhancement of contractions, whereas progesterone might have no effect on calcium channels but increase Ca++ storage in intracellular site(s). In the luteal phase and pregnancy, estrogen and progesterone may cooperatively regulate the mobilization of Ca++ available for the mechanical reaction.

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