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# Functional and biochemical evidence for diazepam as a cyclic nucleotide phosphodiesterase type 4 inhibitor

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**1** The responses of the electrically-driven right ventricle strip of the guinea-pig heart to diazepam were recorded in the absence and in the presence of different selective cyclic nucleotide phosphodiesterase (PDE) inhibitors.

**2** Diazepam, at concentrations ranging from 1 $\mu$M to 100 $\mu$M, was devoid of effect on the contractile force in this preparation.

**3** Conversely, diazepam (5 $\mu$M – 100 $\mu$M) produced a consistent positive inotropic response in the presence of a concentration (1 $\mu$M), that was without effect in the absence of diazepam, of either of the selective PDE 3 inhibitors milrinone or SK&F 94120, but not in the presence of the selective PDE 4 inhibitor rolipram.

**4** This effect of diazepam was not $\gamma$-aminobutyric acid (GABA)-dependent, since it was neither mimicked nor potentiated by GABA, and was not affected by either a high concentration (5 $\mu$M) of the antagonists of the benzodiazepine/GABA/channel chloride receptor complex, picrotoxin, flumazenil and $\beta$-carboline-3-carboxylic acid methyl ester ($\beta$CCMe), or by the inverse agonists, $\beta$-carboline-3-carboxylic acid N-methylamide ($\beta$CCMa) and methyl 6,7-dimethoxy-4-ethyl-$\beta$-carboline-3-carboxylate (DMCM, 0.1 $\mu$M). Furthermore, a specific antagonist of the peripheral benzodiazepine receptors, PK 11195 (5 $\mu$M), did not influence the effect of diazepam.

**5** Biochemical studies with isolated PDEs, confirmed that diazepam selectively inhibits type 4 PDE from guinea-pig right ventricle rather than the other PDEs present in that tissue. The compound inhibited this enzyme in a non-competitive manner. Diazepam was also able to inhibit PDE 5, the cyclic GMP specific PDE absent from cardiac muscle, with a potency close to that shown for PDE 4.

**6** Diazepam displaced the selective type 4 PDE inhibitor, rolipram from its high affinity binding site in rat brain cortex membranes, and also potentiated the rise in cyclic AMP levels induced by isoprenaline in guinea-pig eosinophils, where only type 4 PDE is present.

**7** The PDE inhibitory properties of diazepam were shared, although with lower potency, by other structurally-related benzodiazepines, that also displaced [$^3$H]-rolipram from its high affinity binding site. The order of potency found for these compounds in these assays was not related to their potencies as modulators of the GABA receptor through its benzodiazepine binding site.

**8** The pharmacological and biochemical data presented in this study indicate that diazepam behaves as a selective type 4 PDE inhibitor in cardiac tissue and this effect seems neither to be mediated by the benzodiazepine/GABA/channel chloride receptor complex nor by peripheral type benzodiazepine receptors.

**Keywords:** Cardiac contractility; phosphodiesterase; diazepam; milrinone; SK&F 94120; rolipram; cyclic AMP; benzodiazepines

## Introduction

In previous work, we showed that diazepam potentiates the inotropic response in the rat heart, as well as the enhancement of intracellular levels of cyclic AMP induced by isoprenaline and forskolin (Martinez *et al*., 1995a) or tyramine (Martinez *et al*., 1995b). Other authors have also found evidence that diazepam increases the effect of adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-producing agents such as noradrenaline (Elgoyhen & Adler-Graschinsky, 1989) or adenosine (York & Davies, 1982). However the mechanism responsible for this action of diazepam is still unknown. One possibility is that diazepam could produce this effect by inhibiting one or more cyclic nucleotide phosphodiesterases, the enzymes responsible for the breakdown of cyclic AMP. In fact, experiments performed in several regions of the brain, indicate that diazepam could inhibit these enzymes (Dalton *et al*., 1974).

Molecular, biological and pharmacological studies have clearly established the existence of seven families of PDE isoenzymes, with marked differences in their tissue distribution (for review see Thompson, 1991). In the heart of several species, including man, type 3 and type 4 PDE are the main isoenzymes responsible for cyclic AMP breakdown (Nicholson *et al*., 1991) and it has been shown that the inhibition of both forms is required effectively to increase cyclic AMP levels and produce a full inotropic response (Shahid & Nicholson, 1990). However, the brain is a tissue deficient in PDE 3 and thus, type 4 PDE is the main enzyme responsible for the hydrolysis of cyclic AMP in this tissue (Thompson, 1991). In fact, a stereospecific high-affinity binding site for rolipram has been demonstrated in brain tissue which appears to be associated with the PDE isoenzyme type 4 family (Schneider *et al*., 1986; Jacobitz *et al*., 1996).

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The aim of the present study was to investigate the mechanism of action through which diazepam exerts its positive inotropic effects in the heart. The possible implication of the benzodiazepine receptors coupled to $\gamma$-aminobutyric acid (GABA)-activated chloride channels and the inhibition of one or more cyclic nucleotide phosphodiesterases have been investigated.

