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Relaxant Effect of Tetrazepam on Rat Uterine Smooth Muscle: Role of Calcium Movement

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Abstract

Tetrazepam is a benzodiazepine derivative clinically used as a muscle relaxant. The aim of the present work was to examine its effect on uterine smooth muscle of the rat in estrus.

Tetrazepam required micromolar concentrations to relax contractile responses induced by KCl and acetylcholine in Ca^{2+} solution, but not oxytocin-induced contraction. In Ca^{2+}-free solution, tetrazepam inhibited Ca^{2+}-induced contractions in depolarized uterus and vanadate-induced contractions.

We suggest that tetrazepam relaxes contractile responses induced by activation of voltage-sensitive calcium channels and receptor-operated calcium channels with little selectivity or that it antagonizes the effect of calcium at subsequent steps, possibly intracellular stores sensitive to vanadate but not sensitive to oxytocin. The inhibition of contraction of rat uterus is not related to high-affinity peripheral benzodiazepine binding sites.

Tetrazepam, a 1,4-benzodiazepine derivative, appears to have a rather unusual profile of activity in that the doses which produce sedation and ataxia are considerably higher than those which produce anxiolytic and muscle relaxant effects (Keane et al 1988a). In-vitro studies have show that tetrazepam exhibits a 4- to 7-fold lower affinity than diazepam for the 'central' benzodiazepine receptor (Haefely et al 1985; Keane et al 1988b). A second class of benzodiazepine sites has been established in a number of peripheral tissues (Marangos et al 1982; Skerrit et al 1982). These non-neuronal or peripheral-type sites are characterized by the specific, high-affinity binding of benzodiazepine Ro5-4864 and the isoquinolinecarboxamide compound PK 11195, a compound that selectively displaces Ro5-4864 from its sites (Le Fur et al 1983; Erne et al 1989). Tetrazepam binds to this PBS (Groh & Müller 1985; Müller et al 1985). The physiological roles of the PBS remain unclear, but it has been suggested that these sites may be linked to voltage-dependent Ca^{2+} channels and that ligands which interact at these sites serve as Ca^{2+}-channel regulators (Taft & De Lorenzo 1984; Bender & Hertz 1985; Mestre et al 1985; Rampe & Triggle 1986).

The molecular mechanisms underlying the pharmacological profile of tetrazepam have been little studied. The purpose of the present work was to examine its effect on rat uterine smooth muscle and the possible interaction with Ca^{2+} channels.

Materials and Methods

Preparation of uterine horns

Female Wistar rats, 150–200 g, were given oestradiol valerate (5 mg kg^{-1}). Twenty-four hours later they were killed by a blow on the head and exsanguinated. Uterine horns were removed and mounted in a 30-mL organ-bath chamber filled with Jalon-Ringer solution. The medium was gassed continuously with 95% O_2 –5% CO_2 and maintained at 31°C.

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Drugs and solutions

Oxytocin (Syntocinon) was purchased from Sandoz; vanadate, acetylcholine from Sigma Chemical Company; potassium chloride was obtained from E. Merck; tetrazepam was generously provided by Sanofi Winthrop. All other reagents were of analytical grade. All drugs were dissolved in distilled water, except PK 11195 which was dissolved in dimethylsulphoxide (DMSO). The final concentration of DMSO in the tissue bath was less than 0.01% and did not affect contraction or relaxation. Tetrazepam was initially dissolved in 1 mL of Tween 80 and diluted further in saline to prepare a 2×10^{-3} M stock solution. The final Tween 80 concentration in the bath did not affect the results, as we confirmed in parallel experiments.

The following solutions were used: Jalon-Ringer solution (mM): NaCl 154, KCl 5·63, $CaCl_2$ 0·648, $NaHCO_3$ 5·95 and glucose 2·77; Depolarizing solution (mM): NaCl 103·3, KCl 56·3 $CaCl_2$ 0·648, $NaHCO_3$ 5·95, glucose 2·77; Tyrode solution (mM): NaCl 136·9, KCl 2·68, $CaCl_2$ 0·9, $NaHCO_3$ 11·9 $MgCl_3$ 1·10, NaH_2PO_4 0·42 and glucose 5·5; Locke-Ringer solution (mM): NaCl 154, KCl 5·63, $CaCl_2$ 2·16, $MgCl_2$ 2·10, $NaHCO_3$ 5·95 and glucose 5·55; Ca^{2+}-free solution: the same composition as the Locke-Ringer solution but $CaCl_2$ and $MgCl_2$ were omitted and EDTA (0.01 mM) was added.

