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Hormonal modulation of benzodiazepines' actions on rat isolated uterus

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Abstract

Effects of various benzodiazepines were investigated in ovariectomized rat isolated uterus which had been chronically pre-treated with different female sex hormones: oestrogen, progesterone and oestrogen + progesterone. Uteri obtained from all groups developed a spontaneous, rhythmic activity. The spontaneous activity observed in control uterus was either inhibited in a concentration-dependent manner by diazepam, 4'-chlorodiazepam, clonazepam or 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195), or was abolished in $[Ca^{2+}]_o$-free solution. Diazepam, 4'-chlorodiazepam, clonazepam and PK 11195 all caused a concentration-dependent relaxation of the $[K^+]_o$-pre-contracted uterus with the relative order of potency: PK 11195 > 4'-chlorodiazepam > diazepam > clonazepam. Administration of $[Ca^{2+}]_o$ (1 μM to 10 mM) caused a concentration-dependent contraction of uterus, bathed in $[Ca^{2+}]_o$-free physiological salt solution obtained from different pre-treatment groups. Incubation with different concentrations (μM) of diazepam, 4'-chorodiazepam, clonazepam and PK 11195 caused a decrease in response to $[Ca^{2+}]_o$-induced contraction in all groups of rat uteri. These results indicate that micromolar benzodiazepine binding sites exist in rat uterus. Diazepam, 4'-chlorodiazepam, clonazepam and PK 11195 caused relaxation of pre-contracted rat uterus and this effect may involve the inhibition of influx of $[Ca^{2+}]_o$ and the relaxing effects of different benzodiazepines observed in this study can be modulated by pre-treatment with different female hormones.

Keywords: Uterus, rat; Sex hormone, female; Diazepam; 4'-Chlorodiazepam; Clonazepam; PK 11195; Ca^{2+} channel

1. Introduction

Benzodiazepines are widely used in clinical practice as hypnotics, anxiolytics and anti-convulsants, as well as central-muscle relaxants. A major advance in the understanding of the mechanisms of benzodiazepine action came in 1977, from the discovery of high affinity binding sites for [^{3}H]diazepam in rat brain (Braestrup and Squires, 1977; Möhler and Okada, 1977). It is now known that benzodiazepines can interact at three distinct receptors or binding sites, namely, the central benzodiazepine receptor, peripheral benzodiazepine receptor and micromolar benzodiazepine binding site (Braestrup and Squires, 1977; Möhler and Okada, 1977; Mestre et al., 1984; Bowling and DeLorenzo, 1982; Taft and DeLorenzo, 1984; Johansen et al., 1985). In addition to modulating the central nervous system functions, certain benzodiazepines e.g. diazepam, clonazepam and 4'-chlorodiazepam can exert pharmacological effects on many peripheral tissues such as cardiac muscle (Mestre et al., 1984, Mestre et al., 1985), vascular smooth muscle (French et al., 1989; Elgoyhen et al., 1993) and uterine smooth muscle (Kazanietz and Elgoyhen, 1990) both in humans and animals. Compared with the central benzodiazepine receptors, the physiological role(s) of the peripheral benzodiazepine receptors and micromolar benzodiazepine binding site is unknown.

Benzodiazepines have been prescribed to women during the late stage of pregnancy to relieve anxiety. Therapeutic doses of benzodiazepines can produce plasma concentrations of drug ranging from 0.1 to 50 μM (Christmas and Maxwell, 1970; Bond et al., 1977; Greenblatt et al., 1978; Van der Kleijn et al., 1981). Even though the central effects of benzodiazepines are carefully monitored, effects of these drugs on peripheral tissues are largely ignored. Benzodiazepines such as diazepam, 4'-chlorodiazepam and clonazepam, at micromolar concentrations, have been shown to relax uterine rings in rats in a concentration-dependent manner (Kazanietz and Elgoyhen, 1990). It is not known, however, what type of receptor is involved in the benzodiazepine-mediated effects on uterus, especially during pregnancy when the morphology and physiological functions of the uterus are under a strong hormonal influ-

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ence e.g. oestrogen and progesterone. Moreover, a complete characterization of the benzodiazepine receptor types in rat uterus using specific agonists (diazepam, 4'-chlorodiazepam and clonazepam) and antagonists (1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195) and flumazenil), and the underlying mechanism(s) of the modulating effects of the various hormones on benzodiazepine actions have not been worked out completely.

The present study was therefore designed to examine effects of various benzodiazepines on rat uterus, which were chronically pre-treated with different female sex hormones, and to characterize the benzodiazepine receptor(s) involved. In binding studies, rat tissues, when compared to other species, have been shown to have receptors with relatively higher affinity for benzodiazepines (Parola et al., 1993) and therefore the rat tissue is a useful preparation in which to characterize the benzodiazepine receptors. On the other hand, a possible coupling of voltage-dependent Ca^{2+} channels and benzodiazepine binding sites has not been resolved and several contradictory reports have been published (Mestre et al., 1986; French et al., 1989; Holck and Osterrieder, 1985; Rampe and Triggle, 1987). In the present study, rat uterus was used because uterine contraction is dependent on the influx of extracellular $[Ca^{2+}]_o$ (Bolton, 1979) and therefore the possible interactions between benzodiazepines and the influx of $[Ca^{2+}]_o$ through Ca^{2+} channels can be studied. Our present results indicate that micromolar 'affinity' benzodiazepine binding sites exist in rat uterus and the inhibitory effects of benzodiazepines may involve the inhibition of influx of $[Ca^{2+}]_o$.The process of $[Ca^{2+}]_o$ influx, as well as the uterine relaxing effects of benzodiazepines can be modulated by pre-treatment with different female sex hormones.

