Uterine Relaxant Effect of Zolpidem: A Comparison with Other Smooth Muscle Relaxants

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Zolpidem is an imidazopyridine sedative-hypnotic which interacts with central benzodiazepine-receptors. To examine its effects on uterine smooth muscle we have compared with those obtained by diltiazem, papaverine and diazepam on different experimental models.

The IC_{50} values obtained indicate similar behaviour of zolpidem and diazepam. They showed more active against the spontaneous contractions and those induced by KCl (60 mM) or by $CaCl_2$ (0.01–10 mM) in Ca^{2+}-free depolarizing medium than against acetylcholine (0.1 mM)-induced contractions. Both of them also showed more effectiveness against the tonic component of the acetylcholine-evoked contraction than against the phasic one. All the drugs tested were less powerful against contractions induced by oxytocin than against those induced by other agonists.

This observation let us speculate that the mechanism of action of zolpidem may be related to an action on Ca^{2+} influx through voltage-dependent Ca^{2+}channels due to an interaction with low affinity receptor located at the plasmalemma as has been suggested for diazepam.

Introduction

Zolpidem is a new, short-acting, imidazopyridine sedative-hypnotic. Like the benzodizepine (BZD) agents, zolpidem interacts with central benzodiazepine-receptors to potentiate GABAergic transmission. However, unlike the traditional hypnotics, it does so selectively, demonstrating a much greater affinity for the ϖ_1 receptor subtype (Langer *et al.*, 1988).

It has been demonstrated that benzodiazepine-receptor ligands interact with Ca^{2+} channels in peripheral tissues, while having another primary site of action (Godfraind *et al.*, 1986).

In an attempt to clarify the mechanism of action of zolpidem in the peripheral tissues, we have compared its inhibitory potency with diltiazem, a classic calcium antagonist; papaverine, a non-specific smooth muscle relaxant and diazepam, a benzodiazepine which has affinity for both peripheral and central benzodiazepine-receptor. We have assayed the uterine relaxants effect of zolpidem in comparison with these drugs on the spontaneous contractions and on those induced by KCl, acetylcholine, oxytocin or $CaCl_2$ in Ca^{2+}-free medium.

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Material and Methods

Preparation of uterine horns

Uterine horns were obtained from virgin female Wistar rats (180–200 g) kept in room with controlled temperature (22 °C). The animals were treated with β-estradiol benzoate (0.5 mg/kg,) 24 h before the experiments and killed by a blow on the head. One segment of each uterine horn was removed and mounted in a 10 ml organ bath filled with physiological solution bubbled with a mixture of 95% O_2- 5% CO_2 and maintained at 31 °C.

Drugs and solutions

Zolpidem (N,N,6- trimethyl-2- (*p*-tolyl)-imidazo [1,2–9] pyridine-3-acetamide) hemitartrate (Synthelabo, Paris), diltiazem (cis-(+)-3- (acetyloxy)-5-[2-(dimethyl-amino)ethyl]-2,3- dihydro-2- (4-methoxy-phenyl)-1,1-benzothiazepin- 4 (5H) one) hydrochloride, papaverine (6,7 dimethoxy- 1- veratrylisoquinoline) hydrochloride, diazepam (7-chloro-1,3-dihydro-1-methyl- 5[phenyl- ds] -2H-1,4- benzodiazepin-2- one), acetylcholine chloride, oxytocin (α-hypophamine) and β-estradiol 3-ben-

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zoate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

All drugs reagents were dissolved in distilled water, except zolpidem and diazepam which were dissolved previously in dimethyl sulfoxide (DMSO) and further diluted in distilled water. Controls received the corresponding solvent.

