ELSEVIER

European Journal of Pharmacology 276 (1995) 201–205

ejp

Vascular and tubular actions of diazepam in isolated and perfused rat kidney

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Received 18 July 1994; revised 29 December 1994; accepted 3 January 1995

Abstract

The effects of diazepam were studied in the isolated rat kidney under conditions of constant flow. Kidneys were perfused with modified Ringer-Krebs solution. Diazepam produced a raised fractional excretion of water and sodium without hemodynamic changes, suggesting a direct effect on tubular transport mechanisms. Diazepam decreased renal perfusion pressure in a concentration-dependent fashion when kidneys were pretreated with either noradrenaline or potassium chloride. Similar responses were observed when 7-chloro-5-[4-chloro-phenyl]-1,3-dihydro-1-mehtyl-2*H*-1,4-benzodiazepin-2-one (Ro 5-4864) or clonazepam was used. These data provide evidence for a relaxant effect of benzodiazepines on preconstricted renal vasculature.

Keywords: Diazepam; Clonazepam; Ro 5-4864; Kidney, isolated, perfused; Renal function; Renal vasculature; (Rat)

1. Introduction

Besides their action on $GABA_A$ receptors, benzodiazepines act on a second binding site in cells, known as the peripheral-type benzodiazepine receptor. Peripheral-type benzodiazepine receptor ligands have been shown to exert pharmacological effects on different peripheral tissues, such as relaxation of vascular (Grupp et al., 1987; Elgoyhen et al., 1993) and duodenal (Escubedo et al., 1992) smooth muscle, reduction of noradrenaline-induced chronotropic responses in rat atria (Elgoyhen and Adler-Graschinsky, 1989) and inhibition of the adenosine uptake system in rat vas deferens (Escubedo et al., 1991).

In rat kidney, peripheral-type benzodiazepine receptors are concentrated in the thick ascending limb of the loop of Henle and in the early distal tubule (Beaumont et al., 1984). In vivo administration of different classes of diuretics selectively increases the appearance of peripheral-type benzodiazepine receptors in different regions of the rat kidney (Lukeman et al., 1988). Inhibition of the benzodiazepine 7-chloro-5-[4-chlorophenyl]-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one (Ro 5-4864) binding by thiazide-like compounds has shown a rank order of potencies similar to that for their enhancement of in vivo natriuresis (Lukeman and Fanestil, 1987). Previous studies carried out in our laboratory have shown an increase in fractional excretion of water, sodium and potassium in rats treated with different doses of diazepam (Monasterolo and Elías, 1993). Probably, these effects could be mediated through peripheral-type benzodiazepine receptors. Nevertheless, they could also be explained as actions of diazepam on the central nervous system, on systemic hemodynamics and (or) on neurotransmitter and hormone release. Thus, the aim of the present study was to investigate the possible *direct* effects of diazepam by using the isolated perfused kidney model. This preparation offers the important advantage of circumventing non-renal factors that influence the in vivo model (Bekersky, 1983). The effects of clonazepam (a central-type benzodiazepine receptor agonist) and Ro 5-4864 (a peripheral-type benzodiazepine receptor agonist) on renal vasculature were also tested. Our results indicated that diazepam promotes a direct effect on

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SSDI 0014-2999(95)00019-4

tubular transport mechanisms which is expressed as diuresis and natriuresis. Diazepam also promotes a concentration-dependent relaxation of renal vasculature when the preparations are pretreated with either noradrenaline or potassium chloride.

2. Materials and methods

2.1. Animals

Male Wistar rats, weighing 220–300 g were housed at 21–24°C with 12 h dark-light cycles and maintained on a standard diet and water ad libitum. The animals were chosen at random for control or test experiments.

