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Diazepam potentiates the positive inotropic effect of isoprenaline in rat ventricle strips: role of cyclic AMP

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Abstract

The responses of the electrically driven right ventricle strip of the rat heart to isoprenaline and other cyclic AMP-related inotropic agents were recorded in the absence and in the presence of diazepam. Isoprenaline, in concentrations ranging from 10 nM to 1 μM, significantly increased, in a concentration-dependent manner, the contractile force in this preparation. Diazepam (10 μM) produced a leftward shift in the isoprenaline concentration-response curve and significantly reduced its EC_{50}. Higher concentrations of diazepam (100 μM) produced no further shift, but reduced the maximum of the concentration-response curve of isoprenaline. Forskolin (0.5–10 μM), which directly stimulates adenyl cyclase, also produced a concentration-dependent increase in cardiac contractility. Diazepam (10 μM) displaced to the left the concentration-response curve for forskolin and reduced its EC_{50}. The cyclic AMP analogous dibutyryl cyclic AMP (0.1–1 mM) produced concentration-dependent positive inotropic effects which were not significantly modified in the presence of diazepam (10 μM). Diazepam (10 μM) significantly enhanced the cyclic AMP production induced by isoprenaline (0.1 μM) and forskolin (10 μM) by about 136% and 35% respectively. These results indicate that diazepam potentiates the positive inotropic effect induced by β-adrenoceptor agonists, probably by increasing cyclic AMP production induced by these agents.

Keywords: Cardiac contractility; Isoprenaline; Diazepam; Forskolin; cAMP

1. Introduction

Diazepam and other drugs of the benzodiazepine group have long been used for their actions on the central nervous system (CNS), namely hypnotics, anxyolitics or anticonvulsants (Greenblatt et al., 1983). However, the functions of some peripheral organs seem to be also affected by the action of these drugs. In fact, binding sites for benzodiazepines have been described in peripheral tissues such as kidney, lung, adrenals as well as in the heart (for review see Verma and Snyder, 1989).

Regarding cardiovascular responses, evidence has been presented that diazepam could affect cardiac contractility. However, there is some controversy about this effect since negative (Daniell, 1975), positive (Castillo-Ferrando et al., 1985) and biphasic effects (Gonzalez et al., 1990; Leeuwin et al., 1993) have been described. The negative inotropic effect seems to be because diazepam potentiates the cardiodepressant effect of adenosine (Clanachan and Marshall, 1980; Kenakin, 1982). However there is not, as yet, an explanation of why diazepam produces some positive inotropic effects. One possibility is that catecholamines, which are the physiological cardiostimulant substances, activate β-adrenoceptors and the subsequent cyclic adenosine monophosphate (cyclic AMP) production (Benovic et al., 1988) could be involved in this effect of diazepam. In fact, some stimulating effects of diazepam are effectively blocked by inhibiting the synthesis of endogenous catecholamines (Söderpalm et al., 1991). Consequently, the aim of the present work was to study whether diazepam modifies the inotropic response elicited by a typical β-adrenoceptor agonist such as isoprenaline on the electrically driven rat right ventri-

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cle strip. This model was chosen because it allows a definitive characterization of the effect of inotropic agents (Doggrell, 1989).

We also tested whether other mechanisms of positive inotropism related to cyclic AMP are modified by the presence of diazepam. To this purpose we analysed the effect of the cyclic AMP analogue dibutyryl cyclic AMP (db cyclic AMP) as well as forskolin, which increases cyclic AMP levels through stimulation of membrane adenylate cyclase activity (Seamon et al., 1981).

2. Materials and methods

2.1. Recording of the contractile response of the electrically driven rat right ventricle strip

Sprague-Dawley rats of either sex, 250–350 g, untreated or pretreated with reserpine (5 $mg \cdot kg^{-1}$ i.p. 24 h before experiment) were stunned and exsanguinated. The chest was opened and the heart was rapidly removed and placed in Tyrode solution saturated with 95% O_2-5% CO_2 and the free wall of the right ventricle was excised. All procedures were performed in the presence of Tyrode solution with the following composition (mM): NaCl 136.9, KCl 5.0, $CaCl_2$ 1.8, $MgCl_2$ 1.5, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, dextrose 5.0. Right ventricular strips were mounted longitudinally between two platinum electrodes under 1 g tension in Tyrode solution maintained at 34°C and gassed with 95% O_2-5% CO_2. The preparations were electrically stimulated (Grass SD-9 stimulator) at a frequency of 1.5 Hz and duration of 4 ms and supramaximal (threshold + 50%) voltage was given for at least 30 min before the start of the experiments. Contractions were measured using a force-displacement transducer (Grass FT-03) and recorded on a Dynograph Beckman polygraph. Only preparations which had a stable basal contractile activity at the end of the stabilization period were accepted for study.