The following physiological solutions were used: Jalon-Ringer solution (mM): NaCl 154, KCl 5.63, $CaCl_2$ 0.65, $NaHCO_3$ 5.95, glucose 2.77 (this solution has a low calcium concentration to reduce the interference of spontaneous contractile activity of the uterus preparation). Ca^{2+}-free Jalon-Ringer solution was prepared as for Jalon-Ringer except for the omission of $CaCl_2$. Locke-Ringer solution (mM): NaCl 154, KCl 5.63, $CaCl_2$ 2.16, $NaHCO_3$ 5.95, $MgCl_2$ 2.10, glucose 5.55.

Experimental procedure

Spontaneous contractions. The uterine horn was immersed in Locke-Ringer solution with a resting tension of 0.5 g until stabilization of spontaneous contractions was reached. This solution containes Ca^{2+} physiological concentrations so that the uterus shows spontaneous activity (Ivorra *et al.*, 1993).

K^+-depolarized uterus. The organ was immersed in Jalon-Ringer solution and equilibrated for 20 min with basal tension of 1 g, then KCl (60 mM) was added. This addition caused a rapid contraction, followed by a slight relaxation and a prolonged contraction. In these experimental conditions, cumulative doses of drugs were administered and dose-related relaxations could be observed.

$CaCl_2$ induced contractions in a depolarizing Ca^{2+}-free medium. To asses the effects of these drugs on the influx of Ca^{2+} through voltage-sensitive channels, Ca^{2+} dose-response curves were established according to Godfraind *et al.* (1968) and Weiss (1981). The uterine horns were incubated for 20 min in Jalon-Ringer solution, then for 1 h in Ca^{2+}-free solution. The preparations were washed at intervals of 15 min. Before adding $CaCl_2$, tissues were exposure to a single dose of KCl (60 mM), in these conditions, this addition did not cause contraction. Two cumulative concentration-response curves to $CaCl_2$ (0.01 to 10 mM) were obtained at 60 min intervals in each preparation (Van Rossum, 1963). After obtaining the first curves, washing until complete relaxation, different concentration of diltiazem (0.01, 0.1 and 1 μM), papaverine (1, 10 and 100 μM), diazepam (1, 10 and 100 μM) and zolpidem (1, 10 and 100 μM) were added to the bath and left in contact with the tissue for 20 min. Then, a second cumulative concentration-response curve to $CaCl_2$ in presence of the tested drugs was obtained.

Each antagonist was tested in separate horns and control experiments were performed using only $CaCl_2$ in the absence of antagonists. The maximal contraction obtained with the first dose-response curve to $CaCl_2$ was taken as 100% and all contractions calculated as a funtion of this value. Each preparation was expose to only one concentration of the antagonist.

Acetylcholine-induced contractions. A uterine horn was incubated in Jalon-Ringer solution with a resting tension of 1 g for 20 min. Acetylcholine (0.1 mM) was added, which induced an initial phasic contraction followed by a plateau with small rhythmic contractions. After obtaining two succesive responses which were almost identical, two experimental procedures were performed as above:

a) Cumulative amounts of diltiazem (0.003–10 μM), papaverine (0.01–3 μM), diazepam (1–300 μM) and zolpidem (0.1–100 μM) were added when the contractile response to acetylcholine was reached. After washing, another addition of acetylcholine induced a contractile response.

b) A control contraction was obtained by addition of acetylcholine 0.1 mM; after washing, the drugs tested were added 15 min before the second addition of acetylcholine. The doses used were: diltiazem (0.01, 0.1 and 1 μM), papaverine (10, 30 and 100 μM), diazepam (30, 100 and 300 μM) and zolpidem (30, 100 and 300 μM).

Oxytocin-induced rhythmic contractions. The uterine horn was incubated in Locke-Ringer solution with a resting tension of 0.5 g for 20 min. Oxytocin (0.01 units ml^{-1}) was added and rhythmic contractions were induced by this agonist. Cumulative amounts of the drugs tested were added to the organ bath. The doses used were: diltiazem (0.1–100 μM), papaverine (0.1–50 μM), diazepam (0.1–300 μM) and zolpidem (0.1–1000 μM).