2. Materials and methods

2.1. Ovariectomy and hormone treatment

Female Sprague-Dawley rats weighing 250–300 g obtained from the Animal House of The Chinese University of Hong Kong were ovariectomized 20–25 days before they were killed. Rats were under ether anaesthesia during the ovariectomy surgical procedure. The abdomen of the rat was cut open and both the left and right uterine horns were identified. The ovary was then ligated, using a surgical suture, and was removed. The abdomen and the skin were sutured and rats were allowed to recover in the Animal House. The experiments were approved by the Animal Research Ethics Committee (The Chinese University of Hong Kong).

Ovariectomized rats received either an equivalent amount of vehicle (corn oil) but no hormone (as controls), oestrogen (1 mg/kg/dose), progesterone (20 mg/kg/dose) or oestrogen (1 mg/kg/dose) + progesterone (20 mg/kg/dose). Doses of hormones used in the present study were chosen according to the report by Castracane and Jordan (1975) on the uterus of overiectomized rats. Rats were given a total of 5 subcutaneous hormone injections. An induction dose of each treatment was administered 6–7 days before the beginning of the organ bath experiments and subsequently, individual rats in each group were given 4 consecutive daily doses of the corresponding hormone treatment before the animals were killed.

2.2. Rat isolated uterus isometric tension measurements

A rat, from different pre-treatment groups, was killed under ether anaesthesia by cervical dislocation. After opening the abdomen, the two uterine tubes were identified, removed and immediately immersed in physiological salt solution with the following composition (mM): NaCl 118.0; KCl 4.7; $CaCl_2$ 2.5; $MgCl_2$ 1.2; NaH_2PO_4 1.0; $NaHCO_3$ 25.0 and glucose 11.1. $[Ca^{2+}]_o$-free physiological salt solution was prepared by excluding $CaCl_2$ from the solution. All the experiments were performed in physiological salt solution with 2.5 mM $[Ca^{2+}]_o$ unless stated. A segment of the uterus (about 1 cm in length) was isolated and cut along the longitudinal axis, therefore converting it into a sheet of smooth muscle. The uterine strip was then suspended in a 10-ml organ bath, bubbled with 95% O_2 and 5% CO_2 and maintained at 37°C. One end of the uterine strip was fixed to a hook in the organ bath and the other was connected to a force-displacement transducer (Grass FT03).

Individual drugs were administered into the organ bath cumulatively and isometric tension measurements were performed by a Machintosh computer using the MacLab Chart v3.3.3 programme. Each preparation was under 1.0 g resting tension and allowed to equilibrate for 50 min. During the equilibration period, the tissue was washed with physiological salt solution every 20 min and the resting tension was readjusted if it was necessary. Dimethyl sulphoxide was used as a solvent for benzodiazepines, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195) and flumazenil stock solutions and therefore an equivalent quantity of the solvent was added into the organ bath. Results have shown that dimethyl sulphoxide apparently did not affect the benzodiazepine effects on the uterus. Due to the lipophilicity of benzodiazepines, only one concentration-response curve, of individual benzodiazepines, was constructed from each preparation. Benzodiazepines and PK 11195 were used at their highest concentrations before the effects of the solvent appeared. The effects of these agonists on uterus at their highest concentrations were then considered to be the maximum inhibition for comparison. Experiments on studying the effects of nifedipine were performed in a dimly-lit room and the organ bath was wrapped in aluminum foil.

2.3. Effects of benzodiazepines and PK11195 on the rhythmic activity of rat uterus

At the beginning of the experiment, uterus from all groups were quiescent. However, a uniform, spontaneous rhythmic activity developed only from control rat uterus at the end of the 50 min equilibration period. After the rhythmic activity had been stabilized in control rat uterus, different concentrations of benzodiazepines or PK11195 were administered into the organ bath. Effects of different benzodiazepines and PK 11195 on the rhythmic activity were recorded and compared. At the end of the experiment, the preparation was washed with drug-free physiological salt solution to observe the reversibility of the effects of benzodiazepines and PK11195 on the spontaneous activity of the uterus.

2.4. Effects of benzodiazepines on high $[K^+]_o$-pre-contracted rat uteri

The preparation was sensitized twice with 40 mM KCl followed by washing several times, before commencing the experiments. The preparation was then pre-contracted with 40 mM KCl. After a steady tonic contraction had been developed, an individual benzodiazepine was administered cumulatively into the organ bath. The relaxing effect of the benzodiazepine was recorded, compared to control responses and 100% relaxation was considered to exist when muscle tension returned to the original baseline level. A concurrent, time-matched control preparation was used for each experiment and corresponding quantities of vehicle was added. Effects of PK11195, flumazenil, γ-aminobutyric acid (GABA), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), bicuculline, propranolol and glibenclamide on the benzodiazepine-induced response were examined by incubating the preparation with each of these drugs individually for at least 15 min before the preparation was challenged with individual benzodiazepine. Experiments were performed in control, oestrogen-treated, progesterone-treated and oestrogen + progesterone-treated rat uterus. Results were recorded and compared to control responses.

2.5. $[Ca^{2+}]_o$-induced contractions of rat uterus bathed in high $[K^+]_o$ physiological salt solution

KCl was added into the organ bath (final bath concentration: 40 mM) containing $[Ca^{2+}]_o$-free physiological salt solution, without adjusting osmolarity and no apparent change of the baseline was observed. Uterine contraction was induced by administration of $CaCl_2$ cumulatively into the organ bath. After the maximal contraction had reached a steady state with a bath concentration of 10 mM $[Ca^{2+}]_o$, the preparation was washed with $[Ca^{2+}]_o$-free physio-