## Methods

### *Recording of the contractile response of the electrically-driven guinea-pig right ventricle strip*

Guinea-pigs 400–600 g 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 of 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 and 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 with 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 included in the study.

### *Experimental protocols*

Cumulative concentration-response curves for either isoprenaline, diazepam, milrinone or rolipram were made by increasing the concentration stepwise as soon as the response to the previous concentration 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 concentration of inhibitor or after 5 min in the absence of response.

The interaction of diazepam with two known PDE 3 inhibitors, milrinone and SK&F 94120, as well as with the PDE 4 inhibitor, rolipram, was studied. For this, each selective PDE inhibitor was left in contact with the tissue for 30 min before construction of the concentration-response curve for diazepam. Only one PDE inhibitor was tested on each guinea-pig ventricle strip, except when milrinone and rolipram were tested in combination. In these experiments a full cumulative concentration-effect curve was constructed for rolipram in the presence of milrinone.

The interaction of diazepam with ispoprenaline was also studied. For this, guinea-pig ventricle strips were treated with isoprenaline $10^{-9}$ M and, following a stabilization period, diazepam $10^{-4}$ M was added.

A control strip from the same right ventricle was generally also used to confirm the reproducibility of the response to a single agent in the absence or in the presence of the vehicle.

### *Purification of phosphodiesterase isoenzymes*

Cyclic nucleotide phosphodiesterases 1 to 4 were purified from guinea-pig heart ventricular tissue and PDE 5 from dog platelets following the procedure described by Gristwood *et al.* (1992).

The isoenzymes were characterized by substrate selectivity and affinity, and by the effect of calcium ions (10 $\mu$M) plus calmodulin (1.2 $\mu$M), cyclic GMP and the selective inhibitors rolipram, SK&F 94120 and zaprinast. PDE isoenzymes were kept frozen at −80°C in the presence of 1 g $l^{-1}$ bovine albumin until used.

### *Phosphodiesterase assay and kinetic analysis*

Cyclic nucleotide phosphodiesterase activities were measured as previously described (Gristwood *et al.*, 1992). Drugs were dissolved in dimethylsulphoxide (DMSO) and the effect of this solvent was taken into consideration in the calculations.

### *Binding to the [$^3$H]-rolipram binding site from rat brain cortex*

The binding of [$^3$H]-rolipram to rat brain membranes was performed according to Schneider *et al.* (1986). At least 6 drug concentrations were assayed in duplicate to generate individual displacement curves.

### *Preparation of guinea-pig eosinophils*

Guinea-pig eosinophils were obtained according to Dent *et al.* (1991). Cell viability, as determined by Trypan Blue exclusion, was greater than 98% and eosinophil purity, as determined by May-Grünwald-Giemsa staining, was greater than 95%.

### *Determination of eosinophil cyclic AMP accumulation*

Freshly prepared eosinophils ($1-2\times10^6$), resuspended in Hank's balanced salt solution (HBSS) containing $Ca^{2+}$ and $Mg^{2+}$, were incubated with the compounds at the indicated concentrations, for 10 min at 37°C. Incubations were continued for a further 2 min in the presence of isoprenaline (10 $\mu$M) and terminated by addition of two volumes of cold ethanol. The samples were centrifuged (2,000 *g*) for 15 min at 4°C and the supernatant transferred to a clean tube. The samples were dried by gassing with nitrogen at 60°C and the pellet was resuspended in water. Cyclic AMP was quantified by use of an enzyme-immunoassay kit from Amersham Life Sciences (U.K.) following the protocol without acetylation.

### *Drugs and chemicals used*

Benzodiazepines were obtained from Roche (Madrid, Spain). The $\beta$-carbolines $\beta$-carboline-3-carboxylic acid methyl ester ($\beta$CCMe) and $\beta$-carboline-3-carboxylic acid N-methylamide ($\beta$CCMa) were obtained from Sigma Chemicals Co (Madrid, Spain). PK 11195 (1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl) - 3 - isoquinoline carboxamide) and DMCM, (6,7 - dimethoxy-4-ethyl-$\beta$-carboline-3-carboxylate) were from RBI (Natick, USA). Percoll was purchased from Pharmacia Biotechnology (Barcelona, Spain). Racemic [$^3$H]-rolipram was a special preparation made by Amersham and had a specific activity of 15.8 Ci $mmol^{-1}$. All other drugs and reagents used were from the same sources previously stated (Gristwood *et al.*, 1992; Martinez *et al.*, 1995b).

Isoprenaline, GABA and picrotoxin were freshly dissolved in normal Tyrode solution. Ascorbic acid (1 $\mu$g $ml^{-1}$) was added to the isoprenaline solution to prevent oxidation. Diazepam, chlordiazepoxide, clonazepam, flunitrazepam, lorazepam, nitrazepam, flumazenil, $\beta$CCMe, $\beta$CCMa, DMCM,

PK 11195, milrinone, SK&F 94120 (5-(4-acetimidophenyl)pyrazin-(1H)-one) and rolipram were dissolved in DMSO (obtained from Probus, Barcelona, Spain) and Tyrode solution (DMSO/Tyrode 2 : 8 vol); this stock solution was diluted into prewarmed and pre-aerated bathing solution to achieve the desired final concentration. The appropriate concentration of drug was added to the organ baths so that the concentration of dimethylsulphoxide in the test solution was less than 0.3% by volume, which is devoid of effect in this preparation.