Statistical analysis

The results were expresed as a percentage of the maximum effect (E_{max}) obtained by agonist addition. A regression of responses against -log C of test compound was performed by the least squares method for each preparation. The concentration needed to produced 50% inhibition (IC_{50}) was obtained from the regression plot and a mean IC_{50} ± 95% confidence interval was calculated for each dose assessed. Results are expressed as the mean value and S. E. M. of 6–8 preparations. The significance of differences between values was determined with an one-way analysis of variance (ANOVA) followed by LSD (least significant difference) test. P values smaller than 0.05 were regarded as significant.

Results and Discussion

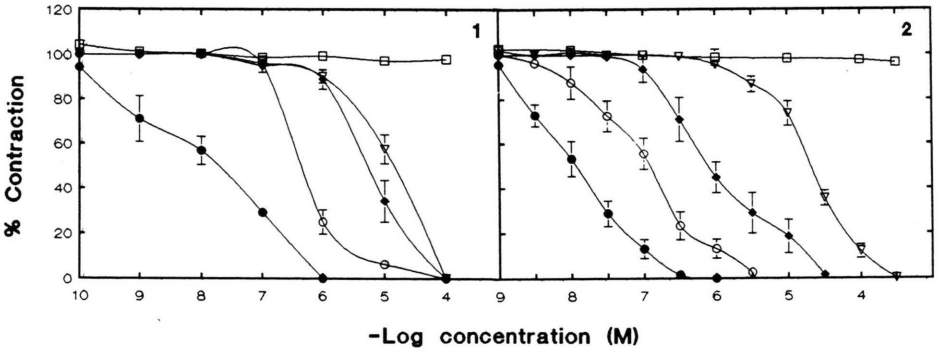
Effects on spontaneous uterine contractions

The addition of cumulative concentrations of diltiazem (0.0001–1 μM), papaverine (0.1–1 μM), diazepam (0.1–100 μM) and zolpidem (0.1–100 μM), diminished both the frequency and the amplitude (represented as % contraction in Fig. 1) of the spontaneous contractions of rat uteri inmersed in Locke-Ringer solution. Papaverine, diazepam and zolpidem at 100 μM, abolished the uterine spontaneous activity. Diltiazem 1 μM also produced a 100% relaxation of the spontaneous contractions.

The IC_{50} values are summarized in Table I. The rank order of potencies of these agents was: diltiazem > papaverine > diazepam > zolpidem. After washing, the contractile response was restored except for the experiments with diltiazem (data not shown).

Relaxant effects of drugs tested on K^+-depolarized rat uterus

All drugs produced dose-dependent relaxations in KCl-depolarized uterus. Fig. 2 shows these dose-response curves and Table I summarizes IC_{50} for each drug tested and the rank of order of potencies of these was: diltiazem > papaverine > diazepam > zolpidem. All the agents tested produced a 100% relaxation of the contraction caused by KCl.



Figs. 1+2. Concentration-response curves of inhibition of spontaneous contractions (1) or KCl 60 mM-evoked contractions (2) obtained after the addition of diltiazem (●), papaverine (○), diazepam (◆) and zolpidem (▽). Vehicle control (□) .

Table I. Values of IC_{50} of diltiazem, papaverine, diazepam and zolpidem against spontaneous contractions (Spont.), and induced by KCl (60 mM), acetylcholine (ACh 0.1 mM) and oxytocin (Oxy 0.01 units ml^{-1}), obtained from the regression plot and expressed as the mean value and S. E. M. of n experiments.