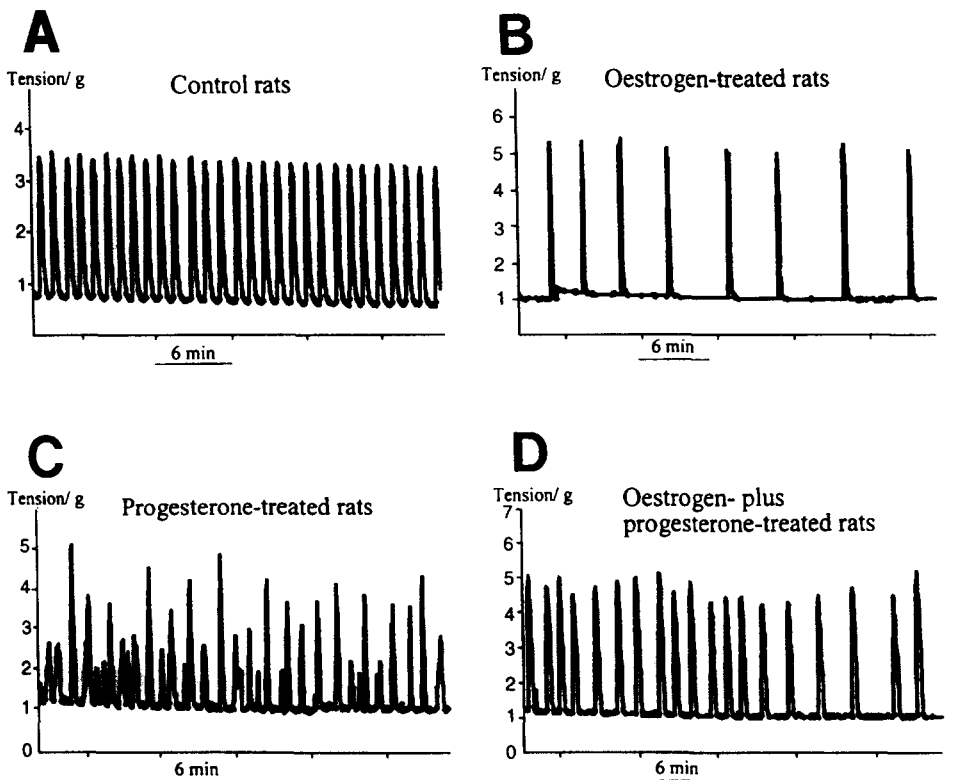

Fig. 1. Representative traces of the spontaneous rhythmic activity recorded from isolated uterus obtained from control (A), oestrogen-treated (B), progesterone-treated (C) and oestrogen + progesterone-treated (D) rats. *Y*-axis scale of each figure has been adjusted to allow for easier visualization of results.

logical salt solution and allowed to relax for at least 30 min. A single dose of individual benzodiazepine or PK 11195 was added into the organ bath 20 min before the construction of the second concentration-response curve to $[Ca^{2+}]_o$. Experiments were performed in controls, oestrogen-treated, progesterone-treated and oestrogen + progesterone-treated rat uterus. Results were recorded and compared to control responses.

2.6. Drugs

4′-Chlorodiazepam, diazepam, clonazepam, GABA, propranolol, DIDS, nifedipine and glibenclamide were obtained from Sigma Chemical Company (St. Louis, MO, USA). Flumazenil and PK11195 were generous gifts from Hoffmann-La Roche (Switzerland) and Rhone-Poulenc Rorer (France), respectively. These drugs were dissolved in dimethyl sulphoxide and prepared as 10 mM stock solutions except for clonazepam, the highest concentration of which was 5 mM. All stock solutions were stored at −30°C. GABA and propranolol were dissolved in de-ionized water whereas DIDS and glibenclamide were dissolved in DMSO. (+)-Bicuculline was purchased from Research Biochemicals International (Natick, MA, USA) and dissolved in de-ionized water. 6α-Methyl-17α-hydroxy-progesterone acetate and β-oestradiol 17-valerate were obtained from Sigma Chemical Company (St. Louis, MO, USA) and were both dissolved in corn oil for subcutaneous injections.

2.7. Statistical analysis of results

Statistical analysis of the results was performed using a two-way analysis of variance and Student's *t*-test for paired and unpaired data where appropriate. Data are given as means ± S.E.M. for n experiments with statistical comparisons at a $P < 0.05$ of significance. When no error bar is presented in a figure, it is smaller than the size of the symbol. The concentration of benzodiazepine required to reduce the contraction by 40% (IC_{40}) was calculated for each individual concentration-response curve using the logarithmic linear regression analysis.

3. Results

3.1. Effects of ovariectomy and hormone treatments on rat uterus

20–25 days after ovariectomy without hormone injection, rat uteri were small, thin-walled and they displayed highly regular spontaneous rhythmic activity (Fig. 1). On the other hand, pre-treatment with oestrogen greatly reduced the frequency of the observed rhythmic activity but the magnitude was greater for approximately the same length of tissue used (Fig. 1). However, the observed rhythmic activity in uteri from progesterone-treated rats was highly irregular and the frequency of contractions were comparable to those observed in control uteri (Fig. 1). After pre-treatment with oestrogen + progesterone, the magnitude of the rhythmic activity was increased and the frequency was reduced, but not by as much as was seen in the rat uteri treated with oestrogen (Fig. 1).

Morphologically, visual observations could not distinguish the uterus obtained from progesterone-treated rats from the control uterus obtained from ovariectomized rats. The endometrium of the progesterone-treated rats, however, appeared to be slightly thickened. Oestrogen treatment markedly thickened the uterine muscle wall and the uterus was filled with a milky watery liquid before they were cut open. Visual observations could not estimate whether the endometrium was thickened or not. However, both the uterine wall and endometrium were definitely thickened in rats pre-treated with oestrogen + progesterone.

3.2. Effects of benzodiazepines on rhythmic contractions of control rat uterus under resting tension

The spontaneous, rhythmic activity observed in control rat uterus was inhibited by PK11195 (10 and 20 μM, $n = 4$), 4′-chlorodiazepam (10 and 20 μM, $n = 5$), diazepam (10 and 20 μM, $n = 6$) and clonazepam (10 and 15 μM, $n = 5$) in a concentration-dependent manner (data not shown). Administration of a Cl^- channel blocker, DIDS 100 μM, on its own had no effect on the spontaneous activity ($n = 3$) and preincubation with this compound had no apparent effect on the inhibitory actions of these benzodiazepines ($n = 4$-6) (data not shown). The inhibitory effects of these compounds, except PK 11195, on the spontaneous rhythmic activity of uterus could be reversed after washing with drug-free physiological salt solution. Administration of a Ca^{2+} channel blocker, nifedipine, also inhibited the uterus' spontaneous activity in a concentration-dependent manner (0.3 nM to 1 μM) ($n = 3$, data not shown).

