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Synergistic interaction of diazepam with 3′,5′-cyclic adenosine monophosphate-elevating agents on rat aortic rings

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Abstract

We investigated the effect of the phosphodiesterase type 4 (PDE4) inhibitory activity of diazepam on the arterial wall. To this purpose, we examined the interaction of diazepam with 3′,5′-cyclic adenosine monophosphate (cyclic AMP)-elevating agents on vasodilatation and cyclic AMP levels in rat aortic rings precontracted with phenylephrine. The involvement of benzodiazepine receptors was also studied. Diazepam (5–100 μM) produced a relaxation of this preparation which was neither mimicked by γ-aminobutyric acid (GABA), nor antagonized by flumazenil and 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195), inhibitors of central or peripheral type benzodiazepine receptors, respectively. The diazepam-induced relaxation was potentiated by the presence of isoprenaline (10 nM), forskolin (50 nM) or milrinone (0.1 μM). Furthermore, diazepam increased the enhancement of cyclic AMP levels induced by these three agents in this tissue. Our results demonstrate a functional and biochemical synergistic interaction of diazepam with cyclic AMP-elevating agents on rat aortic rings.

Keywords: Diazepam; cAMP; Phosphodiesterase inhibitor; Benzodiazepine receptor; Isoprenaline; Aortic ring, rat

1. Introduction

Diazepam has been reported to have a direct relaxant effect on isolated blood vessels (Kazanietz and Elgoyhen, 1990) and to reduce blood pressure in animals (Daniell, 1975) as well as in human subjects (Sunzel et al., 1988). However, the mechanism responsible for these effects has not been established (French et al., 1989; Kazanietz and Elgoyhen, 1990).

We recently demonstrated that diazepam exerts an inhibitory activity on cyclic nucleotide phosphodiesterase type 4, which is involved in the breakdown of 3′,5′-cyclic adenosine monophosphate (cyclic AMP) (Collado et al., 1998). This has functional relevance since a synergistic interaction has been shown between diazepam and cyclic AMP-producing agents in tissues where phosphodiesterase 4 is present, such as the heart of rat (Martínez et al., 1995) and guinea-pig (Hara et al., 1998) or guinea-pig eosinophils (Collado et al., 1998).

It is well known that many agents able to elevate intracellular levels of cyclic AMP can induce vasodilatation (Murray, 1990). Intracellular accumulation of cyclic AMP can be induced by drugs which inhibit cyclic nucleotide phosphodiesterases, mainly phosphodiesterase 3 and phosphodiesterase 4 since they preferentially hydrolyse cyclic AMP in the arterial wall (Beavo et al., 1994; Rose et al., 1997). In fact, relaxant responses have been obtained in rat aortic rings after inhibition of phosphodiesterase 3 or phosphodiesterase 4 (Komas et al., 1991). Therefore, the inhibitory activity on phosphodiesterase 4 exerted by diazepam could be involved in the vascular effects of this drug. To examine this hypothesis, we studied the effect of diazepam on the smooth muscle of the rat aorta, where phosphodiesterase 4 is present (Rose et al., 1997). The possible involvement of benzodiazepine receptors of the central type, which are coupled to γ-aminobutyric acid (GABA)-activated Cl^- channels, as well as of the peripheral type, which are not linked to the GABA receptor complex (Teuber et al., 1999; Parola and Yamamura, 1993), was also investigated.

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2. Materials and methods

Sprague–Dawley rats of either sex (250–350 g) were decapitated. The thoracic aorta was removed, cleaned of extraneous connective and fatty tissue and cut into rings 3 to 5 mm long. Isometric contractile responses were determined by placing the rings in organ baths containing 30 ml Krebs solution of the following composition (mM): NaCl 118, KCl 5, $CaCl_2$ 1.25, $MgSO_4$ 1.19, KH_2PO_4 1.14, $NaHCO_3$ 25 and dextrose 10. Tissues were then maintained under an initial resting tension of 2.0 g and allowed to equilibrate for 2 h before addition of test compounds. Tension was measured isometrically with a force transducer (FT-03, Grass Instrument, Quincy, MA, USA).