Drugs	n	IC_{50} [M] Spont.	KCl	ACh	Ox
Diltiazem	7	$2.12 \pm 0.81 \times 10^{-8}$	$2.02 \pm 0.68 \times 10^{-8}$	$2.12 \pm 0.63 \times 10^{-7}$	$3.15 \pm 0.91 \times 10^{-6}$
Papaverine	8	$7.13 \pm 0.67 \times 10^{-7}$	$2.42 \pm 0.49 \times 10^{-7}$	$7.28 \pm 2.24 \times 10^{-7}$	$2.21 \pm 0.14 \times 10^{-5}$
Diazepam	6	$7.33 \pm 1.50 \times 10^{-6}$	$2.50 \pm 1.27 \times 10^{-6}$	$3.48 \pm 0.77 \times 10^{-5}$	$4.24 \pm 1.47 \times 10^{-5}$
Zolpidem	8	$6.23 \pm 0.22 \times 10^{-6}$	$2.67 \pm 0.39 \times 10^{-5}$	$4.29 \pm 1.36 \times 10^{-6}$	$1.47 \pm 0.35 \times 10^{-4}$

Effect of preincubation with the drugs on $CaCl_2$-induced contractions in a depolarizing Ca^{2+}-free medium

Cumulative concentration-response curves in response to $CaCl_2$ (0.01 to 10 mM) on rat uteri immersed in K^+-depolarizing Ca^{2+}-free solution were reproducible at 60 min intervals. Fig. 3 shows the mean cumulative concentration-response curves for $CaCl_2$ alone and in the presence of different concentrations of diltiazem (0.01, 0.1 and 1 μM), papaverine (1, 10 and 100 μM), diazepam (1, 10 and 100 μM) and zolpidem (1, 10 and 100 μM). The ED_{50} and E_{max} values are summarized in Table II. Papaverine and diltiazem produced a parallel and concentration-dependent rightward displacement of the dose-response curve to $CaCl_2$ with significantly reducing the maximal response with all doses tested.These data indicate that these drugs block $CaCl_2$-induced contraction non-competitively. Diazepam and zolpidem only at 100 μM produced displacement of the dose-response curve with significantly reducing the maximal response.

Relaxant effects on acetylcholine-induced contraction of rat uterus

Addition of diltiazem (0.003–10 μM), papaverine (0.01–30 μM), diazepam (1–300 μM) and zolpidem (0.1–100 μM), during the plateau of contraction by acetylcholine (0.1 mM), produced dose-dependent relaxation; hence, dose-response curves were constructed by addition of cumulative doses of these drugs. These dose-response curves are represented in Fig. 4. The IC_{50} values are summarized in Table I. The rank of order of potencies: diltiazem > papaverine > zolpidem > diazepam. After washing, a new additon of acetylcholine (0.1 mM) produced a contraction that was similar to the first except for the experiences using diltiazem in this case, the second contraction was significantly different in magnitude and morphology from the first.

When the uterus was preincubated with different doses of the agents tested: diltiazem (0.01, 0.1 and 1 μM), papaverine (10, 30 and 100 μM), diazepam (30, 100 and 300 μM) and zolpidem (30, 100 and 300 μM), 15 min before the addition of acetylcholine (0.1 mM), inhibition of the phasic