### *Data analysis*

Data from functional and phosphodiesterase inhibition studies were treated as previously described (Gristwood *et al*., 1992; Martinez *et al*., 1995b).

For the kinetic analysis the cyclic AMP concentration was varied, although the [$^3$H]-cyclic AMP concentration remained constant. $V_{max}$ and $K_m$ values were calculated by use of the Michaelis-Menten equation and graphed as a Lineweaver-Burk plot and as described by Dixon (1953). The values presented are the average ± s.e.mean of at least three independent assays.

$IC_{50}$ values for binding studies were calculated from the concentration-response curves by non-linear regression by use of the programme Inplot, from GraphPad Software. The data presented are the average ± s.e.mean of the values obtained from, at least, three different curves.

For the calculations of cyclic AMP levels, those obtained in the presence of isoprenaline were considered as 100% and the values obtained in the presence of compounds were corrected accordingly. $EC_{50}$ values were calculated by adjusting the data to a sigmoid curve by use of the programme Inplot. Four independent measurements made on two different days were obtained for each drug concentration.

## Results

### *Effects on contractile force*

Diazepam at concentrations ranging from 5 $\mu$M to 100 $\mu$M, was devoid of any effect on the force of contraction of electrically-driven strips from guinea-pig heart right ventricle ($n=4$). On the contrary, isoprenaline (10 nM to 1 $\mu$M) increased, as expected, the amplitude of contraction of the same preparation in a concentration-dependent manner. The maximal effect, which amounted to 240 ± 12.5% over the basal contractility, was obtained at a concentration of 1 $\mu$M ($n=4$). The positive inotropic effect of isoprenaline was rapidly reversed after washout.

Under the same conditions the selective PDE type 3 inhibitor milrinone (5 $\mu$M or 50 $\mu$M), increased the force of contraction by 18.3 ± 4.2% and 23.5 ± 5.3% ($n=4$), respectively, of the isoprenaline maximum. In contrast, the PDE type 4 inhibitor rolipram at the same range of concentrations, was without effect. However, in the presence of 1 $\mu$M milrinone, a concentration which was devoid of effect by itself, rolipram ($n=4$) caused concentration-dependent increases in right ventricle force of contraction that amounted 27 ± 6%, 36 ± 5.2% and 50 ± 8.3% of the isoprenaline maximum, at 10 $\mu$M, 30 $\mu$M and 50 $\mu$M, respectively.

### *Interaction of diazepam with PDE inhibitors*

The effect of diazepam in the presence of the PDE 3 inhibitors milrinone or SK&F 94120 was studied. Each of these agents was used at a concentration of 1 $\mu$M, that was previously found to be devoid of effect by itself in this preparation. Diazepam (5 – 100 $\mu$M), which is also devoid of effect on its own (see above), effectively enhanced the contractility of this preparation in the presence of either milrinone ($n=4$) or SK&F 94120 ($n=4$). However, diazepam did not show any inotropic effect in the presence of 1 $\mu$M of the selective PDE type 4 inhibitor rolipram ($n=4$), (Figure 1).

To establish whether the effect of diazepam in the presence of either milrinone or SK&F 94120 could be attributed to activation of the benzodiazepine/GABA/channel chloride receptor complex, we studied whether this effect would be modified by the presence of GABA (100 $\mu$M) or by the antagonists picrotoxin, flumazenil and $\beta$CCMe (5 $\mu$M) and the inverse agonists $\beta$CCMa (5 $\mu$M) and DMCM (0.1 $\mu$M) of this complex. Each of these agents was added to the organ bath 3 – 5 min before the concentration-response curve for diazepam was determined. No effects were observed, either alone ($n=3$ for each agent) or in the presence of milrinone (1 $\mu$M, again $n=3$ for each agent) or SK&F 94120 ($n=3$ for each agent). Furthermore, none of these agents altered the effect of diazepam in the presence of either milrinone (1 $\mu$M, $n=3$) or SK&F 94120 (1 $\mu$M, $n=3$). The specific antagonist of the peripheral benzodiazepine receptors PK 11195 (5 $\mu$M), had no effect alone ($n=3$), and did not alter the effect of diazepam in the presence of milrinone (1 $\mu$M, $n=3$) or SK&F 94120 (1 $\mu$M, $n=3$).