In some aortic rings, the endothelium was removed by gently rubbing the inner surface with a wooden stick. Endothelium removal was confirmed by the lack of relaxation in response to 20 μM acetylcholine in rings precontracted with 1 μM phenylephrine. In contrast, acetylcholine 20 μM induced complete relaxation of endothelium-bearing preparations.

2.1. Experimental protocols

Tissues were contracted with 1 μM phenylephrine. When the contractile response reached a steady state, diazepam was added cumulatively. The relaxation produced by each concentration of diazepam was measured 5 min later and the value was expressed as a percentage of the initial phenylephrine-induced tone.

In experiments where interactions with benzodiazepine receptor-related agents were studied, each agent was left in contact with the tissue for 10 min before the cumulative concentration-curve for diazepam was recorded. The role of nitric oxide (NO) in the effect of diazepam was studied with N^w-L-arginine methyl ester (L-NAME), and that of the endothelium was studied by comparing the effect of diazepam in rat aortic rings with and without endothelium. L-NAME was added at the concentration of 300 μM to each bath 10 min before the addition of phenylephrine.

For the study of the effects of diazepam in combination with some cyclic AMP- related agents, an appropriate concentration of isoprenaline, forskolin or milrinone was added when the contraction elicited by phenylephrine had stabilized, i.e. after 20 min. Ten minutes later, a concentration–response curve for diazepam was determined.

2.2. Measurement of cyclic AMP

Concentrations of cyclic AMP were measured by radioimmunoassay (^{125}I-tyrosine-methylester-succinyl-cyclic AMP, Diagnostic Pasteur, France), according to the manufacturer's instructions. These experiments were carried out with groups of rings of thoracic rat aorta. Each ring was taken from a different animal. The production of cyclic AMP induced by a 2-min exposure to isoprenaline, forskolin or milrinone was measured either under control conditions or in the presence of 10 μM of diazepam added 5 min before and during the exposure. After incubation with drugs, the tissue was immediately frozen. Then, the preparation was weighed and homogenized in cold perchloric acid 1.5 ml (0.3 mol l^{-1}) with a Polytron homogenizer and centrifuged (12,000 rpm, 4 °C, 15 min). The supernatants were treated with potassium phosphate until pH 6.2 was reached. The sensitivity of the assay was 2 pmol ml^{-1}. Intra- and inter-assay coefficients of variation were 7.7% and 8.2%, respectively. The antibody cross-reacted 100% with 3′,5′-cyclic AMP and less than 0.3% with other nucleotides. Cyclic AMP concentrations are expressed as pmol g^{-1} of tissue.

2.3. Drugs and chemicals used

Diazepam and flumazenil were generously supplied by Roche (Madrid, Spain). Forskolin, isoprenaline, milrinone, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-Methylpropyl)-3-isoquinolinecarboxamide (PK 11195), γ-aminobutyric acid (GABA) and N^w-L-arginine methyl ester (L-NAME) were obtained from Sigma (Madrid, Spain) and dimethyl sulphoxide (DMSO) was obtained from Probus, Barcelona, Spain.

Isoprenaline, GABA and L-NAME were freshly dissolved in normal Tyrode solution. Ascorbic acid (1 μg ml^{-1}) was added to the isoprenaline solution to prevent oxidation. Diazepam, flumazenil, PK 11195 and milrinone were dissolved in DMSO and Tyrode solution (2 DMSO:8 Tyrode); this stock was diluted into prewarmed and preaerated bathing solution to achieve the desired final concentration.

2.4. Statistical analysis

Results are expressed as mean values ± S.E.M. Responses are expressed as a percentage of the maximum increase in tension. Student's *t*-test or one-way analysis of variance (ANOVA), followed by Tukey's method for multiple comparisons were used. The criterion for significance was that P values should be less than 0.05.