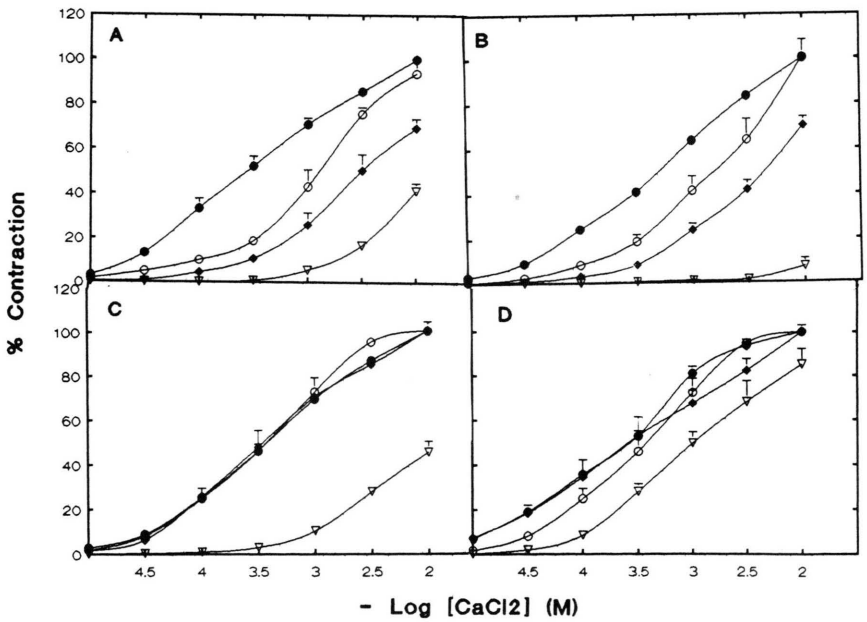
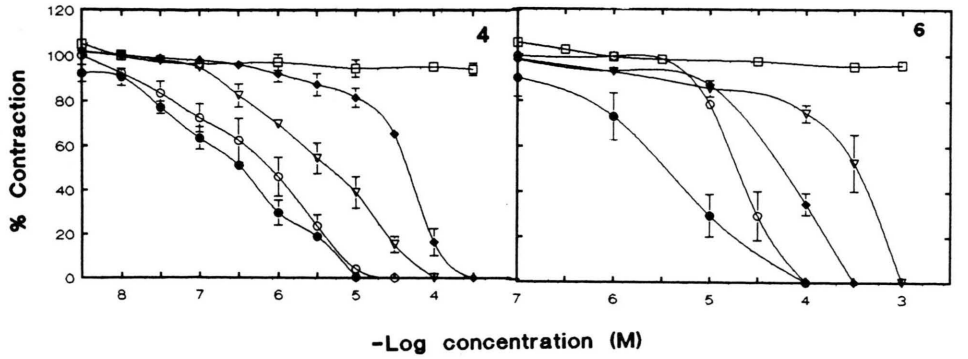


Fig. 3. $CaCl_2$ -induced contraction in Ca^{2+}-free medium. Control (●) or in presence of diltiazem (A) 0.01 μM (○), 0.1 μM (◆) and 1 μM (▽), papaverine (B) 1 μM (○), 10 μM (◆) and 100 μM (▽), diazepam (C) 1 μM (○), 10 μM (◆) and 100 μM (▽) and zolpidem (D) 1 μM (○), 10 μM (◆) and 100 μM (▽).



Figs. 4+6. Dose response relaxation curves in uterus previously contracted with acetylcholine 0.1 mM (4) or oxytocin 0.01 units ml^{-1} (6) obtained after the addition of different agents, diltiazem (●), papaverine (○), diazepam (◆) and zolpidem (▽). Vehicle control (□).

Table II. Parameters of dose-response curves of contraction induced by cumulative doses of $CaCl_2$ (0.01–10 mM) in Ca^{2+}-free depolarizing medium, in the absence (control) or in the presence of diltiazem 0.01 μM, 0.1 μM and 1 μM; papaverine 1 μM, 10 μM and 100 μM; diazepam 1 μM, 10 μM and 100 μM and zolpidem 1 μM, 10 μM and 100 μM. Values of ED_{50} were obtained from the regression plot. Maximal effect (E_{max}) values obtained as a percentage with respect to control curve. Values expressed as the mean value and S. E. M. of n experiences. * $p < 0.01$, ** $p < 0.05$.