### *Interaction with isoprenaline*

Isoprenaline $10^{-9}$ M or diazepam $10^{-4}$ M alone were devoid of effect on the force of contraction of the electrically-driven guinea-pig heart right ventricle strip. However, in combination

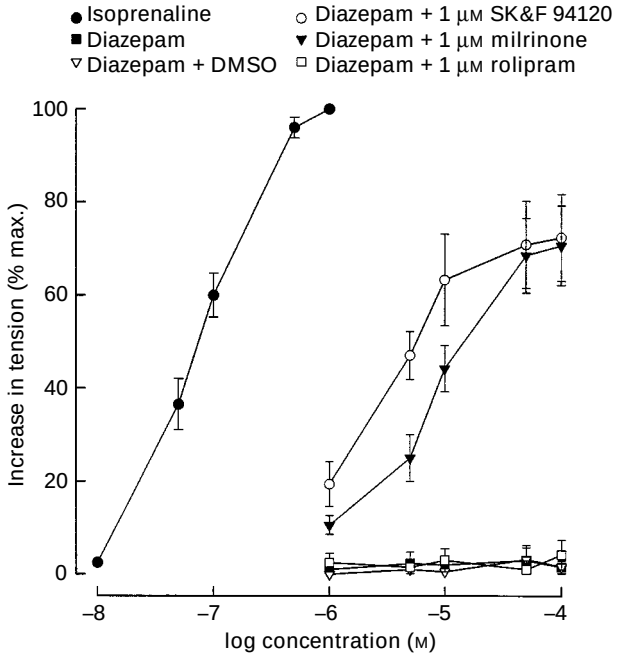


**Figure 1** Cumulative concentration-response curves for the inotropic activity of diazepam alone and in the presence of 1 $\mu$M of either of the selective PDE 3 inhibitors milrinone or SK&F 94120, the selective PDE 4 inhibitor rolipram or the PDE inhibitors solvent, dimethyl sulphoxide (DMSO). Concentrations of DMSO were the same as those present in the PDE inhibitors solutions. Increases in tension are plotted as percentages of the maximum response to isoprenaline. Each point represents the mean and ± s.e.mean (vertical lines) of four experiments.

the two drugs caused an increase of 68.7±9.5% ($n=4$) indicating a synergistic interaction. The diazepam solvent applied in the same concentrations as that present in the diazepam solutions did not cause significant changes in the inotropic effect of isoprenaline.

### *Inhibition of phosphodiesterase isoforms*

Table 1 shows the $IC_{50}$ values obtained for diazepam and the reference PDE 4 inhibitor rolipram as inhibitors of PDE isoenzymes. Diazepam inhibited PDE 4 with an $IC_{50}$ of 8.7±1.6 μM ($n=5$, Figure 2a). In contrast, GABA and the GABA antagonist picrotoxin, produced less than 10% inhibition of PDE 4 activity at doses up to 2 mM (data not shown). Diazepam also inhibited PDE 5 with a similar potency to that obtained for PDE 4 (Table 1). The potency of diazepam for the other PDE isoenzymes tested (PDEs 1, 2 and 3) was at least 6 fold lower than for PDE 4. In particular, only a marginal effect was observed for PDE 3, which precluded the calculation of an $IC_{50}$ for diazepam.

Additionally, the effect of several benzodiazepines, structurally related to diazepam, on the activity of phosphodiesterases 3, 4 and 5 was determined. As shown in Table 1, most of the analogues tested inhibited PDE 4, although out of the seven compounds tested diazepam showed the highest potency as PDE 4 inhibitor. The effect of the same diazepam analogues on PDE 3 and PDE 5 was variable, but in all cases lower than that of diazepam.

### *Mechanism of action of diazepam as inhibitor of PDE 4*

The mechanism of inhibition of guinea-pig cardiac PDE 4 by diazepam was characterized as described in Methods in two independent assays which produced similar results. In Figure 3a a Linweaver-Burk plot is presented to illustrate the variation of $1/V_{max}$ (y-axis intercept) and $1/K_M$ (x-axis intercept) for this enzyme as a function of diazepam concentration. $K_M$ was barely affected by diazepam, whereas $V_{max}$ was clearly concentration-dependently reduced by the drug. This indicates that diazepam acts as a non-competitive inhibitor of PDE 4. The same conclusion was obtained by plotting the same data according to Dixon (1953), as shown in Figure 3b. A value for $K_i$ (x-axis intercept in absolute value) of 11 μM was obtained with this plot. Under the same experimental conditions rolipram behaved as a competitive inhibitor of the PDE 4 isoenzyme, as previously described by Reeves *et al.* (1987) for the same enzyme preparation (data not shown).