2.2. Experimental protocols

Cumulative concentration-response curves for isoprenaline as well as for forskolin and db cyclic AMP were made by increasing the concentration stepwise as soon as the response to the previous dose had levelled off. Drugs were added to the organ bath (30 ml capacity) in volumes less than or equal to 0.1 ml. Concentrations of drugs were increased after a steady-state response had been attained with the previous concentrations or after 5 min in the absence of response. Following this procedure, the tissues were washed with fresh bathing medium to remove the agonist. The bath was washed out several times in a 60 min period, in order to restore the resting level. After washout, a second concentration-response curve was established for the agonist in the presence of diazepam. Diazepam was left in contact with the tissue for 30 min before construction of the second concentration-response curve. To avoid an order effect, experiments were carried out according to a balanced crossover design (Kenakin, 1993). Each preparation was exposed to only one inotropic agent. A 'control' strip from the same right ventricle was generally also used to confirm the repeatability of the response to a single agonist in the absence or in the presence of the vehicle used to dissolve diazepam.

2.3. Cyclic AMP analysis

To study the effect of diazepam on intracellular cyclic AMP levels, the right ventricle of the rat was cut out as previously described and was allowed to stabilize for about 60 min in Tyrode solution. Diazepam was added to the media 30 min before the incubation time with either isoprenaline (0.1 μM) or forskolin (10 μM).

Cyclic AMP was determined by means of a cyclic AMP ^3H assay system following the indications of the manufacturer (Amersham International, Amersham, UK). Tissues were extracted in 0.3 M perchloric acid (in a ratio 1:10, w/v), with a Polytron homogenizer. Extracts were centrifuged at 12000 rpm for 15 min, using a Biofuge A centrifuge (Heraus, Germany). Supernatants were treated with potassium hydroxide solution until pH 7.5 was reached. The samples were centrifuged once and the supernatants were used for cyclic AMP analysis. Proteins were determined according to a Lowry modified procedure (Hartree, 1972).

2.4. Drugs and chemicals used

The following drugs were used: isoprenaline, dibutyryl cyclic AMP, forskolin and reserpine were obtained from Sigma Chemicals Co. (Spain). Diazepam was generously supplied by Roche (Spain). The cyclic AMP ^3H assay system was obtained from Amersham International, UK.

Isoprenaline was freshly dissolved in normal Tyrode solution containing ascorbic acid (1 $\mu g \cdot ml^{-1}$) to prevent oxidation. Diazepam and forskolin were dissolved in dimethyl sulphoxide (obtained from Probus, Barcelona, Spain) and Tyrode solution (2 dimethyl sulphoxide : 8 Tyrode); this stock was diluted into prewarmed and preaerated bathing solution to achieve the desired final concentration. The appropriate concentration of drug was added to the organ baths so that the concentration of dimethyl sulphoxide in the test solution was less than 0.3% by volume which is devoid of effect in this preparation.

2.5. Analysis of data

Results are expressed as fractions of the increase in force of contraction produced by a maximun dose of the agonist (mean ± S.E.M.). The values for the curve 'before diazepam' were then corrected for time-dependent changes in sensitivity occurring during the experiment by application of correction factors derived from the appropriate control experiment. Correction factors were obtained by expressing the mean total tension at each concentration on the second curve of control experiments as a fraction of the total value for the corresponding concentration on the first curve (Herepath and Broadley, 1990).

To ascertain whether the effect of a positive inotropic agent is modified by the presence of diazepam, we measured the corresponding EC_{50} of a given drug in the absence and in the presence of diazepam. EC_{50} was determined from individual curves by linear interpolation between two points on either side of the 50% response.