Experimental procedure

To assess the effect of tetrazepam on the influx of calcium through voltage-sensitive channels, two different kinds of experiment were designed:

K^+-depolarized uterus. The organ was immersed in Jalon-Ringer solution and equilibrated for 20 min under a resting tension of 0·5 g. After the equilibration period, the preparation was contracted by changing the solution in the bath to a depolarizing solution. This addition caused a rapid contraction, followed by a slight relaxation and a prolonged contraction plateau. When the plateau was reached, tetrazepam was added in progressively increasing cumulative concentrations (10^{-8}–10^{-4} M) at 15-min intervals since preliminary experiments demostrated that this was the time needed to produce a steady-state relaxation. The experiment was repeated in the presence

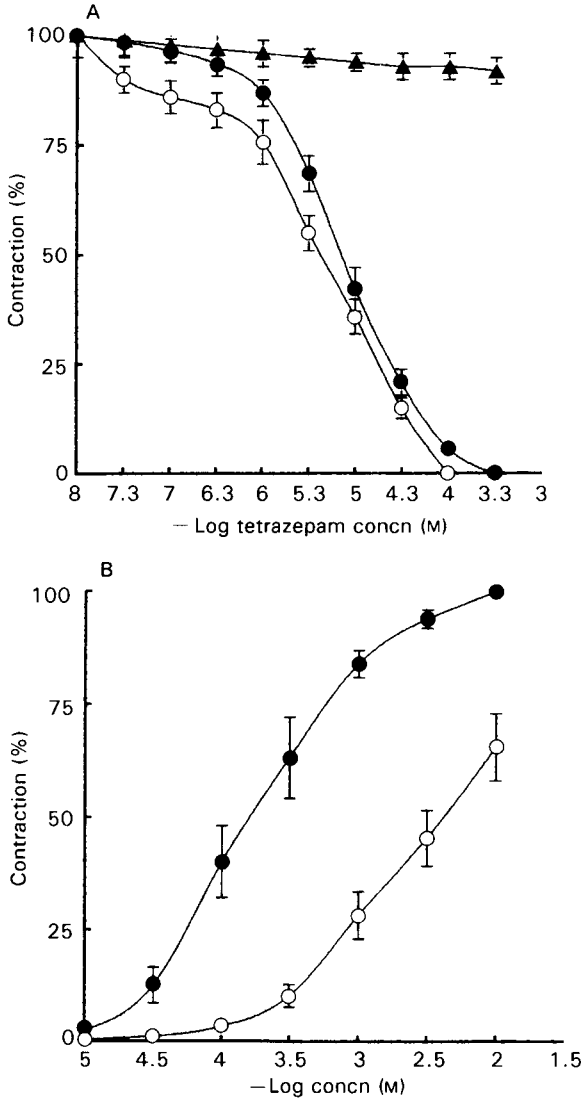

FIG. 1. A. Tetrazepam-induced relaxation in depolarized uterus. ▲ Vehicle, ● control, ○ with 1 μM PK 11195. B. Inhibition of $CaCl_2$ concentration-related contractions of rat uterus by (10^{-5} M) tetrazepam, in Ca^{2+}-free solution. ● Control, ○ with tetrazepam.

of PK 11195 (10^{-6} M). The concentration of PK 11195 used was that previously shown to block peripheral-type benzodiazepine receptors (Le Fur et al 1983). After washing, further addition of depolarizing solution induced a contractile response.