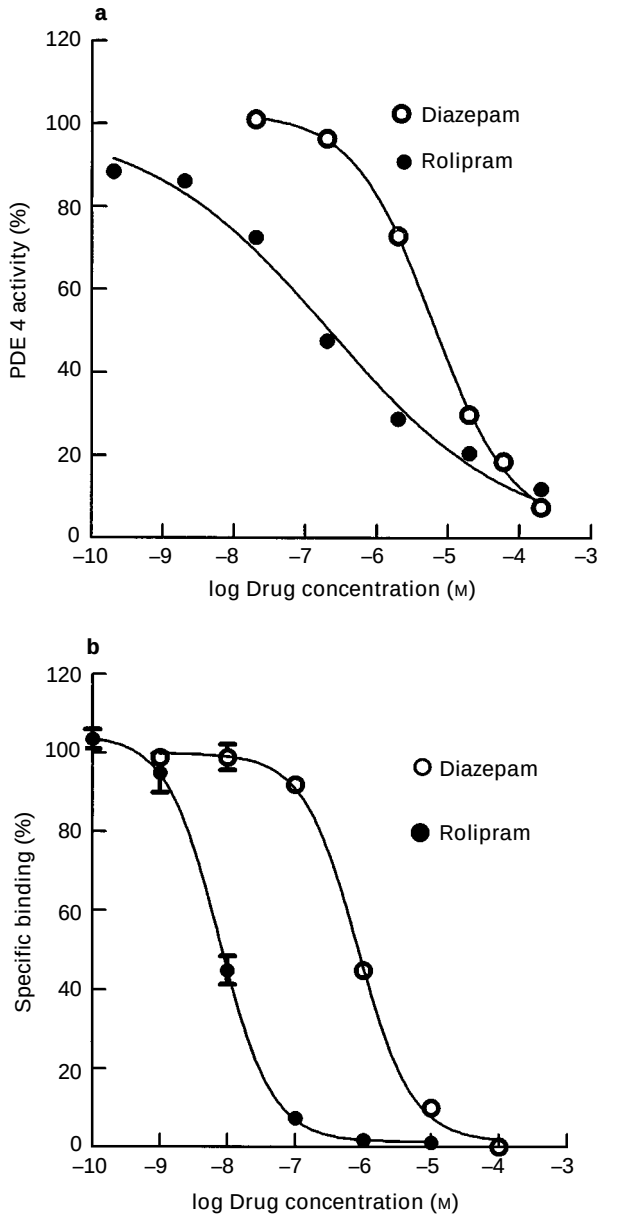


**Figure 2** Inhibition of guinea-pig cardiac ventricle PDE 4 (a) and displacement of [$^3$H]-rolipram from its high affinity binding site in rat brain (b) by diazepam and rolipram. Data shown correspond to a representative assay run in duplicate. Error bars fell within the size of symbols.

**Table 1** Potencies of rolipram, diazepam and diazepam-related benzodiazepines as inhibitors of PDE isoenzymes and as displacers of [$^3$H]-rolipram from its high affinity binding site

| *Compound* | *PDE1* | *PDE2* | *PDE3* | *PDE4* | *PDE5* | *[$^3$H]-rolipram binding site* |
|---|---|---|---|---|---|---|
| Rolipram | (11±5%) | (40±1.5%) | 242±11 | 0.32±0.09 | 125±36 | 0.058±0.0004 |
| Diazepam | 95.5±6.7 | 56.0±5.2 | (46.7±1.9%) | 8.7±1.6 | 10.9±1.4 | 0.81±0.27 |
| Chlordiazepoxide | n.t. | n.t. | (49.7±4.9%) | 36.6±6 | 87±1 | (48.8±4.1%) |
| Clonazepam | n.t. | n.t. | (46±1%) | 137±22 | (17±3%) | (19.0±9.1%) |
| Flunitrazepam | n.t. | n.t. | (35±8%) | 64±14 | 66±19 | (14.7±2.7%) |
| Lorazepam | n.t. | n.t. | (38±23%) | (30.5±4.2%) | (29±8%) | (41.8±33%) |
| Nitrazepam | n.t. | n.t. | 60±12 | 88±12 | 64±1 | (6.1±3.1%) |
| Flumazenil | n.t. | n.t. | (30.5±1.5%) | 126±14 | 62±1 | (16.8±9.7%) |

Values are expressed as $CI_{50}$±s.e.mean in μM concentration, or as % inhibition/displacement (values within parentheses) at the highest concentration tested in each assay (200 μM for PDE assays and 10 μM for the binding assay, respectively). Data correspond to at least three independent assays run in duplicate. n.t. indicates not tested.

Diazepam being a non-competitive inhibitor, the $K_i$ value of this drug for PDE 4 is equal to its $IC_{50}$ (8.7 $\mu$M) according to Cheng & Prusoff (1973). This is in close agreement with the value obtained from the Dixon plot.

### *Displacement of [$^3$H]-rolipram from rat cortex membranes*

The $IC_{50}$ values obtained for diazepam, rolipram and several diazepam analogues as displacers of [$^3$H]-rolipram from its high affinity binding site in rat brain cortex membranes are shown in Table 1.

Both diazepam and rolipram were able to displace potently [$^3$H]-rolipram (Figure 2b), whereas the other benzodiazepines tested showed much less potency than diazepam in this assay. The $IC_{50}$ obtained for diazepam in this assay ($0.81 \pm 0.27$ $\mu$M) is 10.7 fold lower than its $IC_{50}$ as PDE 4 inhibitor. Under the same experimental conditions rolipram exhibited a ratio of 55 fold, also in favour of the [$^3$H]-rolipram displacement.