Statistical comparisons of EC_{50} values as well as the effect of a given drug concentration 'before and after' diazepam were made by Student's t-test.

3. Results

3.1. Isoprenaline + diazepam

In reserpinized rats

Experiments aimed to study the interaction betweeen isoprenaline and diazepam were performed in reserpinized rats to prevent the effect of endogenous catecholamines. Isoprenaline (10 nM–1 μM) increased the amplitude of contraction of rat isolated right ventricular strips in a concentration-dependent manner, producing a maximal effect, which amounted to 95.8 ± 8.7% over the basal contractility, at 1 μM. The positive inotropic effect of isoprenaline was rapidly reversed after washout. Diazepam at a concentration of 10 μM, which is virtually devoid of any effect on ventricular contractility, was applied to the preparation about 30 min before the second concentration-response curve for diazepam was made. This concentration of diazepam produced a leftward shift in the isoprenaline concentration-response curve (Fig. 1) and also reduced the EC_{50} value (μM) for isoprenaline from 0.08 ± 0.014 for isoprenaline alone to 0.02 ± 0.004 in the presence of diazepam. These two values were statistically different ($P < 0.05$). The effect of diazepam could not be attributed to its solvent dimethyl sulphoxide, since this solvent applied in the same concentrations as those present in the diazepam solutions did not cause significant changes in the inotropic effect of isoprenaline (Fig. 1).

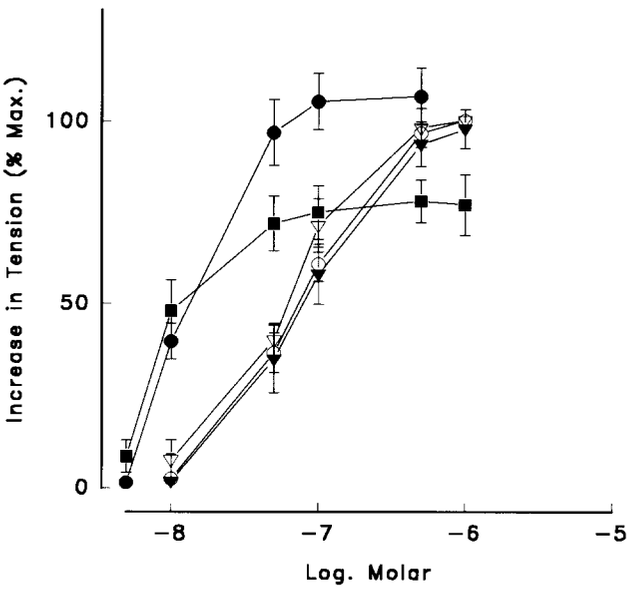

Fig. 1. Effect of diazepam on the increase in tension of electrically driven right ventricular strips of reserpinized rats (5 mg·kg^{-1} i.p., reserpine) in response to isoprenaline. Each tissue was used to make two curves for isoprenaline, in the absence ($n = 17$) and, after washing, in the presence of one of the follow concentrations of diazepam: 1 μM (▽, $n = 3$), 10 μM (●, $n = 6$), 100 μM (□, $n = 4$) or the diazepam solvent dimethyl sulphoxide (▼, $n = 4$). Concentrations of dimethyl sulphoxide were the same as those present in the diazepam solution. Increases in tension are plotted as percentages of the maximum response. Each point represents the mean value ± S.E.M. (vertical bars).

Similar experiments were carried out in the presence of higher and lower concentrations of diazepam. Each preparation was exposed to only one concentration of diazepam. A 10 times higher concentration of diazepam (100 μM) produced no further shift of the concentration-response curve for isoprenaline; the EC_{50} values (μM) for isoprenaline in the presence of 10 (0.02 ± 0.004) and 100 μM (0.014 ± 0.007) diazepam did not differ significantly ($P > 0.05$). However, this concentration of diazepam significantly reduced by 24 ± 7% the maximal response to isoprenaline ($P < 0.05$) (Fig. 1). Diazepam at 1 μM was virtually devoid of effect upon right ventricle strip responses to isoprenaline (Fig. 1). The EC_{50} values (μM) for isoprenaline alone (0.082 ± 0.014) and in the presence of 1 μM diazepam (0.061 ± 0.01) did not differ significantly ($P > 0.05$).