3. Results

3.1. Effect of diazepam on phenylephrine-induced contractions of rat aortic rings

Fig. 1 shows an example of the response to the addition of diazepam to the bathing solution of an aortic ring that had been precontracted with 1 μM phenyleprine. As shown, diazepam caused a relaxant effect in this preparation. Similar experiments were carried out on seven prepara-

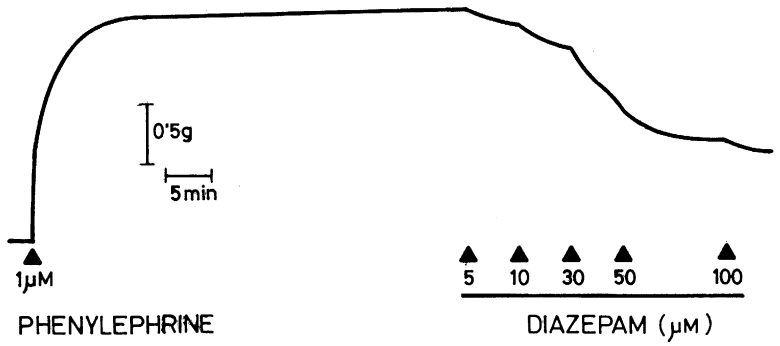

Fig. 1. Isometric force recording, from a representative experiment, showing the effect of the addition of diazepam to the organ bath containing a ring of rat aorta precontracted with phenylephrine.

tions and the results are summarized in Fig. 2. As can be seen, diazepam (5–100 μM) produced a concentration-dependent relaxant effect and the 50% relaxation (IC_{50}) was attained at 69.0 ± 6.5 μM. DMSO, the vehicle of diazepam, produced per se a 9% and 14% relaxation at concentrations of 0.3% and 0.6%, respectively. These concentrations of DMSO were equivalent to those achieved when 50 and 100 μM of diazepam were used.

To establish whether the diazepam-induced relaxation of phenylephrine- precontracted rat aortic rings could be attributed to activation of the benzodiazepine/GABA/channel Cl^- receptor complex, we studied whether this effect is mimicked by GABA or prevented by the antagonist of this complex, flumazenil. GABA (1–100 μM) did not elicit any effect on this preparation. Also, flumazenil (5 μM), which is devoid of effect when applied alone, did not counteract the relaxant effect of diazepam, and the IC_{50} of diazepam in the presence of flumazenil 61.3 ± 8.4 μM was not statistically different to that obtained with diazepam alone (69.0 ± 6.5 μM, $P > 0.05$). The specific antagonist of the peripheral benzodiazepine receptors PK 11195 (5 μM) neither had an effect on its own ($n = 5$) nor altered the effect of diazepam in this preparation ($n = 5$) (Fig. 2).