K^+-depolarized uterus in Ca^{2+}-free medium. The uterine horns were bathed for 20 min in Jalon-Ringer solution with resting tension of 0·5 g. The solution was replaced by Ca^{2+}-free high-K^+ (60 mM) solution containing EDTA (0·01 mM), with a 30-min period of frequent washing. Concentration–response cumulative curves with $CaCl_2$ (10^{-5}–10^{-2} M) were obtained for the preparations suspended in Ca^{2+}-free solution containing 0·01 mM EDTA. In these conditions control curves, and also curves in presence of tetrazepam (10^{-5} M), were obtained after a 15-min preincubation period of the tissue with this drug. Increasing the incubation time from 5 min to 15 or 30 min did not cause a more pronounced inhibitory effect on this calcium-dependent contraction.

We also studied the action of tetrazepam on oxytocin- and acetylcholine-induced contractions to ascertain what effect it has on the influx of calcium through receptor-operated calcium channels.

Oxytocin-induced rhythmic contractions

A uterine horn was incubated in Locke-Ringer solution with a resting tension of 0·5 g for 20 min, oxytocin (0·01 UI mL^{-1}) was added, inducing rhythmic contractions. When these were stable the tetrazepam (10^{-8}–10^{-4} M) was added to the organ bath.

Acetylcholine-induced contraction

The uterine horn were equilibrated for 90 min in Tyrode solution under a resting tension of 1 g. The organ was contracted submaximally by addition of acetylcholine (2×10^{-7} M). This addition induced only a single transient contraction of the uterus. The doses of tetrazepam tested were (10^{-8}, 5×10^{-8}, $2{\cdot}5 \times 10^{-7}$, 5×10^{-7} and 10^{-6} M) and in all cases these doses were added 5 min before exposure to agonist.

Vanadate-induced Ca^{2+}-free contraction

In order to determine the possible action of tetrazepam on intracellular Ca^{2+}, the organ was inmersed in Locke-Ringer solution and equilibrated for 20 min with a resting tension of 0·5 g. Subsequently, the solution was replaced by Ca^{2+}–Mg^{2+}-free solution with EDTA (0.01 mM) and the incubation was continued for 60 min. A sustained contractile response to vanadate (0·3 mM) was obtained and cumulative amounts of tetrazepam (10^{-8}-10^{-4} M) were added at 15-min intervals.

Statistical analysis

Results are expressed as the mean ± s.e.m. of 5 or more preparations (n) obtained from different animals. Statistical significance of differences between the means was assessed using Student's *t*-test for unpaired data; $P < 0{\cdot}05$ was considered to represent a significant difference. The concentration needed to produce 50% inhibition (IC50) was obtained from the regression plot and a mean IC50 ± 95% confidence interval was calculated for each dose assessed.

Results

Effects of tetrazepam on contractions induced by KCl or $CaCl_2$

Tetrazepam produced concentration–dependent relaxation in KCl-depolarized uterus. The range of concentrations was (10^{-8}–10^{-4} M) and the IC50 value was $8{\cdot}0 \pm 0{\cdot}6 \times 10^{-6}$ M (n = 6). The mean cumulative inhibitory concentration–response curves are shown in Fig. 1A and the data are presented in Table 1. In the presence of PK 11195 (10^{-6} M), tetrazepam also completely relaxed the KCl-induced contraction with an IC50 value ($8{\cdot}2 \pm 2{\cdot}3 \times 10^{-6}$ M) not significantly different from that obtained in the absence of PK 11195.

The cumulative concentration-response curves for $CaCl_2$ alone and with tetrazepam (10^{-5} M) are shown in Fig. 1B (n = 6). Tetrazepam produced rightward shifts of the cumulative concentration-response curves to $CaCl_2$ (10^{-5}–10^{-2} M) with a significant reduction at the maximal concentration: the pD_2 values were $3{\cdot}79 \pm 0{\cdot}14$ in the absence of tetrazepam and $2{\cdot}42 \pm 0{\cdot}25$ ($P < 0{\cdot}001$) in its presence.

Table 1. IC50 values (mM) of tetrazepam to inhibit the contractions induced by 0·2 μM acetylcholine, 60 mM KCl (in absence and presence of 1 μM PK 11195) and 0·3 mM vanadate.