In non-reserpinized rats

The inotropic effect of isoprenaline was also studied in four experiments with non-reserpinized rats. Isoprenaline (10 nM–1 μM) increased cardiac contractility in a dose-dependent manner, this effect being similar to that obtained in reserpinized rats. In fact, the maximum inotropic response to isoprenaline in untreated

rats was virtually identical with that obtained in reserpinized rats. The EC_{50} value (μM) was slightly higher in untreated rats, but was not significantly different from that of reserpinized rats ($P > 0.05$; untreated 0.1 ± 0.019, reserpinized 0.082 ± 0.014).

Diazepam (10 μM) consistently produced a leftward shift of the isoprenaline concentration-response curve to approximately the same extent as that observed for reserpinized rats. These results suggest that, in this preparation, pretreatment with reserpine does not modify the inotropic effect of isoprenaline either alone or in the presence of diazepam.

3.2. Effects of isoprenaline and diazepam on the tissue levels of cyclic AMP

To know whether the diazepam-induced potentiation of the inotropic effect of isoprenaline elicited under our experimental conditions is due to changes in tissue levels of cyclic AMP, we studied whether diazepam could modify either the basal or the stimulated concentration of cyclic AMP in the isolated right ventricle of the rat.

The production of cyclic AMP was stimulated by 0.1 μM isoprenaline, which caused approximately a 50% increase in basal contractility (see Fig. 1). Diazepam (10 μM) seemed to increase the tissue levels of cyclic AMP (5.45 ± 0.7 pmol/mg protein) but this difference was devoid of statistical significance when compared with the control value (3.7 ± 0.8 pmol/mg protein) ($P > 0.05$). However, this concentration of diazepam significantly increased the cyclic AMP production induced by isoprenaline by approximately 136%. The vehicle used as solvent for diazepam did not produce any statistically significant change in the effect of isoprenaline on the tissue level of cyclic AMP in our experiments (Fig. 2).

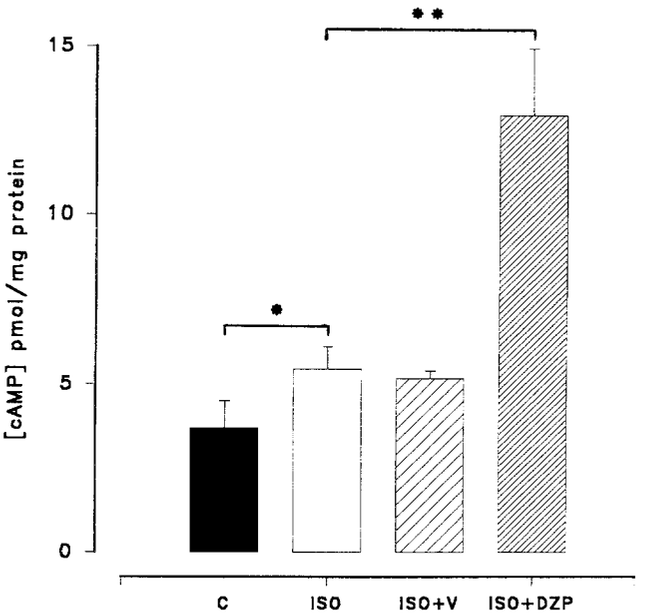

Fig. 2. Effects of isoprenaline (ISO) in the absence and in the presence of diazepam (DZP) on the tissue levels of cyclic AMP. The mean ± S.E.M. of cyclic AMP levels (pmol per mg of protein) is given for three experiments per group. Diazepam (10 μM) or the diazepam vehicle (V) was added to the media 30 min before and during 1 min incubation with isoprenaline (0.1 μM). The value for ISO was significantly different from that for control (C) and the value for ISO + DZP was significantly different from that for ISO at $^{*}P < 0.05$ and $^{**}P < 0.01$ respectively when tested by Student's t-test.