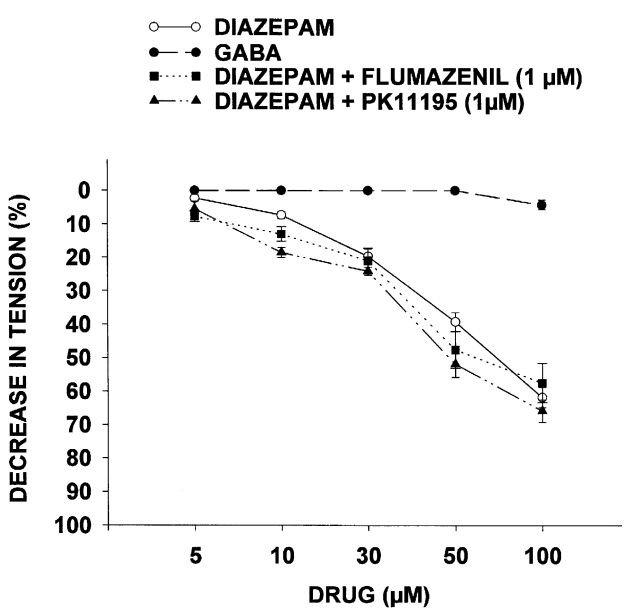

Fig. 2. Concentration–relaxation curves of GABA and diazepam on phenylephrine (1 μM)-precontracted rat aortic rings. The effect of diazepam was tested alone and in the presence of the antagonists of benzodiazepine receptors of central and peripheral type, flumazenil and PK 11195, respectively. The relaxant effect of diazepam alone was significantly different ($P < 0.05$) from that of γ-aminobutyric acid (GABA). However, the effect of diazepam in the presence of flumazenil or PK 11195 was not significantly different from that obtained with diazepam alone. Significant differences were determined by ANOVA followed by Tukey's test. Each point represents the mean value ± S.E.M. of five to seven experiments.

3.2. Role of NO

In order to examine the role of NO, we studied whether the absence of functional endothelium or the presence of the nitric oxide synthase inhibitor L-NAME modified the diazepam-induced relaxation of phenylephrine-precontracted rat aortic rings.

The removal of the endothelium did not modify the relaxation induced by diazepam and the IC_{50} values for diazepam in the presence (69.0 ± 6.5 μM) or in the absence (77.2 ± 8.3 μM) of functional endothelium were not statistically different ($P > 0.05$). However, after a 10-min pre-exposure to L-NAME, the relaxation of rat aortic rings induced by diazepam was significantly reduced (Fig. 3), and the IC_{50} value for diazepam increased from 69.0 ± 6.5 to 100.3 ± 7.2 μM in the absence and in the presence of L-NAME, respectively ($P < 0.05$).

3.3. Potentiation of the relaxant effect of diazepam by cyclic AMP-elevating agents

The influence of pretreatment by compounds which increase levels of cyclic AMP (isoprenaline, a β–adrenoceptor agonist; forskolin, an adenylate cyclase activator, and milrinone, a phosphodiesterase 3 inhibitor) was investi-

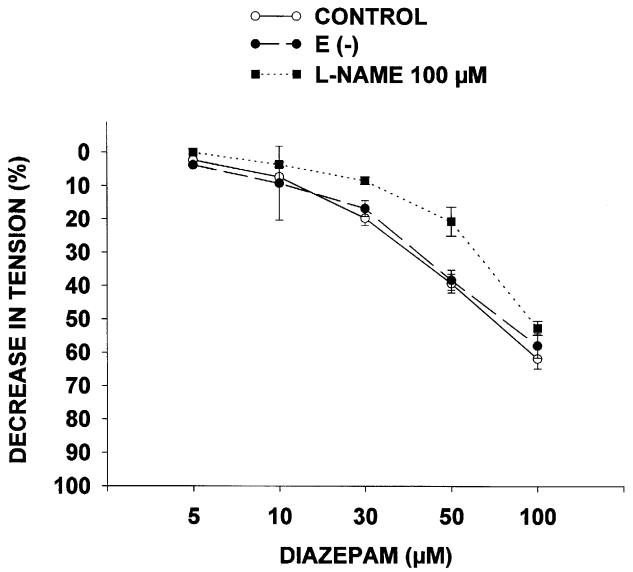

Fig. 3. Relaxant effect of diazepam on phenylephrine-precontracted rat aortic rings with and without (E(−)) functional endothelium and in the absence or presence of N^{ω}-L-arginine methyl ester (L-NAME). $P < 0.05$ when comparing diazepam in the presence and in the absence of L-NAME. No significant difference was found between the effect of diazepam obtained in preparations with and without functional endothelium (ANOVA followed by Tukey's test). Values are means ± S.E.M.; $n = 5–8$ experiments.