2.2. Isolated rat kidney preparation

The animals were anaesthetized with sodium pentobarbital (50 mg/kg body weight i.p.). The right kidney was prepared and perfused as previously described (Elías et al., 1985,1987). The perfusion medium (pH = 7.4) consisted of Ringer-Krebs solution supplemented with dextran 2% (Sigma Chemicals Co., average molecular weight 82 200) as a colloid-osmotic agent, glucose (10 mM), sodium pyruvate (5 mM), sodium lactate (5 mM) and creatinine (400 mg/l). The medium also contained 0.5 mM cysteine, 0.5 mM glutamic acid and 2.3 mM glycine to prevent the loss of glutathione from specific regions of the kidney (Epstein et al., 1982; Brezis et al., 1983; Torres et al., 1986). The medium was constantly bubbled with O_2-CO_2 (19:1, v/v). The recirculating perfusate volume was 100 ml. The entire system operated under thermostatic control at 37°C. Perfusion through the isolated kidney in situ was performed with the use of a peristaltic pump (Masterflex, USA) at a constant rate measured with a flowmeter (Gilmont Instruments, USA) inserted in the arterial line. Perfusion pressure was continuously measured at the tip of the arterial cannula by means of a pressure transducer (Gould P231D, USA) and was recorded on a multi-pen recorder (Rikadenki, Japan).

After 20–25 min of equilibration 5-min urine collection periods were started and 'arterial' perfusate samples were taken at midpoint.

2.3. Experimental protocols

Effects of diazepam on the isolated rat kidney function

Group I: This group consisted of control preparations to assess their viability throughout the perfusion.

Group II: After a 5-min control clearance period, four consecutive 5-min clearance periods were studied in the presence of different concentrations of diazepam. The final concentrations of diazepam (0.05, 2, 54 and 230 μM) were obtained by adding cumulative amounts of diazepam into the perfusion medium. Diazepam was prepared freshly in absolute ethanol. Further dilutions were made with double-distilled water.

Glomerular filtration rate was estimated from the creatinine clearance. Fractional excretion of water (FE_{H2O} = urine volume per min/glomerular filtration rate), glucose (FE_{Glu} = glucose clearance/glomerular filtration rate) and sodium (FE_{Na} = sodium clearance/glomerular filtration rate) were determined. A group of preparations was run with the addition of the corresponding volumes of diluents.

Effects of diazepam, clonazepam and Ro 5-4864 on the noradrenaline-preconstricted vasculature of the isolated rat kidney

Group I: After a control clearance period, noradrenaline was infused into the perfusion medium to induce vasoconstriction. A perfusate noradrenaline concentration of 1 μM was provided throughout the experiment. This group was used as control in the presence of noradrenaline.

Group II: After a control clearance period, 15-min stabilization with noradrenaline 1 μM was allowed. Then, cumulative amounts of diazepam were added every 5 min to the perfusate to attain the final concentrations: 2, 54, 230 and 580 μM.

Perfusion pressure measurements were performed when the recording graph reached a steady state after the addition of each dose of diazepam.

Group III: The same scheme as described for group II was followed. In this group the benzodiazepine, Ro 5-4864 (5–150 μM), a peripheral-type benzodiazepine agonist, was studied. Ro 5-4864 was prepared in absolute ethanol and then diluted in double-distilled water.

Group IV: The same scheme as described for group II was followed. In this group the benzodiazepine, clonazepam (2–500 μM), a central-type benzodiazepine receptor agonist, was studied. Clonazepam solution was prepared like the other benzodiazepines solutions.

A group of preparations was run in the presence of the corresponding volumes of diluent.

Effects of diazepam on the potassium chloride (KCl)-preconstricted vasculature of the isolated rat kidney

The same scheme and diazepam concentrations as described for the noradrenaline protocol were used. In this series of experiments the vasoconstriction was induced by 40 mM KCl in equimolar replacement for sodium chloride. Perfusion pressure changes in the presence of different concentrations of diazepam (2–580 μM) were determined. A control group in the presence of the corresponding volumes of diluent was run.