Agonist	Tetrazepam (μM)	IC50 (μM)
Acetylcholine (0·2 μM)	0·01–1	0·25 ± 0·05
KCl (60 mM)	0·01–10 (without PK 11195)	8·00 ± 0·60
	0·01–10 (with PK 11195)	8·16 ± 2·26*
Vanadate (0·3 mM)	0·01–10	2·07 ± 0·63

* Not significant vs the value obtained without PK 11195.

Effects of tetrazepam on contractions induced by oxytocin or acetylcholine

Addition of 0·01 units mL^{-1} oxytocin to the uterine horn induced a rhythmic contractile response with stable freuency and amplitude. The addition of cumulative doses (10^{-8}–10^{-4} M) of tetrazepam did not modify either frequency or amplitude (data not shown).

The addition of acetylcholine (2×10^{-7} M) induced a single transient submaximal uterine contraction (70% Emax). In the presence of tetrazepam this contraction was inhibited in a dose-dependent manner (Fig. 2) and the IC50 value was $2.50 \pm 0.05 \times 10^{-7}$ M (Table 1).

Effects of tetrazepam on vanadate-induced Ca^{2+}-free contraction

Vanadate (0·3 mM) induced a sustained response as long as the uterus was exposed to the agonist. When tetrazepam was added in cumulative amounts (10^{-8}–10^{-4} M), dose-dependent relaxation was obtained (Fig. 3) and the IC50 value was $2.1 \pm 0.6 \times 10^{-6}$ M (Table 1).

Discussion

The aim of the present work was to examine the mechanism of tetrazepam-induced relaxation in rat uterine smooth muscle. In order to do this, we tested the action of tetrazepam in different experimental models, some involving Ca^{2+} influx by voltage-operated channels (VOCs) and the others involving the opening of receptor-operated Ca^{2+} channels (ROCs).

Our results have shown that when extracellular calcium is present, tetrazepam inhibits sustained K^+-induced contraction and that in Ca^{2+}-free solution tetrazepam inhibits cumulative doses of $CaCl_2$ in depolarized uterus. This behaviour indicates that the relaxant mechanism of tetrazepam may be mainly related to Ca^{2+} influx, possibly through VOCs.

It has been demonstrated that a variety of agents interact with Ca^{2+} channels while having another primary site of action. This is the case of benzodiazepine-receptor ligands (Godfraind et al 1986). Also, it has been known that the rat peripheral-type benzodiazepine receptor has low affinity for benzodiazepine ligands, and exhibites high affinity for PK 11195 (Parola et al 1991). The relaxant effects of tetrazepam appear to be unrelated to peripheral-type benzodiazepine receptors because pretreatment with PK 11195 did not modify this effect. Further support for this conclusion comes from observations that peripheral-type benzodiazepine receptors were recently localized in the mitocondrial outer membrane (Anholt et al 1986; French et al 1989) whereas the VOC and ROC are localized in the cell membrane.

The mechanism underlying the rise in cytoplasmic Ca^{2+} induced by acetylcholine and oxytocin is complex and probably involves both voltage-dependent and receptor-operated calcium channels (Bolton 1979; Edwards et al 1986; Savineau et al 1990), as well as release of intracellular Ca^{2+} ions (Villar et al 1986; Anselmi et al 1987; D'Ocón et al 1987). Tetrazepam relaxes uterine contraction induced by acetylcholine in a dose-dependent manner, which is mediated in part through receptor-operated calcium channels, but did not modify oxytocin-induced rhythmic contractions. Contractile responses

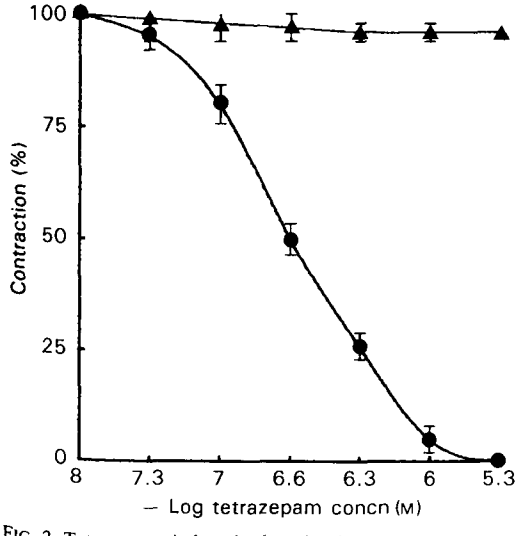

FIG. 2. Tetrazepam-induced relaxation in rat uterus precontracted with acetylcholine (2×10^{-7} M). ▲ Vehicle, ● with tetrazepam. Each value is expressed as a percentage of the initial maximal contraction in response to the agonist. Each data point represents the mean ± s.e.m.of at least six measurements.