3.3. Diazepam + db cyclic AMP

In order to see whether the effect of cyclic AMP on cardiac contractility is potentiated by diazepam we studied the interaction between this drug and the cyclic AMP analogue db cyclic AMP.

db cyclic AMP (0.1–1 mM) elicited a weak inotropic response in this preparation. In fact, the highest concentration of db cyclic AMP, which amounted to 1 mM, only increased cardiac contractility, compared to the control, by $26.6 \pm 10\%$ ($n = 3$). Diazepam did not potentiate the positive inotropic effect of db cyclic AMP but instead it reduced it, since in the presence of diazepam, db cyclic AMP (1 mM) increased contractility by only $12.2 \pm 10\%$. However, because of the large scatter of results, the difference between these two values was devoid of statistical significance ($P > 0.05$).

3.4. Diazepam + forskolin

To examine whether diazepam modifies the effect of an agent which directly stimulates adenyl cyclase such as forskolin, dose-response curves for this agent were made in the absence and in the presence of diazepam. Forskolin produced a concentration-dependent increase in cardiac contractility. Diazepam 10 μM, which is devoid of effect in this preparation, however, produced a leftward displacement of the dose-response curve for forskolin (Fig. 3A). The EC_{50} value (μM) of forskolin on its own (3.2 ± 0.6) was also significantly reduced in the presence of diazepam (0.97 ± 0.3) ($n = 4$). This change in the EC_{50} is statistically significant ($P < 0.05$).

The effect of diazepam does not seem to be a consequence of its solvent since dimethyl sulphoxide applied in the same concentrations as those present in the diazepam solution did not significantly modify the concentration-response curve for forskolin.

3.5. Effects of forskolin and diazepam on tissue levels of cyclic AMP

Forskolin (10 μM) produced a 4-fold increase in basal (control) levels of cyclic AMP in the right ventri-

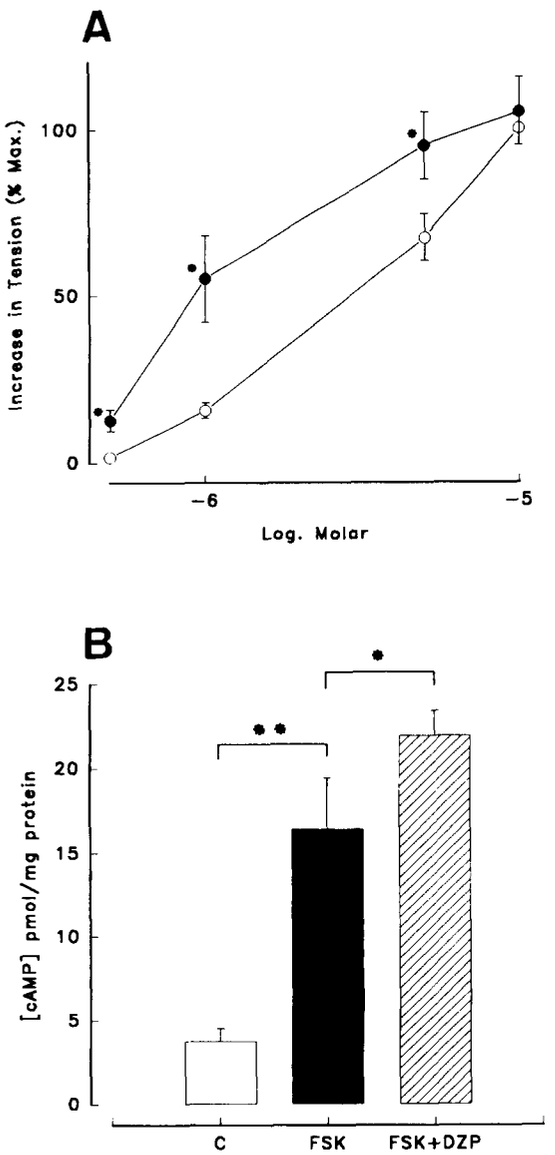

Fig. 3. (A) Cumulative concentration-response curves for forskolin alone (○) and in the presence (●) of diazepam (10 μM) on electrically driven right ventricular strips of the rat ($n = 4$). * $P < 0.05$, when compared with forskolin alone (Student's t-test). Further details as in legende to Fig. 1. (B) Effects of forskolin (10 μM) in the absence and in the presence of diazepam (10 μM) on the tissue levels of cyclic AMP ($n = 3$). The diazepam vehicle (dimethyl sulphoxide) did not significantly modify the effect of forskolin on cyclic AMP levels. * $P < 0.05$ and * * $P < 0.01$ (Student's t-test). Further details as in legend to Fig. 2.

cle of the rat. In the presence of diazepam (10 μM), the same concentration of forskolin still produced a further increase in cyclic AMP levels (Fig. 3B).