After the experiments were concluded, the renal vascular responsiveness was always tested by adding

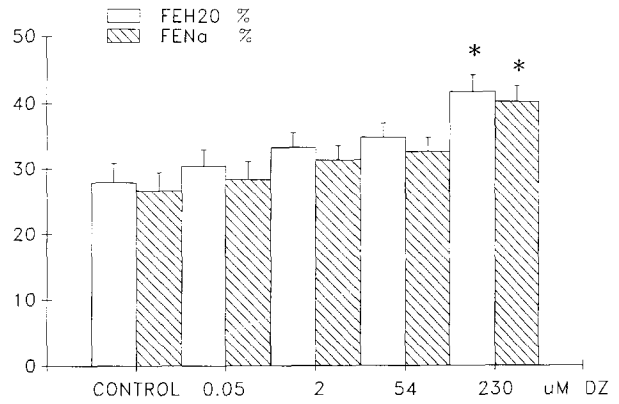

Fig. 1. Rates of water and sodium excretion from rat isolated kidneys exposed to diazepam treatment. Ordinate represents percentage of fractional excretion. Values are expressed as means ± S.E.M. ($n = 6$). * Significantly different ($P < 0.05$) when compared with control period.

noradrenaline into the perfusion medium to give a concentration of 10 μM. At the end of each perfusion the kidney was promptly removed from the circuit and weighed.

2.4. Analytical methods

The volume of urine collected was determined by gravimetry. Determinations of creatinine and glucose in urine and perfusate samples were carried out by standard methods using commercial kits (Wiener Lab., Argentina) and sodium concentration was measured by flame photometry.

2.5. Statistical analysis

Results are presented as the means ± S.E.M. Statistical significance of the differences between values was assessed by multifactor analysis of variance followed by Scheffe's multiple range test; P values less than or equal to 0.05 were considered significant.

2.6. Chemicals

All chemicals were of the highest grade available commercially. (±)-Noradrenaline hydrochloride was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Diazepam and clonazepam were kindly donated by Productos Roche (Buenos Aires, Argentina) and Ro 5-4864 (7-chloro-5-[4-chloro-phenyl]-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one) by R. Hoffmann-La Roche (Basel, Switzerland).

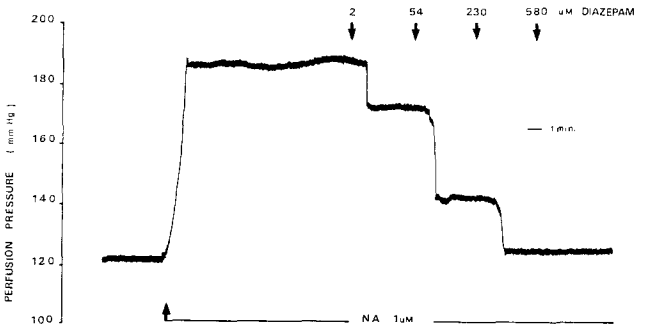

Fig. 2. A typical recording of perfusion pressure illustrating the vasodilator effect of sequentially increasing concentrations of diazepam on the noradrenaline-pretreated isolated rat kidney.

3. Results

3.1. Effects of diazepam on isolated rat kidney function

The values for parameters of renal function shown in Table 1 remained unchanged during the whole experiment in the control preparations. Renal function data obtained during control periods in all the experimental groups did not differ from those in Table 1, and were not affected by the addition of the vehicle.

The highest concentration of diazepam increased FE_{H2O} and FE_{Na}. These data are shown in Fig. 1. On the other hand, hemodynamic parameters and FE_{Glu} remained unchanged with diazepam treatment.

3.2. Effects of diazepam, clonazepam and Ro 5-4864 on the preconstricted vasculature of the isolated rat kidney

In the control group, the vasoconstriction (estimated by perfusion pressure) achieved with either noradrenaline 1 μM or KCl 40 mM remained unchanged throughout the experiment and was not modified by the addition of the corresponding volumes of diluent.