Drugs	*n*	E_{max}(%)	ED_{50} [M]
$CaCl_2$	–	–	$3.09 \pm 0.15 \times 10^{-4}$
$CaCl_2$+Diltiazem			
0.01 μM	6	93.67±7.85	$1.21 \pm 0.61 \times 10^{-3}$*
0.1 μM	6	69.36±4.13	$4.71 \pm 1.79 \times 10^{-3}$**
1 μM	6	41.38±3.39	$2.83 \pm 0.71 \times 10^{-2}$**
$CaCl_2$+Papaverine			
1 μM	7	110.36±7.85	$1.65 \pm 0.36 \times 10^{-4}$*
10 μM	6	70.06±3.91	$4.18 \pm 0.44 \times 10^{-3}$**
100 μM	8	7.14±3.79	
$CaCl_2$+Diazepam			
1 μM	7	107.89±2.04	$4.00 \pm 1.31 \times 10^{-4}$
10 μM	6	100.29±3.88	$3.76 \pm 0.35 \times 10^{-4}$
100 μM	6	45.39±4.88	$2.63 \pm 0.89 \times 10^{-2}$**
$CaCl_2$+Zolpidem			
1 μM	8	102.44±3.75	$3.31 \pm 1.91 \times 10^{-4}$
10 μM	6	108.91±3.20	$4.29 \pm 1.17 \times 10^{-4}$
100 μM	6	85.09±7.17	$1.76 \pm 0.31 \times 10^{-3}$*

peak and of tonic contraction with respect to the contractile response obtained in the absence of these drugs, were observed. However the tonic response was more sensitive than the phasic one (Fig. 5).

Modification of uterine response to oxytocin

Addition of oxytocin 0.01 units ml^{-1} to uterine horn incubated in Locke-Ringer solution induced rhythmic contractile response with stable frequency and amplitude. The addition of cumulative amounts of diltiazem (0.1–100 μM), papaverine (0.1–50 μM), diazepam (0.1–300 μM) and zolpidem (0.1–100 μM), diminished both the frequency and amplitude of the contractions. Fig. 6 shows the decrease of the amplitude of contraction (% contraction). The parameters of these curves are summarized in Table I. All the agents at the higher dose tested completely abolished the contractile response to oxytocine 0.01 units ml^{-1}. After washing, complete recovery of rhythmic contractions induced by oxytocin was observed except for the experiments carried out with diltiazem.

The IC_{50} values obtained indicate less effectiveness of all tested drugs against acetylcholine on spontaneous contractions and on those evoked by KCl, although they are smaller than those obtained against oxytocin. Diltiazem, diazepam and zolpidem were more active against the tonic component than against the phasic phase of the acetylcholine-induced contraction; this results suggest a major influence on the Ca^{2+} influx from extracellular medium through VOCs. On the other hand, papaverine was less specific inhibiting the acetylcholine-induced contraction, this result is in according with its mechanism of action (Cumiskey and Feigenson, 1983).