3.3. Effects of elevated $[K^+]_o$ on rat uterus under resting tension

Rat uterus obtained from all groups of rats, contracted in response to elevated $[K^+]_o$ in the organ bath. The contractile response was phasic with $[K^+]_o$ concentrations < 30 mM and became tonic with higher $[K^+]_o$ concentrations. 40 mM $[K^+]_o$ elicited a contractile response which had both phasic and tonic components and a steady state contraction was achieved within 20 min. In all groups of rat uterus, 40 mM $[K^+]_o$ produced about 85–90% of the maximum contraction ($n = 4$ for each group, data not shown). The sizes of the 40 mM $[K^+]_o$-induced contractions were 2–4 g and 4–6 g (Fig. 1) in control and hormone-pre-treated groups, respectively. Even though the

size of the 40 mM $[K^+]_o$-induced contractions were bigger (4–6 g) in all hormone-treated uterus (uterus was 1 cm in length), the effects of benzodiazepine-mediated uterine relaxation when expressed as percentage inhibition were not different from hormone-treated uterus ($n = 8$; data not shown) which were shorter in length (about 0.4 cm) with smaller contractions (2–4 g) induced by the 40 mM $[K^+]_o$. Therefore, the same lengths of uterus (1 cm) were obtained from all groups of rats and were used in the latter experiments. High $[K^+]_o$-induced uterine contraction, observed in all groups, was completely abolished in $[Ca^{2+}]_o$-free physiological salt solution. High $[K^+]_o$-induced contraction was inhibited by nifedipine, a well known voltage-dependent Ca^{2+} channel blocker, in a concentration-dependent manner with an IC_{50} of 9.0 ± 1.2 nM (Fig. 2) (control rats, $n = 5$).

3.4. Relaxing effect of diazepam on rat uterine strips pre-contracted with 40 mM KCl

3.4.1. Control rats

Diazepam, an agonist which acts on both the central and peripheral benzodiazepine receptors, caused an inhibition of contraction of the pre-contracted uterus in a concentration-dependent fashion with an IC_{40} of 23.5 ± 2.1 μM (maximum inhibition: 70.8 ± 3.9%, Fig. 2) ($n = 7$).

Flumazenil (1 nM to 10 μM) ($n = 4$) and GABA (1 nM to 100 μM) ($n = 4$) itself had no apparent effect on the pre-contracted rat uterus (data not shown). However, PK 11195, a putative peripheral benzodiazepine receptor antagonist, applied alone caused a concentration-dependent inhibition of contraction of pre-contracted rat uterus (Fig. 2) ($n = 6$). Incubation with either 10 μM flumazenil ($n = 3$), GABA 10 μM ($n = 3$), PK11195 1 μM ($n = 4$), DIDS 100 μM ($n = 4$), bicuculline 10 μM ($n = 3$), propranolol 1 μM ($n = 4$) or glibenclamide 200 nM ($n = 3$) had no significant effect on the diazepam-induced relaxation (data not shown). All these drugs, on their own, had no effect on the pre-contracted uterus in all groups of rats ($n = 3$ for each drug, data not shown).

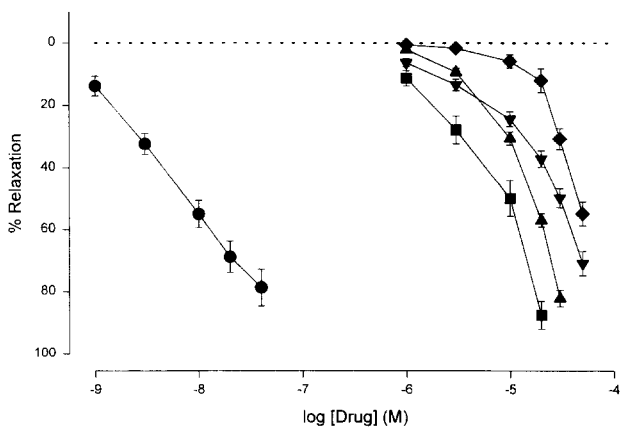

Fig. 2. Relaxing effects of nifedipine (●), PK 11195 (■), 4′-chlorodiazepam (▲), diazepam (▼) and clonazepam (◆) on control rat uterus pre-contracted with 40 mM $[K^+]_o$. Each point represents the mean ± S.E.M. The dotted line represents 0%.

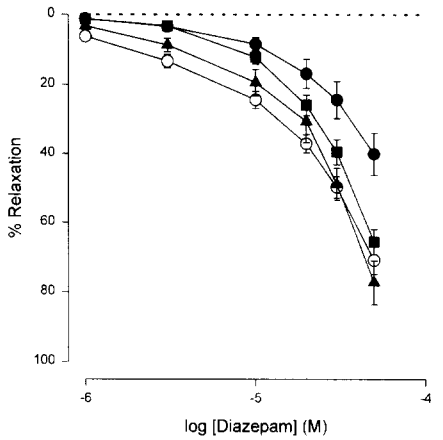

Fig. 3. Relaxing effects of diazepam on control (○), oestrogen-treated (●), progesterone-treated (▲) and oestrogen + progesterone-treated (■) rat uterus. Each point represents the mean ± S.E.M. The dotted line represents 0%.

3.4.2. Oestrogen-treated rats

Diazepam-induced uterus relaxation was significantly smaller than the results observed in control uterus which received no hormone pre-treatment. The maximum relaxation was 40.1 ± 6.2% (Fig. 3) ($n = 6$, $P < 0.05$).