When added in sequentially increasing concentrations diazepam, Ro 5-4864 and clonazepam induced a concentration-dependent relaxation of the noradrenaline-preconstricted renal vasculature. A typical record-

Table 1
Functional parameters of isolated rat kidney during control periods

Perfusion flow (ml/min)	PP (mm Hg)	GFR (ml/min/g)	FE_{H2O}% (UV/GFR)	FE_{Na}% (Cl_{Na}/GFR)	FE_{Gl}% (Cl_{Gl}/GFR)
22.66 ± 0.61	127 ± 6	0.196 ± 0.029	27.86 ± 2.92	26.65 ± 2.79	11.08 ± 1.14

Values shown are means ± S.E.M.; $n = 6$. PP = perfusion pressure; GFR = glomerular filtration rate; UV = urine volume/min; Cl_{Na} = sodium clearance; Cl_{Gl} = glucose clearance.

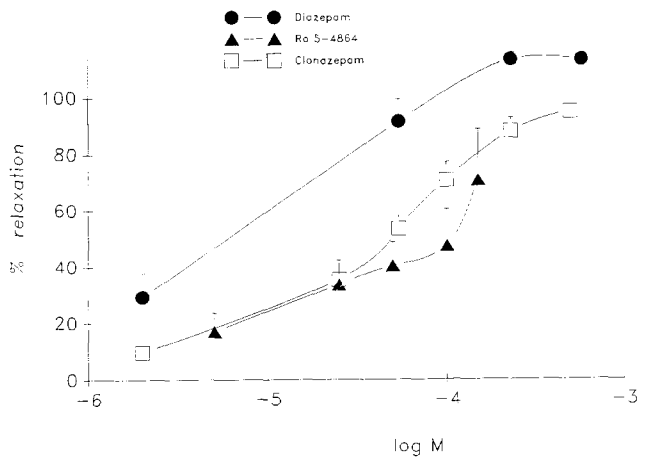

Fig. 3. Effects of diazepam, Ro 5-4864 and clonazepam on noradrenaline-preconstricted renal vasculature. Values are expressed as means ± S.E.M. ($n = 4$–8). 100% relaxation represents the complete reversal of the increase in perfusion pressure produced by noradrenaline (1 μM). Basal (126 ± 4 mm Hg, $n = 17$) and noradrenaline-induced (245 ± 6 mm Hg, $n = 17$) perfusion pressure did not differ between experimental groups.

ing of the modifications in the perfusion pressure produced by different concentrations of diazepam is presented in Fig. 2. Results obtained with diazepam, Ro 5-4864 and clonazepam are collected in Fig. 3. This figure shows differences in potency among the drugs. Diazepam seems to have a higher potency (10-fold approximately) than Ro 5-4864 and clonazepam. Nevertheless, these relative potencies should be considered

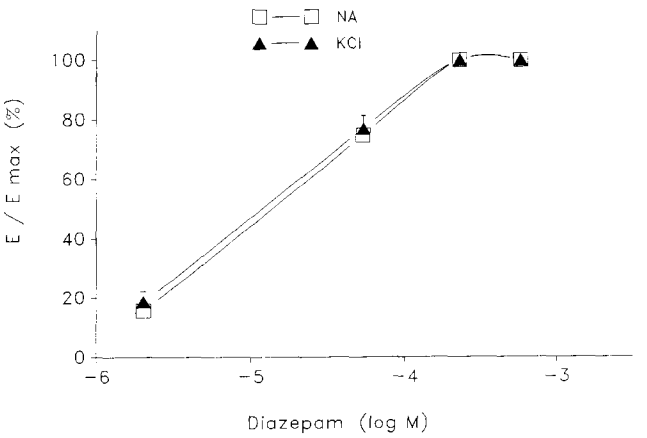

Fig. 4. Effects of diazepam on the noradrenaline- and potassium chloride-preconstricted renal vasculature. Values are expressed as means ± S.E.M. ($n = 4$–5). Ordinate represents the ratio between the diminution in perfusion pressure observed after the addition of a dose of diazepam and the maximal diminution in perfusion pressure observed in the same experiment. When the maximal diazepam-induced relaxation was reached (in both noradrenaline and potassium chloride pretreatment) the perfusion pressure value was similar to that observed in the control period. Basal perfusion pressure (129 ± 5 mm Hg, $n = 9$) had similar values in both experimental groups. Noradrenaline-induced perfusion pressure was 239 ± 7 mm Hg, $n = 5$. KCl-induced perfusion pressure was 232 ± 11 mm Hg, $n = 4$.

with caution since, because of testing the whole organ, apparent differences could actually be related to pharmacokinetic factors affecting the drugs' responses and not exclusively to the affinity of the drugs to the putative receptor.