### *Effect of diazepam on the isoprenaline-induced cyclic AMP accumulation in guinea-pig eosinophils*

The incubation of guinea-pig peritoneal eosinophils with isoprenaline raised the cyclic AMP levels from the basal values of $0.14 \pm 0.04$ amol per cell to $0.73 \pm 0.2$ amol per cell. This latter value was considered for the following calculations. Diazepam was able to increase, in a concentration-dependent manner, the level of cyclic AMP by more than 13 fold, reaching a maximal effect at a concentration of 100 $\mu$M (Figure 4). An $EC_{50}$ value of $22 \pm 1.8$ $\mu$M ($n=4$) was obtained for this effect. As shown in Figure 4 a similar profile, although with higher potency, was obtained for the standard PDE 4 inhibitor rolipram, that yielded an $EC_{50}$ of $0.16 \pm 0.01$ $\mu$M ($n=6$).

## Discussion

It is known that diazepam can increase cardiac contractility (Kenakin, 1982). This effect seems to be mediated through an enhancement of intracellular cyclic AMP levels (Martinez *et al*., 1995a), but the mechanism responsible for this enhancement was, until now, unclear. The present study demonstrated that diazepam can inhibit PDE 4, one of the four cyclic nucleotide phosphodiesterases found in cardiac tissue, whereas its potency as inhibitor of the other three isoenzymes, and in particular PDE 3, was much lower. Since PDE 4 and PDE 3 are the main isoenzymes responsible for the hydrolysis of cyclic AMP in cardiac tissue (Nicholson *et al*., 1991), our results suggest that diazepam increases cardiac contractility through the inhibition of PDE 4, which in turn leads to the previously described increases in cyclic AMP concentration found in cardiac tissue after diazepam treatment (Martinez *et al*., 1995a).

The results from functional experiments with cardiac ventricle strips showed a marked potentiation of diazepam inotropic effect by the selective PDE 3 inhibitors milrinone and

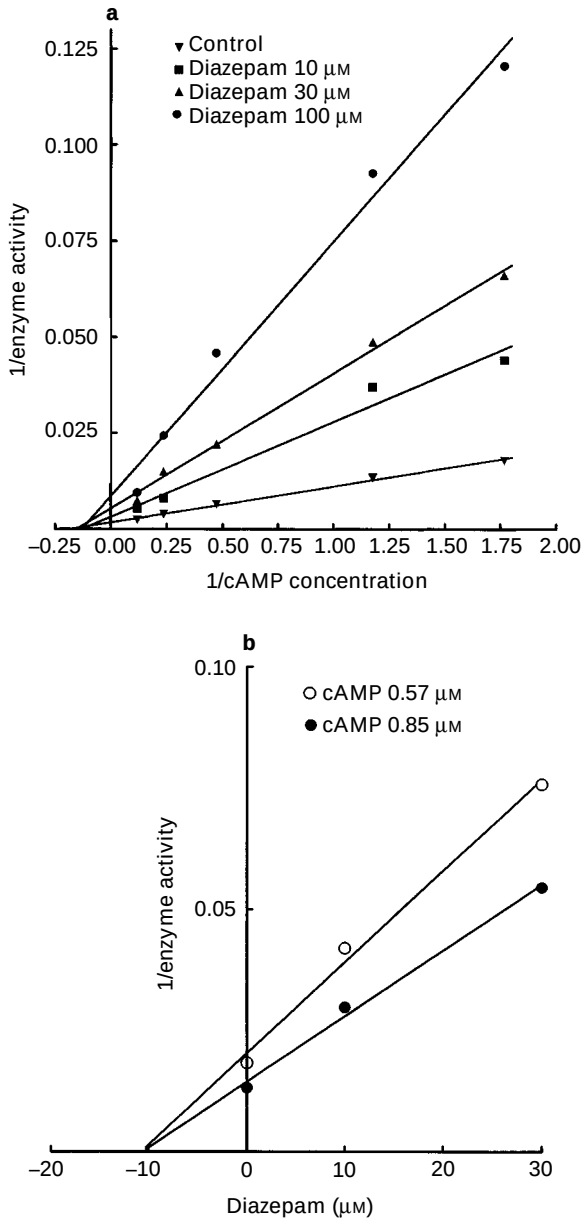


**Figure 3** Kinetic analysis of the effect of diazepam on PDE 4 cyclic AMP hydrolisis. Linweaver-Burk plots (a) and Dixon plot (b). Data are the values obtained from a representative experiment run in duplicate.

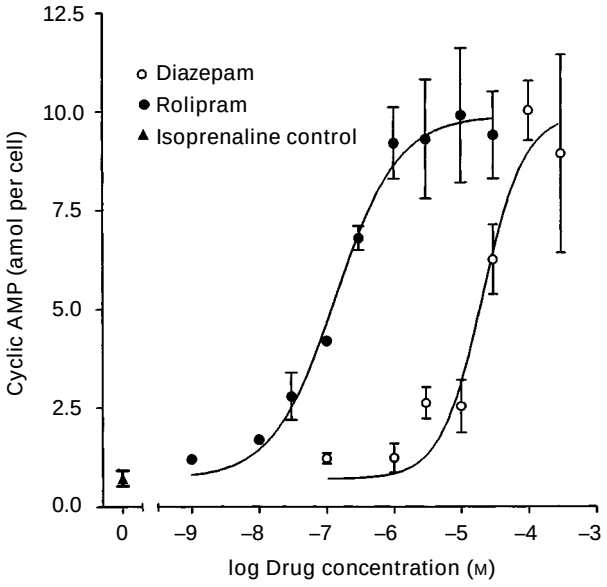


**Figure 4** Effect of diazepam and rolipram on the isoprenaline-induced cyclic AMP accumulation in guinea-pig isolated eosinophils. Data shown are the average of four (diazepam) and six (rolipram) independent assays; vertical lines show s.e.mean.