4. Discussion

The present results demonstrated that diazepam 10 μM potentiates the positive inotropic response as well as the cyclic AMP production elicited by isoprenaline in the isolated right ventricle of the rat. This concentration of diazepam, although in some preparations it induced a positive inotropic effect, is devoid of a statistically significant effect on the contraction force of electrically driven right ventricular strips of the rat (Hernández, 1991). Rats were pretreated with reserpine (5 $mg \cdot kg^{-1}$ i.p., 24 h before), to prevent the contribution of endogenous catecholamines which could interfere with the effect of some agents on the isolated right ventricle of the rat (Hernández et al., 1994). This treatment schedule produces a total depletion of adrenergic stores in the rat heart (Rice et al., 1987) and abolishes the inotropic effect of the indirect sympathomimetic drug tyramine in this preparation (Martinez et al., 1995). However, endogenous catecholamines do not seem to interfere with the inotropic response to isoprenaline in the absence and in the presence of diazepam since, in our results, this response was virtually the same in non-reserpinized and in reserpinized rats. This agrees with previous findings indicating that pretreatment with reserpine produces supersensitivity to the chronotropic (Adler-Graschinsky et al., 1972; Rice et al., 1987), but not to the inotropic responses to isoprenaline (Rice et al., 1987).

Since diazepam is not a water-soluble substance we used dimethyl sulphoxide to dissolve this compound. Dimethyl sulphoxide has shown some pharmacological activity in several tissues (Cherki-Vakil and Meiri, 1991; Schreiberg and Slapke, 1991). In fact, in the innervated cardiac atrium of guinea pig, dimethyl sulphoxide at 3% produced a moderate increase in inotropism which was very marked when the final bath concentration was 6% (Sams et al., 1966). However, in our system the concentration of dimethyl sulphoxide was about 0.3%, which is very much lower than those mentioned above and neither caused any direct inotropic effect nor produced any significant change in the dose-response curve for isoprenaline.

Diazepam is considered a cardiodepressant agent mainly due to its effects on the CNS (Chai and Wang, 1966). However, there are several reports from in vitro studies indicating that diazepam could directly affect cardiac contractility. In fact, concentrations of diazepam similar to those used in the present work produced a positive inotropic effect on isolated left atria of either guinea-pig (Kenakin, 1982) or rat (Castillo-Ferrando et al., 1985) as well as in the Langendorff rat heart (Leeuwin et al., 1993). No explanation has been provided as yet for this positive inotropic effect of diazepam.

It is well established that catecholamines are powerful positive inotropic agents which are normally present in the heart (Fujii and Vatner, 1986). Therefore, diazepam may also act by potentiating the excitatory actions of catecholamines on cardiac contractility. The

present study demonstrates that an isoprenaline–diazepam interaction exists in the rat heart.

The possibility that diazepam could facilitate the protein phosphorylation process through cyclic AMP-dependent protein kinase (Evans, 1986) was tested by studying the inotropic effect of the cyclic AMP analogous db cyclic AMP in the absence and in the presence of diazepam. Under our experimental conditions db cyclic AMP elicited a weaker inotropic response than isoprenaline. This result is, in some way, expected since db cyclic AMP is less potent in stimulating cyclic AMP-dependent protein kinase than cyclic AMP (Miller et al., 1973). In our study diazepam did not increase the inotropic effect of db cyclic AMP.