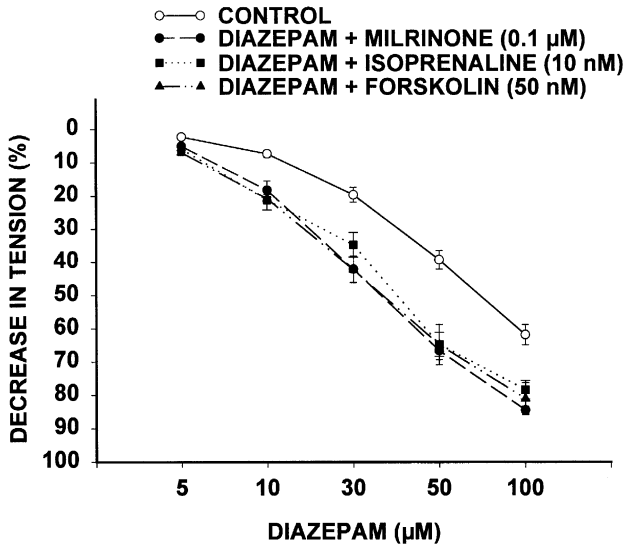

Fig. 4. Relaxation of phenylephrine-precontracted rat aortic rings by diazepam in the absence and in the presence of isoprenaline, forskolin or milrinone. Relaxant responses to diazepam in the presence of each of these agents were significantly different (ANOVA followed by Tukey's test) from those elicited by diazepam alone. Values are means ± S.E.M.; $n = 5–8$ experiments.

gated on relaxation responses elicited by diazepam. A concentration of each compound devoid of an effect on its own in this preparation was used to look for potentiation of the effect of diazepam.

Fig. 4 shows the effect of these drugs on the rat aortic relaxation induced by diazepam. Each of these three agents enhanced, by approximately the same magnitude, the relaxant effect of diazepam and also reduced its IC_{50} value from 69.0 ± 6.5 µM for diazepam alone to 43 ± 2.1 µM, 41.3 ± 4.9 µM and 39.4 ± 4.1 µM in the presence of 10 nM isoprenaline, 50 nM forskolin and 100 nM milrinone, respectively ($P < 0.05$ for each value when compared to diazepam alone).

3.4. Effects of diazepam in combination with isoprenaline, forskolin and milrinone on the tissue levels of cyclic AMP

These experiments were designed to connect functional effects with the intracellular levels of cyclic AMP in aortic rings. To this purpose, we studied the effect of diazepam in the absence and in the presence of either isoprenaline (0.1 µM), forskolin (0.3 µM) or milrinone (10 µM) on cyclic AMP levels in this preparation. The concentrations used of these three agents caused approximately a 50% relaxation in our preparation (data not shown), and are close to their IC_{50} values in rat aorta reported in the literature (Komas et al., 1991; Eckly et al., 1994; Delpy et al., 1996).

The cyclic AMP content in aortic rings pretreated with phenylephrine (1 µM) was 95.7 ± 12 pM g^{-1} ($n = 8$), which was not significantly modified by the presence of 10 µM diazepam (86.6 ± 5 pM g^{-1}, $n = 8$, $P > 0.05$). However, diazepam enhanced the effect of isoprenaline, forskolin and milrinone on cyclic AMP levels in this preparation (Fig. 5). The vehicle used as solvent for diazepam neither produced any significant effect on tissue