SK&F 94120, but not by the selective PDE 4 inhibitor rolipram. The existence of synergism on cardiac contractility resulting from the interaction between PDE 3 and PDE 4 inhibitors is well known (Shahid & Nicholson, 1990) and has been used as simple procedure to establish the isoenzyme selectivity of PDE inhibitors (Gristwood *et al*., 1992). Thus, one possible explanation for the results obtained with diazepam could be that the compound is, like rolipram, an inhibitor of PDE 4. Further indirect evidence for PDE inhibitory activity is provided by the finding that diazepam potentiates inotropic responses to isoprenaline. Indeed, diazepam clearly increased the contractile force of guinea-pig heart ventricle strips in the presence of a concentration of isoprenaline which is devoid of effect on its own. This is in agreement with previous results demonstrating a potentiation of the functional effects of isoprenaline through PDE 4 inhibition (Qian *et al*., 1993).

The biochemical studies confirmed that diazepam, like rolipram, can inhibit PDE 4 whereas its effect on PDE 3 is negligible. The effect of diazepam on the other PDE isoenzymes present in guinea-pig cardiac muscle (PDEs 1 and 2) was significantly lower than for PDE 4.

It has also been found that diazepam is an inhibitor of PDE 5, with a potency close to the one found for PDE 4. Since PDE 5 is absent from cardiac muscle (for review see Nicholson *et al*., 1991) the cardiac effects observed with diazepam are unlikely to be due to its interaction with this enzyme. Therefore, our data indicate that diazepam can be considered a relatively selective inhibitor of PDE 4 in cardiac tissue. The kinetic mechanism of inhibition of PDE 4 by diazepam has been determined, and found to be of a non-competitive nature. This is also the case for other PDE 4 inhibitors with chemical structures unrelated to diazepam, such as the recently described di-substituted catechol CP-80,633 (Cohan *et al*., 1996).

The action of diazepam as a PDE 4 inhibitor at the functional level has been checked by looking at its effect on the potentiation of the isoprenaline-induced cyclic AMP accumulation in guinea-pig eosinophils (Dent *et al*., 1991). Only phosphodiesterase inhibitors with affinity for PDE 4 are active in this assay, since PDE 4 is the only isoenzyme present in these cells (Souness *et al*., 1991). Diazepam is able to increase the cyclic nucleotide levels in this assay by more than 13 fold and shows a potency close to its potency as inhibitor of the isolated enzyme, as is the case for rolipram.

The affinity of diazepam and related drugs for the high affinity binding site for rolipram described in rat brain (Schneider *et al*., 1986) has also been checked. The affinity of rolipram for this binding site is several orders of magnitude higher than its $K_i$ as inhibitor of the catalytic activity of PDE 4, and similar discrepancies exist for many other PDE 4 inhibitors. It has been shown that certain peripheral actions of PDE inhibitors, in tissues where PDE 4 predominates, are more closely correlated with their ability to displace [$^3$H]-rolipram than with inhibition of PDE 4 (Harris *et al*., 1989; Barnette *et al*., 1995). Diazepam behaves also in this assay as a typical PDE 4 inhibitor, displacing [$^3$H]-rolipram with an affinity approximately 11 fold higher than its potency as an inhibitor of the enzyme. This finding is consistent with previous results indicating that diazepam displaces [$^3$H]-rolipram from its high affinity binding site in the rat forebrain membrane fraction (Schneider *et al*., 1986).

The present results demonstrate that diazepam inhibits the PDE 4 isoenzyme and this could explain the potentiation of the effect of cyclic AMP-producing agents, shown for diazepam by several authors (York & Davies, 1982; Elgoyhen & Adler-Graschinsky, 1989; Martinez *et al*., 1995a,b).

The effect of diazepam on cardiac contractility does not seem to be related to its interaction with the described GABA receptor, since it is neither mimicked, nor potentiated by GABA itself and is not antagonized by picrotoxin or flumazenil. Additionally, neither the neutral antagonist $\beta$CCMe (Skerritt & Macdonald, 1984), nor the inverse agonists DMCM and $\beta$CCMa (Petersen & Jensen, 1984), affected the enhancement of cardiac contractility induced by diazepam, in the presence of either of the two PDE 3 inhibitors (milrinone and SK&F 94120) used in the present study. Further evidence in support of this comes from the biochemical data obtained for some of the above mentioned compounds as inhibitors of cyclic nucleotide phosphodiesterases. In contrast to diazepam, GABA and picrotoxin, two substances that bear no structural relationship with diazepam, had no effect on guinea-pig PDE 4. On the other hand, most of the diazepam structurally-related benzodiazepines tested showed different affinities for PDE 4 and other phosphodiesterases as well as for the [$^3$H]-rolipram binding site. Interestingly, this was the case irrespective of their agonistic or antagonistic nature at the GABA receptor complex. Finally, the rank order of potency for the different diazepam analogues as PDE 4 inhibitors or [$^3$H]-rolipram displacers was unrelated to their affinities for the GABA receptor benzodiazepine binding site, as well as to functional responses believed to be mediated through this receptor (Mohler & Okada, 1977).