The possibility that diazepam facilitates the effect of isoprenaline on adenyl cyclase by increasing cyclic AMP production was investigated. To this purpose we studied the interaction between forskolin and diazepam. It is well known that forskolin is an activator of the catalytic subunit of adenylate cyclase and by this mechanism increases cyclic AMP production (Seamon et al., 1981). In our study, diazepam also potentiated the inotropic effect of forskolin. This suggests that the diazepam-induced positive inotropic effect could be due to an increase in cyclic AMP production induced by directly (forskolin) or indirectly (isoprenaline) acting agents. This idea is further supported by the fact that diazepam sharply increased the isoprenaline-induced increase in cyclic AMP concentration in the rat heart. Consistent with these findings, evidence suggesting a mechanism of potentiation by diazepam of cyclic AMP producing agents has been presented. York and Davies (1982) reported an increase in adenosine-induced cyclic AMP production in slices of cerebral cortex. Also, a significant increase in noradrenaline-induced cyclic AMP levels that reached up to 50% of the control value has been described in the isolated right atria of the rat. Surprisingly, an antagonism of the chronotropic effects of noradrenaline was obtained in the presence of diazepam (Elgoyhen and Adler-Graschinsky, 1989). In contrast, in our study there was consistent functional and biochemical evidence for a potentiation by diazepam of the effect of isoprenaline on cardiac contractility and cyclic AMP production in the isolated right ventricle of the rat.

The mechanism by which diazepam potentiates the effect of cyclic AMP-producing agents is still far from clear. It does not seem to be a calcium-dependent phenomenon. In fact, the effect of diazepam on nomotopic cardiac automaticity in the rat is not modified either by hypercalcic Tyrode's solution or by the presence of the Ca^{2+}-entry blocker diltiazem (Ruiz et al., 1989a). Furthermore, the positive inotropic effect of ouabain, which is a consequence of an increase in intracellular Ca^{2+} concentration (Smith, 1988), is not modified by the presence of diazepam in this preparation (unpublished observations). Our results indicate that diazepam strongly potentiates β-adrenoceptor agonists but that the potentiation of agents directly acting on the adenylate cyclase enzyme is very much weaker. For example, diazepam increased isoprenaline-induced cyclic AMP production by about 136% and that induced by forskolin by about 35%. One possibility is that diazepam could increase the affinity of β-adrenoceptors for agonists, as has been described for some other agents (for review see Wolfe and Molinoff, 1988). However, further research is still required to establish the actual mechanism responsible for this effect.

Our results indicate that, in addition to the previously described cardiodepressant effect of diazepam (Kenakin, 1982; Hernández, 1991), this drug could increase catecholamine-induced cyclic AMP production, thus potentiating its positive inotropic effect. Supporting this idea is the fact that the β-adrenoceptor blocker, propranolol, antagonized the inotropic effects of both diazepam and midazolan in the Langendorff heart preparation of the rat (Leeuwin et al., 1993). These two actions are opposite and could be related to the biphasic effect on cardiac contractility reported for diazepam in the isolated rat heart (Leeuwin et al., 1993). It could, therefore, be speculated that the cardiodepressant effect of diazepam, which is clearly manifested at higher concentrations (Hernández, 1991), is counteracted by the cardioexcitatory effect of the diazepam-induced potentiation of both directly (forskolin) or indirectly (isoprenaline) acting adenylcyclase-activating agents. Our results agree with this view since diazepam (10 μM) produced a leftward shift of the concentration-response curve for isoprenaline. However, a 10 times higher concentration of diazepam (100 μM) produced no further shift, but reduced the maximum of the concentration-response curve for isoprenaline on cardiac contractility. There are some results which support this idea. For example, in the isolated guinea-pig left atria, diazepam (10 μM) produces a positive inotropic effect, but higher concentrations (50–100 μM) reduce contractile force in this preparation (Kenakin, 1982). Also, diazepam (0.2 mg/kg) increases heart rate as well as the incidence of bupivacaine-induced malignant cardiac arrhythmias in rats (Gregg et al., 1988). However, higher concentrations of diazepam have been reported to produce some antiarrhythmic effects in the spontaneously beating isolated right ventricle of the rat (Ruiz et al., 1989b) as well as in the dog heart following coronary artery occlusion (Pinto et al., 1991).

In conclusion, our results indicate that diazepam potentiates the positive inotropic effect induced by isoprenaline in the right ventricle strip of the rat heart, probably by enhancing cyclic AMP production. This effect of diazepam is obtained with a concentration similar to that (0.1–10 μM) producing clinical effects

in humans (Hillestad et al., 1974; Browne, 1982) and could be related to the cardiac sympathomimetic or β-adrenergic-like effects produced by diazepam in some patients (Kumagai et al., 1991). In fact, benzodiazepine receptors have been characterized in the living human heart by positron emission tomography (Charbonneau et al., 1986). However, the clinical relevance of these findings demands further investigation.

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