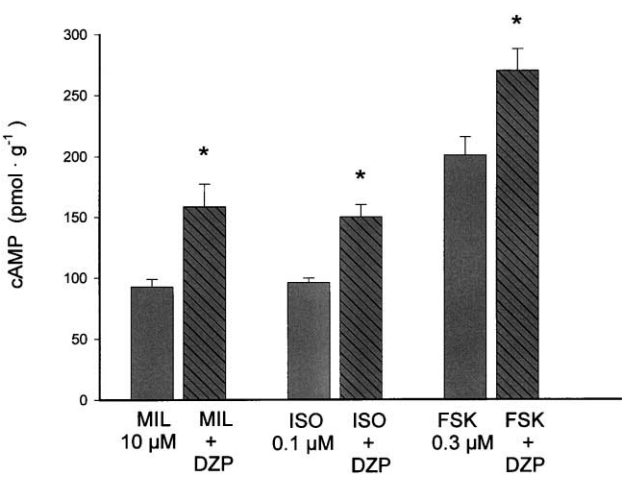

Fig. 5. Changes in cyclic AMP levels in rat aortic tissue incubated with phenylephrine (1 µM). The effects of milrinone (MIL), isoprenaline (ISO) or forskolin (FSK) and the combination of each agent with diazepam (DZP, 10 µM) were studied in matched aortic rings. * $P < 0.05$ when comparing the effect of each agent alone and in the presence of diazepam. Student's *t*-test. Values are means ± S.E.M. of five experiments.

cyclic AMP levels on its own, nor altered the effects of the agents used in combination with diazepam (data not shown).

4. Discussion

Our results show that micromolar concentrations of diazepam induce a concentration-dependent relaxation of rat aortic rings precontracted by phenylephrine. These results agree with previous "in vitro" observations, indicating a vasodilator effect of diazepam (French et al., 1989; Kazanietz and Elgoyhen, 1990).

We recently reported that diazepam inhibits the activity of phosphodiesterase 4 (Collado et al., 1998). Phosphodiesterase activity in rat aorta is mainly due to phosphodiesterase type 3 and type 4, which account for more than 70% of the total cyclic AMP phosphodiesterase activity measured (Rose et al., 1997). The vasodilator effect of phosphodiesterase 4 inhibitors is well established (for review see Polson and Strada, 1996). In fact, the standard phosphodiesterase 4 inhibitor rolipram produces a relaxant effect in rat aortic rings similar to that obtained with diazepam (Komas et al., 1991). The IC_{50} value for the vasorelaxant effect of diazepam, in the present study, was 69.0 ± 6.5 μM, which appears high in comparison with its K_i value for inhibition of phosphodiesterase 4 (10 μM) (Collado et al., 1998). This discrepancy between functional and biochemical effects has been previously observed for other phosphodiesterase 4 inhibitors. For example, IC_{50} values for relaxation of thoracic rat aorta were found to be 30 and 152 μM for denbufylline and rolipram (K_i for phosphodiesterase 4 inhibition 0.46 and 0.76 μM, respectively) (Komas et al., 1991; Lugnier and Komas, 1993). The underlying reason is not known, but it has been attributed to the existence of other phosphodiesterase isoenzymes, namely phosphodiesterase 3, in the vascular wall, which are also involved in cyclic AMP breakdown (Polson and Strada, 1996).

The observed potentiation of the relaxant effect of diazepam by adenylyl cyclase stimulators such as isoprenaline or forskolin is consistent with the involvement of the phosphodiesterase 4 inhibitory activity of diazepam in this effect. Indeed, both isoprenaline and forskolin have been shown to potentiate the rolipram-induced relaxation of rat aorta (Komas et al., 1991). The vasorelaxant effect of diazepam was also potentiated by the presence of the phosphodiesterase 3 inhibitor milrinone in our study. This agrees with the well-known enhancement of rat aorta relaxation induced by phosphodiesterase 4 inhibitors when phosphodiesterase 3 is inhibited (for review, see Polson and Strada, 1996). This synergistic interaction is even more evident in cardiac tissue, where phosphodiesterase 4 inhibitors are devoid of any inotropic effect on their own but increase cardiac contractility in combination with phosphodiesterase 3 inhibitors (Nicholson et al., 1991). It is interesting to note that diazepam also induces a positive inotropic response in the right ventricle of guinea-pig in the presence of milrinone, but not when applied alone (Collado et al., 1998).