3.4.3. Progesterone-treated rats

Diazepam caused a concentration-dependent inhibition of contraction of the pre-contracted uterus with an IC_{40} of 31.7 ± 2.6 μM and maximum inhibition of 65.5 ± 3.4% (Fig. 3) ($n = 8$). Compared to control rat uterus, the diazepam-induced relaxation with concentrations of 1–30 μM was significantly attenuated ($n = 8$) but the maximum inhibition was similar (65.5 ± 3.4 vs. 70.8 ± 3.9%) ($n = 8$).

3.4.4. Oestrogen + progesterone-treated rats

In contrast to oestrogen-treated and progesterone-treated rat uteri, combined treatment of oestrogen + progesterone did not cause any significant change of the diazepam-induced maximum relaxation of the uterus, compared to control rat uterus (IC_{40}: 24.5 ± 4.1 vs. 23.5 ± 2.1 μM, maximum inhibition: 77.2 ± 6.3 vs. 70.8 ± 3.9%) ($n = 8$) (Fig. 3).

3.5. Relaxing effect of clonazepam on rat uterine strips pre-contracted with 40 mM KCl

3.5.1. Control rats

Clonazepam, an agonist acting on the central benzodiazepine receptors, caused a concentration-dependent (1–50 μM) inhibition of contraction (IC_{40}: 37.8 ± 2.8 μM, maximum inhibition: 54.8 ± 3.8%) ($n = 5$) (data not shown).

Similar to diazepam, incubation with GABA 1 μM ($n = 4$), PK11195 1 μM ($n = 4$), flumazenil 10 μM ($n = 4$), glibenclamide 200 nM ($n = 3$), bicuculline 10 μM ($n = 3$), propranolol 1 μM ($n = 4$) or DIDS 100 μM ($n = 3$) had no apparent effect on clonazepam-induced uterus relaxation (data not shown).

3.5.2. Oestrogen-treated rats

Similar to diazepam, clonazepam-induced uterus relaxation observed in oestrogen-treated uterus was significantly attenuated when compared to controls (maximum inhibition: 28.1 ± 0.9 vs. 54.8 ± 3.8%) ($n = 6$, $P < 0.05$) (data not shown).

3.5.3. Progesterone-treated rats

In progesterone-treated rat uterus, only the maximum inhibition of contraction induced by clonazepam but not the IC_{40} was sightly enhanced when compared to controls (maximum inhibition: 64.8 ± 3.0 vs. 54.8 ± 3.8%) ($n = 6$, $P < 0.05$) (data not shown).

3.5.4. Oestrogen + progesterone-treated rats

Similar to diazepam, combined treatment of the rat uterus with oestrogen + progesterone had no apparent effect on the clonazepam-induced inhibition of uterine contraction (IC_{40}: 37.7 ± 6.5 vs. 38.5 ± 4.4 μM, maximum inhibition: 52.5 ± 6.5 vs. 54.8 ± 3.8%) ($n = 5$) (data not shown).

3.6. Relaxing effect of 4′-chlorodiazepam on rat uterine strips pre-contracted with 40 mM KCl

3.6.1. Control rats

4′-Chlorodiazepam, a peripheral benzodiazepine receptor agonist, caused a concentration-dependent inhibition of uterine contraction (IC_{40}: 13.5 ± 0.7 μM, maximum inhibition: 82.1 ± 2.7%) ($n = 4$) (data not shown). Similar to diazepam and clonazepam, the relaxant effect of 4-chlorodiazepam was not affected by 10 μM flumazenil ($n = 3$), GABA 1 μM ($n = 4$), PK11195 1 μM ($n = 3$), glibenclamide 200 nM ($n = 4$), bicuculline 10 μM ($n = 4$), propranolol 1 μM ($n = 4$) or DIDS 100 μM ($n = 3$) (data not shown).

3.6.2. Oestrogen-treated rats

In contrast to both diazepam and clonazepam, oestrogen pre-treatment had no apparent effect on the 4′-chlorodiazepam-induced inhibition of contraction (IC_{40}: 15.8 ± 1.8 vs. 13.5 ± 0.7 μM, maximum inhibition: 85.7 ± 3.3 vs. 82.1 ± 2.7%) ($n = 6$) (data not shown).

3.6.3. Progesterone-treated rats

Progesterone pre-treatment only slightly, but significantly, enhanced the maximum inhibition induced by 30 μM 4′-chlorodiazepam (maximum inhibition: 93.2 ± 3.1 vs. 82.1 ± 2.7%) ($n = 6$, $P < 0.05$) (data not shown).

3.6.4. Oestrogen + progesterone-treated rats

Similar to clonazepam, oestrogen + progesterone pre-treatment had no apparent effect on 4′-chlorodiazepam-mediated inhibition of contraction (IC_{40}: 12.4 ± 0.2 vs. 13.5 ± 0.7 μM) ($n = 6$) (data not shown).

3.7. Effects of hormone pre-treatment on $[Ca^{2+}]_o$-induced contractions in uterine strips bathed in 40 mM $[K^+]_o$ $[Ca^{2+}]_o$-free physiological salt solution

In control rat uterus, administration of $[Ca^{2+}]_o$ caused a concentration-dependent contraction (EC_{50} = 0.17 ± 0.04 mM) ($n = 12$). Two concentration-response curves were constructed with each uterine strip. In the time-matched control uterus, both curves were recorded without any benzodiazepine or PK11195 incubation. The second curve recorded was either not different from the first one (considered as 100%) ($n = 6$) or in some cases it was slightly potentiated with an increase in the maximum of the curve being obtained (EC_{50} = 0.19 ± 0.05 mM, maximum effect = 112.0 ± 2.8%) ($n = 15$) (data not shown).