Finally, the peripheral, non GABA-related benzodiazepine binding site does not seem to be involved in our results, since the specific peripheral antagonist PK 11195 (Parola & Yamamura, 1993) had no influence on the functional responses of diazepam.

All these results support the contention that other cellular mechanisms in addition to the important facilitation of GABA-mediated chloride conductance, such as for instance cyclic nucleotide phosphodiesterases inhibition, may contribute to the effects of diazepam.

The activity of diazepam as an inhibitor of PDE 4 described in the present work was obtained with concentrations of about 2.5 $\mu$g ml$^{-1}$, which are higher than those obtained with doses recommended for the chronic treatment of anxiety (2–30 mg day$^{-1}$), which are in the range of 0.02 to 1.01 $\mu$g ml$^{-1}$ (Greenblatt *et al*., 1981). However, this agent produces high tolerance and physical dependence with the subsequent potential for drug abuse which could lead to plasma concentrations higher than those considered as anxiolytic (Robertson, 1994). On the other hand, diazepam is also used at higher doses for the control of 'status epilepticus' (up to 3 mg kg$^{-1}$ infused over 24 h) or for the induction of anaesthesia, which could produce serum concentrations of $6.01 \pm 1.21$ $\mu$g ml$^{-1}$ (Samuelson *et al*., 1981; Reynolds 1996). Furthermore, brain concentrations of diazepam could be greater than corresponding plasma concentrations (De Lorenzo *et al*., 1981). Therefore, the inhibitory PDE 4 activity of diazepam described in the present work was obtained with concentrations which indeed could be reached under clinical conditions and this could have some relevance, especially in the brain where this is the main isoenzyme in the hydrolysis of cyclic AMP (Nicholson *et al*., 1991).

The effect of diazepam on phosphodiesterases could also be part of the mechanisms involved in some experimental and clinical effects of benzodiazepines which are not well understood. Interestingly, diazepam has some effects, such as antidepressant activity (Tiller & Schweitzer, 1992), airway smooth muscle relaxation (Koga *et al*., 1992) and hyperglycaemia (Rehri *et al*., 1994), which are shared by PDE 4 inhibitors (for review see Thompson, 1991). Furthermore

inhibition of PDE 4 can elicit some anti-inflammatory effects (Teixeira *et al*., 1997), and it is known that diazepam inhibits antigen-induced eosinophil infiltration into guinea-pig conjunctiva (Kawata *et al*., 1966) as well as other immune-cell functions, such as phagocytosis of human polymorphonuclear leukocytes and monocytes or human natural killer cell activity (for review see Pawlikowski, 1993).

Finally, several studies have indicated that some of the functional effects of PDE 4 inhibitors correlate with their affinity for the [$^3$H]-rolipram binding site rather than with their potency as inhibitors of cyclic AMP breakdown (Harris *et al*., 1989; Barnette *et al*., 1995). We have shown that diazepam is 10 fold more potent as displacer of [$^3$H]-rolipram than as an inhibitor of PDE 4. Therefore, plasma concentrations of diazepam as low as 0.25 $\mu$g ml$^{-1}$, that fall within the range found during the therapeutic use of diazepam, could be exerting a significant effect on PDE 4-mediated responses in diazepam-treated patients.

One of the characteristic features of PDE 4 inhibitors when administered to susceptible animal species or man is the induction of nausea and vomiting (Scott *et al*., 1991; Heaslip & Evans, 1995). This is believed to be a general property of PDE 4 inhibitors, and appears to correlate better with the ability of the compounds to interact with the high affinity binding site for [$^3$H]-rolipram than with their potency as inhibitors of the catalytic action of the enzyme (Duplantier *et al*., 1996). The fact that diazepam is some 30 fold less potent than rolipram as inhibitor of the enzyme and, in particular, its relative lower affinity for the [$^3$H]-rolipram binding site compared with rolipram (PDE 4/binding site ratios of 10.7 fold and 55 fold, respectively) may account for the absence of episodes of nausea and vomiting associated with the clinical use of diazepam.

In conclusion, a specific interaction of diazepam with phosphodiesterase type 4 in the heart has been established as the most likely mechanism mediating the previously described effects of diazepam in this tissue. This property is shared, to a lesser extent, with other benzodiazepines structurally related to diazepam, but not by other agents known to be active at the GABA receptor complex. Diazepam and its analogues are also able to inhibit another phosphodiesterase isoenzyme, PDE 5, that is absent from the heart. The interaction between the known effects of diazepam on the CNS and those caused by its ability to inhibit PDE 4, described in this study, cannot be ruled out, and a further investigation is necessary to establish the clinical relevance of these findings.

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