Diazepam at 10 μM, which caused 8% relaxation, did not increase cyclic AMP levels. Such a lack of correlation between cyclic AMP content and functional effects has also been demonstrated for rolipram, which produces a dose-dependent relaxant effect without increasing cyclic AMP levels in rat aorta (Komas et al., 1991; Lindgren et al., 1990). The reason for this discrepancy is still unknown, but it has been hypothesized to be due to the possible existence of different intracellular pools of cyclic AMP, as suggested in cardiomyocytes (Delpy et al., 1996). According to this hypothesis, some agents enhance cyclic AMP in a particular subcellular compartment where the functional response takes place, but this increase in cyclic AMP is not great enough to be detected when measured biochemically (Kenakin, 1993). Our results, however, showed that the effect of diazepam 10 μM on cyclic AMP tissue levels became evident when cyclic AMP production was stimulated by isoprenaline or forskolin and when phosphodiesterase 3 was inhibited by milrinone. Similar findings have been obtained with rolipram, which fails to modify the cyclic AMP content in rat aorta when applied alone (Lindgren et al., 1990), but it increases the effects of isoprenaline or milrinone on cyclic AMP levels in this tissue (Lindgren et al., 1991). Such data indicate that diazepam mimics the effects of the standard phosphodiesterase type 4 inhibitor rolipram and support the notion that phosphodiesterase 4 inhibitory activity is involved in the effect of diazepam. Diazepam also possesses inhibitory activity on phosphodiesterase 5 and this may contribute to its relaxant effect on rat aortic rings (Komas et al., 1991). However, this property does not seem to play a role in the interactions described here since phosphodiesterase 5 inhibitors do not enhance the vasodilator or biochemical effects of cyclic AMP-elevating agents such as isoprenaline or forskolin (Delpy et al., 1996; Polson and Strada, 1996).

Elimination of the endothelium did not affect the diazepam-induced relaxant response. This agrees with the clinical observation that diazepam increases myocardial blood flow in patients with and without coronary artery disease (Ikram et al., 1973). However, our results indicate some involvement of NO in the relaxant effect of diazepam, since the relaxant effect was reduced in the presence of a selective inhibitor of NO production. This suggests that diazepam could stimulate the release of NO directly from smooth muscle cells. Although not addressed directly in the present paper, it is likely that the source of this muscle NO is the inducible isoform of NO synthase, which is expressed in normal aorta at low levels or at higher levels following endothelial denudation (Binko et al., 1999).

According to our results, the effect of diazepam is not GABA-dependent, since it was neither mimicked by GABA nor antagonized by flumazenil, which is an effective inhibitor of the effects mediated by the benzodiazepine/GABA/channel Cl^- receptor complex (Haefely et al., 1985). This agrees with other non-GABA-related actions produced by diazepam and supports the contention that cellular mechanisms different from those involving GABA-mediated chloride conductance may contribute to the effects of benzodiazepines (see Baldessarini, 1996 for review). Finally, the micromolar concentrations of diazepam required to induce relaxation could indicate an involvement of the peripheral, non-GABA-related, benzodiazepine binding site (Zisterer and Williams, 1997). However, this does not seem to be the case, because the specific peripheral antagonist PK 11195 (Parola and Yamamura, 1993) did not influence the functional responses elicited by diazepam.

The present results demonstrate a direct relaxant effect, as well as functional and biochemical synergistic interactions with cyclic AMP-elevating agents, of diazepam on rat aortic rings. The relaxant effects were apparent at concentrations similar to those associated with a diazepam overdose. Diazepam is a commonly abused prescription drug (Buckley et al., 1995) and its relaxant effect may well be involved in the hypotension that is seen in patients who take an overdose of the drug (Parfitt, 1999).

The synergistic interaction of diazepam with cyclic AMP-elevating agents described in the present work could also be of interest in clinical practice, particularly in anesthesia, where this combination is likely (McCaughey and Mirakhur, 1997). For example, hypotension associated with milrinone is enhanced by the concomitant administration of diazepam during surgical anesthesia in patients undergoing coronary bypass surgery (Verdu et al., 1999).

In summary, we propose that the phosphodiesterase 4 inhibitory activity of diazepam could be involved in its vasorelaxant effect. Further investigations, however, will be necessary to establish the clinical relevance of these findings.

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