In all the hormone-pre-treated (oestrogen, progesterone, oestrogen + progesterone) rat uterus, there was a change in the shape of the concentration-response curves to $[Ca^{2+}]_o$ and the degree of change of $[Ca^{2+}]_o$-induced contraction was found to be greater over the $[Ca^{2+}]_o$ concentration range of 3 μM to 0.3 mM when compared to controls ($n = 5$–12) (data not shown). The maximum contraction induced by 10 mM $[Ca^{2+}]_o$ was however similar in all groups of rat uteri ($n = 5$–12).

3.8. Effects of different concentrations of benzodiazepines and PK 11195 on $[Ca^{2+}]_o$-induced uterine contraction in control rat uteri

In control rats, diazepam caused a concentration-dependent inhibition of $[Ca^{2+}]_o$-induced contractions. At low

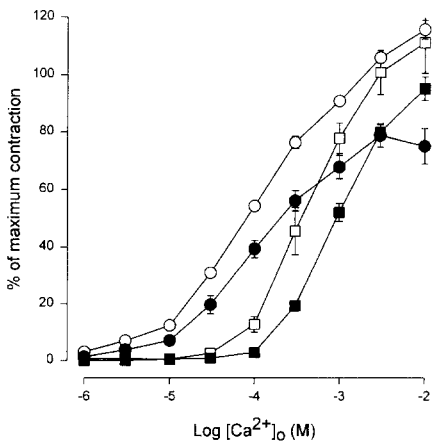

Fig. 4. Effect of increasing $[Ca^{2+}]_o$ concentrations on control (circles) and oestrogen-treated (squares) rat uteri. Uteri were bathed in 40 mM $[K^+]_o$ $[Ca^{2+}]_o$-free physiological salt solution. Each point represents the mean ± S.E.M. (○ = control; ● = plus 20 μM diazepam; □ = oestrogen-treated; ■ = plus 20 μM diazepam).

Table 1
Summary of the effects of incubation with diazepam (20 μM), 4′-chlorodiazepam (4′-Chloro, 10 μM), clonazepam (40 μM) and PK 11195 (7 μM) on $[Ca^{2+}]_o$-induced contractions in rat uterus

Drug	Control rats	Oestrogen-treated rats	Progesterone-treated rats	Oestrogen + progesterone-treated rats
Diazepam	75.2 ± 6.1 (5)	94.7 ± 3.9 (5)	68.3 ± 5.3 (6)	100.6 ± 5.3 (5)
4′-Chloro	55.4 ± 7.9 (6)	96.0 ± 10.4 (7)	79.3 ± 6.1 (5)	94.7 ± 5.1 (6)
Clonazepam	72.8 ± 6.7 (6)	83.3 ± 5.7 (5)	79.4 ± 5.5 (5)	98.3 ± 5.5 (5)
PK 11195	71.5 ± 4.4 (4)	77.2 ± 6.2 (5)	55.4 ± 6.6 (5)	82.4 ± 5.8 (4)

Results are expressed as means ± S.E.M. of maximum response (%). Numbers in parentheses indicate the number of experiments.

concentrations (3 and 10 μM) of diazepam, there was a trend of $[Ca^{2+}]_o$ concentration-response curve shifted to the right. At higher diazepam concentrations (20 and 30 μM), there was a definite decrease in response of rat uterus to $[Ca^{2+}]_o$ ($n = 6$) (data not shown). Similar effects on $[Ca^{2+}]_o$-induced contractions were recorded with 4′-chlorodiazepam (3, 10 and 20 μM, $n = 5$), clonazepam (10, 20 and 40 μM, $n = 5$) and PK11195 (3, 10 and 30 μM, $n = 4$) (data not shown).

3.9. Effects of a single dose of benzodiazepines and PK 11195 on $[Ca^{2+}]_o$-induced uterine contraction

A single dose of an individual benzodiazepine which was equivalent to the corresponding IC_{40} concentration was used in this study. In control rats, a single dose of diazepam, 20 μM, caused a decrease in response of uterus to $[Ca^{2+}]_o$. A rightward shift of the $[Ca^{2+}]_o$-induced concentration-response curve and a decrease in the maximum contraction (maximum effect: 75.2 ± 6.1 vs. 128.8 ± 2.2%) (Fig. 4) ($n = 5$). Similar observations in the $[Ca^{2+}]_o$-induced concentration-response curve were recorded with clonazepam 40 μM ($n = 5$, see Table 1), 4′-chlorodiazepam 10 μM ($n = 5$, see Table 1) and PK 11195 7 μM ($n = 5$, see Table 1).

Similar to control rats, in oestrogen-treated uterus, 20 μM diazepam caused a rightward shift of the $[Ca^{2+}]_o$-induced concentration-response curve and a decrease in the maximum contraction (maximum effect: 94.7 ± 3.9 vs. 110.7 ± 10.7%) (Fig. 4) ($n = 4$). Similar observations were recorded with clonazepam 40 μM ($n = 4$, see Table 1), 4′-chlorodiazepam 10 μM ($n = 4$, see Table 1) and PK 11195 7 μM ($n = 4$, see Table 1) on uterus pre-treated with either progesterone or oestrogen + progesterone (see Table 1).

4. Discussion

The uterus is a preparation in which tension development is mainly dependent on the influx of extracellular Ca^{2+} rather than Ca^{2+} release from the intracellular pools (Bolton, 1979). Rhythmic contractions of rat uterus observed in the present study could be inhibited by a well known Ca^{2+} channel blocker, nifedipine, and was abolished in $[Ca^{2+}]_o$-free physiological salt solution. All the benzodiazepines (diazepam, 4′-chlorodiazepam and clonazepam) and PK11195 tested in our study showed an inhibition of the spontaneous, rhythmic activity. The present results suggested that these compounds may inhibit the influx of $[Ca^{2+}]_o$ through the voltage-dependent Ca^{2+} channels in rat uterus. The complete reversal of the inhibitory effects caused by the benzodiazepines but not PK 11195 on the rhythmic activity can exclude the possibility of a toxic effect caused by the benzodiazepine compounds on rat uterus. On the other hand, the irreversibility of the PK 11195 effect may represent a different nature of binding to the receptors. It has been shown that PK 11195 binds to the histidine residue of the peripheral benzodiazepine receptors (Benavides et al., 1984) in a entropy-driven manner (Le Fur et al., 1983a, Le Fur et al., 1983b) whereas the binding of other benzodiazepines to the receptors is enthalpy-driven.