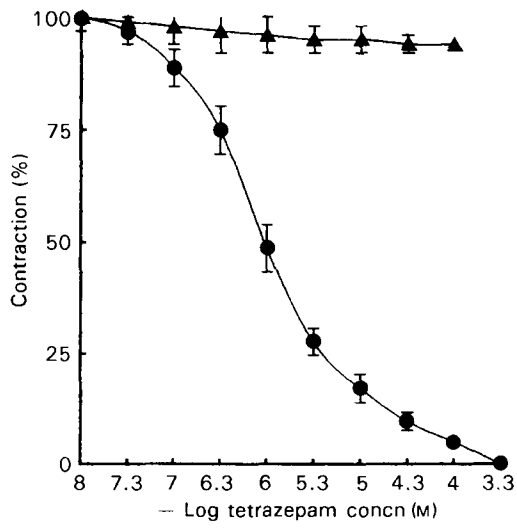

FIG. 3. Tetrazepam-induced relaxation in rat uterus precontracted with vanadate in Ca^{2+}-free EDTA-containing solution. ▲ Vehicle, ● with tetrazepam. Each data point represents the mean ± s.e.m. of at least six measurements.

induced by oxytocin involve the activation of a specific receptor, increasing phosphatidylinositol turnover and the subsequent release of Ca^{2+} from intracellular stores, and the opening of receptor-operated Ca^{2+} channels, according to Edwards et al (1986), and depend partially on the release of eicosanoids (Hall 1983; Hidalgo et al 1987). It has been demostrated that oxytocin induces a more specific release of intracellular Ca^{2+} than does vanadate (D'Ocón et al 1989). Tetrazepam has no effect on the release of Ca^{2+} from intracellular stores sensitive to oxytocin, but relaxed vanadate contractions in Ca^{2+}-free solution in a dose-dependent manner. This behaviour indicates that the relaxant effects of tetrazepam may be mainly related to the release of intracellular calcium from stores sensitive to vanadate. Moreover, the sustained contraction induced by vanadate of rat uterus in Ca-free EDTA-containing solution can be related to the release of Ca^{2+} from intracellular storage sites (D'Ocón et al 1989) and this action can be explained by the results of previous studies showing vanadate to be a potent inhibitor of Ca-Mg ATPase (Mironneau et al 1984; Nechay 1984) and Na-K ATPase (Grover et al 1981). Therefore, the relaxant action of tetrazepam may interfere intracellularly with the mechanism of vanadate but not with that involving oxytocin. It is probable that this mechanism is ATP-dependent and is not related to phosphatidylinositol turnover.

The present findings suggest that tetrazepam relaxes contractile responses induced by activation of VOCs and ROCs by a non-selective mechanism, since the range of doses needed to inhibit KCl- and acetylcholine-induced contraction was similar in both cases. Micromolar concentrations of tetrazepam appear to be required in order to attain the uterine-relaxant effect. This effect is unrelated to the high-affinity benzodiazepine binding site and is probably mediated through inhibition of the calcium channels. Tetrazepam presents a sufficient structural similarity with a calcium antagonist (diltiazem) and may interact with the diltiazem site associated with the Ca^{2+} channels. It is possible that tetrazepam reduces calcium entry at a number of different channels or that it antagonizes the effect of calcium at a later stage, possibly an intracellular site, and that inhibition of contraction of rat uterus is not related to the high-affinity PBR. This finding may be of some clinical relevance since benzodiazepines have been reported to attain high levels in plasma (Kaplan 1980) and we noticed that tetrazepam at micromolar concentrations affects uterine muscle tone.

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