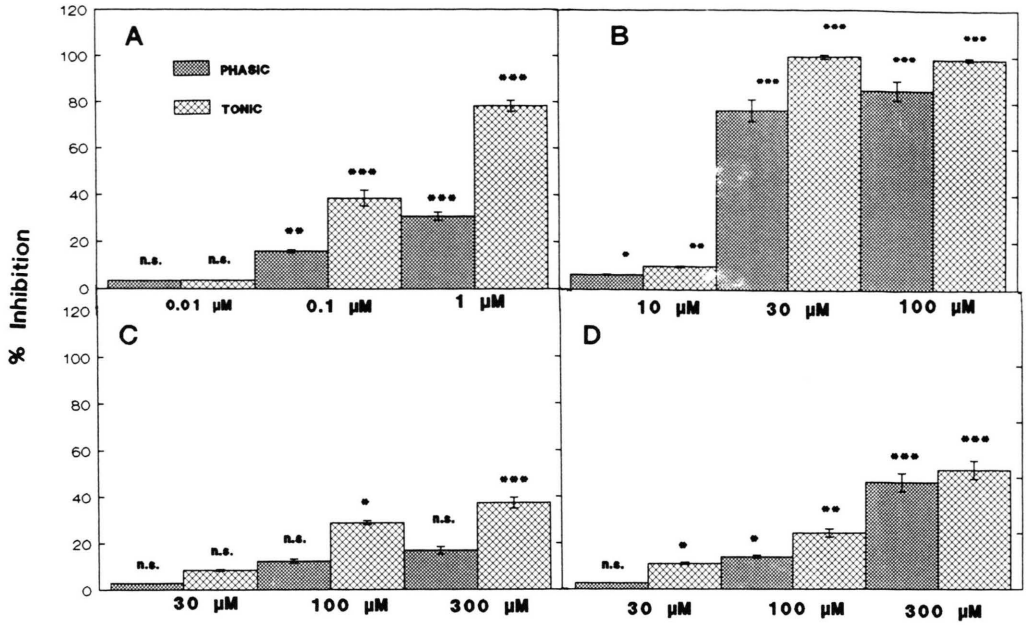


Fig. 5. Inhibitory effects in presence of diltiazem (A), papaverine (B), diazepam (C) and zolpidem (D) on the contractile response evoked by acetylcholine 0.1 mM. The results are expressed as a percentage of the response induced by the agonist before treatments. Histograms represented are means of 6–8 observations with SE mean shown by vertical bars. Signifficant differences from control tissues: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Diltiazem is a calcium antagonist with selectivity for the entry of calcium through the VOCs (Cauvin *et al.*, 1983; Hurwitz, 1986; Nayler and Horowitz, 1983). Besides, the most of the calcium entry blockers have an additional intracellular site of action, related to an increase of Ca^{2+} efflux or to stimulation of Ca^{2+} uptake (Spedding, 1983; Saida and Van Breemen, 1983; Cohen *et al.*, 1984), this justify the inhibitory potency of diltiazem on acetylcholine and oxytocin-induced contractions. Diltiazem appeared like the most powerful drug tested with a great difference in these experiences independent of the agonist used to contract the uterus, and was the only able to inhibit irreversiblely the uterine contractions.

Papaverine is a non-specific smooth muscle relaxant (Cumiskey and Feigenson, 1983) that increase the efflux and uptake of Ca^{2+} by intracellular organelles (Imai and Kitagwa, 1981; Koike and Takayanagi, 1981; Huddart *et al.*, 1984). This action could be related to an increase in cAMP that leads to the accumulation of calcium in endoplasmic reticulum and induces phosphorilation of myosin light chain kinase, which causes smooth muscle relaxation (Cumiskey and Feigenson, 1983; Calixto and Loch, 1985).

Benzodiazepines have been reported to have relaxant effects on different preparations of smooth muscle as the guinea-pig trachea (Raeburn *et al.*, 1988; Herrera *et al.*, 1996), the rat aorta (French *et al.*, 1988; Pérez-Guerrero *et al.*, 1996a), the skinned rat urinary bladder (Marti-Cabrera *et al.*, 1994) and the rat uterus (Kazanietz and Elghyhen, 1990; Pérez-Guerrero *et al.*, 1996b). It has been demonstrated that benzodiazepines interact with Ca^{2+} channels while having another primary site of action (Godfraind *et al.*, 1986). Diazepam is an agonist with affinity for both peripheral and central sites (Schoemaker *et al.*, 1983).

Diazepam showed more activity against the spontaneous contractions and those induced by KCl (60 mM) or $CaCl_2$ in Ca^{2+}-free medium and this behaviour was likely to zolpidem. This data support that the mechanism underlying by micromolar concentrations inhibition of different benzodiazepines is an action on Ca^{2+} influx through Ca^{2+} channels due to an interaction with low affinity receptor located at the plasmalemma of

different isolated preparations (Raeburn *et al.*,1988; De Lorenzo *et al.*, 1981; Fares *et al.*, 1987).

The present findings suggest that zolpidem shows a similar behaviour to diazepam on rat uterus and the the mechanism underlying the relaxant effect of zolpidem may be similar to diazepam and this hypotesis could explain our results and our current studies.

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