Instead of directly inhibiting the influx of $[Ca^{2+}]_o$, benzodiazepines and PK 11195 could also possibly hyperpolarize the uterine cells, by increasing the inward Cl^- currents for example, thus diminishing the probability of voltage-dependent Ca^{2+} channel opening, therefore inhibiting the spontaneous activity of the uterus. However, this effect is unlikely because in the presence of the Cl^- channel blocker DIDS (100 μM) (Stutts et al., 1992), the inhibitory effects of these benzodiazepines and PK 11195 on rat uterus were unaffected. An electrophysiological study is being carried out to explore the interactions between benzodiazepines and K^+ channels ($I_{K(del)}$ and $I_{K(Ca)}$) and our preliminary results suggest that $I_{K(Ca)}$ channels were not involved in the benzodiazepine-mediated uterus relaxation.

The possible involvement of the opening of ATP-sensitive K^+ channels in benzodiazepine-induced relaxation was examined using the ATP-sensitive K^+ channel blocker, glibenclamide. Pre-treatment with glibenclamide (200 nM) had no apparent effect on diazepam-, clonazepam- and 4-chlorodiazepam-induced uterus relaxation. Therefore, it seems that the opening of ATP-sensitive K^+ channels could not be responsible for the observed benzodiazepine-induced relaxation.

In the present study, the involvement of the activation

of β-adrenoceptors was also examined. Similar to glibenclamide results, propranolol 1 μM did not affect the 4′-chlorodiazepam-, clonazepam-, diazepam- or PK 11195-induced relaxation of $[K^+]_o$-contracted rat uterine strips. Therefore, it is unlikely that activation of β-adrenoceptors and a subsequent increase in cAMP production can account for the relaxant effects of benzodiazepines observed in the present study. However, our present results are in contrast to the recent publication by Gimeno et al. (1994) which reports that an increase in cyclic nucleotides e.g. cAMP, play a significant role in the vasorelaxing effects of peripheral benzodiazepines on rat isolated aorta.

To determine what type(s) of benzodiazepine receptors are involved, the specific central benzodiazepine receptor antagonist flumazenil (Hunkeler et al., 1981) was used. Flumazenil (10 μM) had no effect on the relaxation of $[K^+]_o$-pre-contracted rat uterus (Kazanietz and Elgoyhen, 1990), caused by 4′-chlorodiazepam, clonazepam or diazepam. Incubation with either GABA, bicuculline (a $GABA_A$ receptor antagonist) (Curtis et al., 1970), or DIDS (a Cl^- channel blocker), also failed to affect the uterine relaxation effects caused by diazepam, 4′-chlorodiazepam and clonazepam. These observations strongly suggested that central benzodiazepine receptors are not involved in mediating the relaxant effects of benzodiazepines in rat uterus.

On the other hand, the involvement of peripheral benzodiazepine receptors can also be ruled out. This is because benzodiazepine-induced relaxation of $[K^+]_o$-pre-contracted rat uteri could not be blocked by the putative peripheral benzodiazepine receptor-specific antagonist PK 11195. Although PK11195 was classified as a peripheral benzodiazepine receptor antagonist, in the present study, PK 11195 not only failed to block the relaxant effects of benzodiazepines but itself had a relaxant effect (Camarasa et al., 1988; Elgoyhen et al., 1993; Gimeno et al., 1994) which was more potent than that of all the benzodiazepines tested. Besides, the central benzodiazepine receptor-specific agonist, clonazepam, whose reported affinity for the peripheral benzodiazepine receptor is some 10000 times lower than that of 4′-chlorodiazepam (Schoemaker et al., 1983), was only three times less potent than 4′-chlorodiazepam (IC_{40}: 13.5 ± 0.7 μM vs. 37.8 ± 2.8 μM) in relaxing rat uterine strips pre-contracted with 40 mM $[K^+]_o$. Moreover, micromolar concentrations of benzodiazepines were required to produce relaxation whereas nanomolar concentrations were required to saturate peripheral benzodiazepine receptors in this tissue (Fares et al., 1987). Such a disparity between the pharmacological potency of benzodiazepines and their affinity for the peripheral benzodiazepine receptors in the rat uterus is consistent with the results obtained from other studies (Hullihan et al., 1983; Awad and Gavish, 1987; Camarasa et al., 1988; Raeburn et al., 1988; French et al., 1989; Elgoyhen et al., 1993). In these studies, the potency of benzodiazepines did not correlate with their affinity for the peripheral benzodiazepine receptors. This 'non-coincidence' of pharmacological concentration-response curve and receptor binding study has been observed in other agonists, e.g. insulin (Cuatrecasas and Hollenberg, 1976). All these results suggested that the relaxant effects of benzodiazepines on the uterine smooth muscle are probably not mediated by the peripheral benzodiazepine receptors.

In addition to the central- and peripheral-type benzodiazepine receptors, a third type of receptors has been proposed with which benzodiazepines bind at micromolar concentrations, and it does not discriminate between central benzodiazepine receptor-specific and peripheral benzodiazepine receptor-specific ligands (Bowling and DeLorenzo, 1982; Taft and DeLorenzo, 1984). In our present study, 4′-chlorodiazepam, diazepam and clonazepam were 'similarly' equipotent and they were only effective in causing uterine relaxation at the micromolar but not nanomolar concentration ranges. It is therefore possible that the micromolar binding sites were involved in benzodiazepine-mediated uterus relaxation.

Chronic pre-treatment of rats with oestrogen alone but not with progesterone significantly attenuated the maximum relaxing effects of clonazepam and diazepam. Pre-treatment with oestrogen + progesterone: the 'attenuation effect' of oestrogen on the relaxing action of diazepam and clonazepam on rat uterus was not observed. It is generally agreed that oestrogen and progesterone produce opposite effects on the mechanical activity of uterine myocardium (Melton and Saldivar, 1965; Marshall, 1959) and this may explain the 'antagonizing effect' of progesterone on oestrogen-mediated effects observed in the present study.

It has been shown that oestrogen pre-treatment can alter the responses of rat isolated detrusor muscle to different agents (Elliott et al., 1992a). In the present study, the oestrogen-mediated attenuation of the clonazepam- and diazepam-mediated effects may be due to the down-regulation of the benzodiazepine receptors and/or decrease in receptor density (Batra and Andersson, 1989). It has been reported that sex hormones can regulate peripheral benzodiazepine receptors (Gavish, 1995) and chronic oestradiol treatment caused a marked decrease in the density of peripheral benzodiazepine binding sites in rat testis (Gavish et al., 1986). However, in the present study only clonazepam (a central benzodiazepine receptor agonist) and diazepam (a central and peripheral benzodiazepine receptor agonist) were affected by oestrogen treatment but not the peripheral benzodiazepine receptor agonist 4′-chlorodiazepam. The reason for this discrepancy is not known but it may be due to tissue differences (Gavish et al., 1986; Awad and Gavish, 1987) or a different type of receptors being involved. Another possibility could be the hypertrophic effect of oestrogen on rat uterus which may affect the access of benzodiazepines to the receptors. Compared to oestrogen, the effects of progesterone on peripheral benzodiazepine receptor density received less attention and has not been fully defined.

On the other hand, it has been proposed that micromolar benzodiazepine binding sites are linked to voltage-sensitive Ca^{2+} channels and activation of these binding sites causes an inhibition of Ca^{2+} uptake in intact nerve-terminal preparations in rats (Taft and DeLorenzo, 1984) as well as attenuating the voltage-gated Ca^{2+} conductance in leech neurones (Johansen et al., 1985). Several studies have suggested that inhibition of $[Ca^{2+}]_o$ influx is the mechanism underlying the relaxant effects of benzodiazepines on smooth muscles (Ishii et al., 1982; French et al., 1989; Elgoyhen et al., 1993). In order to examine whether benzodiazepines affect the process of influx of $[Ca^{2+}]_o$, $[Ca^{2+}]_o$-induced uterus contraction experiments were performed in all groups of rats. Pre-treatment with different hormones resulted in similar changes in the 'shape' of $[Ca^{2+}]_o$ concentration-response curves. The sensitivity of hormone-treated rat uteri was considerably lower than control uteri at low $[Ca^{2+}]_o$ and the difference was greatest at $[Ca^{2+}]_o$ from 0.001 mM to 0.3 mM. Also, with $[Ca^{2+}]_o$ between 0.1 mM and 1 mM, the slope of $[Ca^{2+}]_o$ concentration-response curve was steeper in hormone-treated uteri. These results suggested that hormone pre-treatment may affect either the sensitivity/response of the uterus to $[Ca^{2+}]_o$ (Elliott et al., 1992b) especially at low concentrations of $[Ca^{2+}]_o$ or the movement of $[Ca^{2+}]_o$ into the uterus muscle as reported by Elliott et al. (1992a, , 1992b) in rat isolated detrusor muscle.

In control rat uterus, $[Ca^{2+}]_o$-induced contractions of $[K^+]_o$-depolarized preparations were also investigated in the presence of different concentrations of individual benzodiazepines (diazepam, 4′-chlorodiazepam and clonazepam) and PK 11195. For diazepam the trend was a reduction in response to $[Ca^{2+}]_o$ and a significant reduction of contraction was observed at 30 μM. Similar results were observed in 4′-chlorodiazepam, clonazepam and PK 11195 experiments. Effects of benzodiazepines on $[Ca^{2+}]_o$-induced contractions in rat uterus have been reported (Granger et al., 1983). These studies suggested that benzodiazepines may modulate the influx of $[Ca^{2+}]_o$ through the voltage-operated Ca^{2+} channels. Similar 'shifts' of the $[Ca^{2+}]_o$ concentration-response curves were observed with benzodiazepines (diazepam, 4′-chlorodiazepam and clonazepam) and PK 11195 in the present study in hormone pre-treated rat uteri, although the 'degree' of shift observed was not the same as in the control rat uteri.

In conclusion, the benzodiazepines: 4′-chlorodiazepam, diazepam and clonazepam, tested in the present study, inhibit $[K^+]_o$-induced contractions in rat uterus in the rank order of potency of 4′-chlorodiazepam > diazepam > clonazepam in control rats. Flumazenil, GABA, bicuculline and PK11195 had no significant effect on the relaxant effects of benzodiazepines, indicating that both central benzodiazepine receptors and peripheral benzodiazepine receptors were probably not involved. On the other hand, micromolar concentration requirements of these benzodiazepines to produce relaxant effects and the 'indiscrimination' of the receptors for different benzodiazepines may suggest that the relaxing effects were mediated by the micromolar benzodiazepine binding sites. Besides, the benzodiazepine effects on the $[Ca^{2+}]_o$-induced contractions in $[K^+]_o$-depolarized rat uterus provide evidence that modulation of $[Ca^{2+}]_o$ influx through voltage-sensitive Ca^{2+} channels is the mechanism by which they act. The relaxant effects of different benzodiazepines on rat uterus can be modulated by pre-treatment with the female sex hormones oestrogen and progesterone. The hormone modulatory effects may be due to a change in the sensitivity of the rat uterus to $[Ca^{2+}]_o$ or a change in the Ca^{2+} channel current density but further study is necessary to clarify the underlying mechanism(s).

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