Diazepam also evoked a concentration-related relaxation when the preparation was pretreated with KCl (Fig. 4).

Diazepam inhibited both noradrenaline- and KCl-induced contractions at similar concentrations (IC_{50}: 10^{-5} M). Perfusion pressure values obtained with the highest doses of diazepam were not different from those observed in the control period (before the addition of noradrenaline or KCl). These data are collected in Fig. 4.

4. Discussion

Data from this study provide evidence for the ability of diazepam (a benzodiazepine interacting with both peripheral- and central-type benzodiazepine receptors) to produce direct effects on renal functions.

Diazepam decreased the reabsorption rates of sodium and water as compared with control periods without promoting hemodynamic changes, thus a direct effect of diazepam on tubular transport mechanisms could be assumed. In this regard, we have observed that diazepam induced diuresis and natriuresis without detectable changes in glomerular filtration rate or *p*-amino hippuric acid clearance after in vivo administration to conscious rats (Monasterolo and Elías, 1993). Moreover, it has been described that the renal molecular species that is the peripheral-type benzodiazepine receptor is closely associated with, if not the same as, the molecular species which recognizes thiazide-type ligands (Lukeman and Fanestil, 1987).

A concentration-dependent relaxation caused by diazepam was observed when the preparations were pretreated with either noradrenaline or KCl. Furthermore, the other benzodiazepines studied, clonazepam with high affinity to central-type benzodiazepine receptors and Ro 5-4864 with high affinity to peripheral-type benzodiazepine receptors, were also able to produce relaxation of the noradrenaline-pretreated renal vasculature. In spite of the lack of study of specific antagonists, the vasodilator effects of the different benzodiazepines assayed could be suspected to be mediated through central- and peripheral-type benzodiazepine receptors. Hernández (1991) has reported that the inhibitory effect of diazepam on cardiac contractility in the rat is mediated by both central- and peripheral-type benzodiazepine receptors. Other in vitro experiments, using different vascular models, showed benzodiazepine inhibition of both noradrenaline and KCl-induced contractions (French et al., 1989). Moreover, the

benzodiazepine-relaxant effect was reported for other smooth muscles such as estrogen-contracted uterine tissue (Kazanietz and Elgoyhen, 1990), guinea-pig trachea (Raeburn et al., 1988) and rat duodenum and vas deferens (Escubedo et al., 1992). In both our and the above-cited work, micromolar concentrations of benzodiazepines were required to obtain their relaxant effects. The need for a micromolar concentration to evoke smooth muscle relaxation was also described for other pharmacological effects (i.e. diminution of the chronotropic responses to noradrenaline in isolated atria – Elgoyhen and Adler-Graschinsky, 1989).

Since diazepam promotes relaxation in both noradrenaline- and KCl-preconstricted renal vasculature it could be supposed that diazepam alters the biochemical machinery involved in smooth muscle contractions. Smooth muscle contractions depend on Ca^{2+} influx. Micromolar concentrations of Ro 5-4864 inhibit Ca^{2+} entry in rat vas deferens preparations during KCl stimulation (Escubedo et al., 1992). Alterations in second messenger systems such as changes in cAMP levels (Elgoyhen and Adler-Graschinsky, 1989) or phosphoinositide breakdown (Escubedo et al., 1992) by benzodiazepines have been investigated. In addition, it was reported that diazepam potentiates the effect of adenosine in rat vas deferens (Escubedo et al., 1991). Possible effects of diazepam on endothelial cells should not be disregarded. Experiments to elucidate the possible mechanisms involved are included in our current investigations.

Acknowledgements

The authors thank Wiener Lab., Rosario, Argentina, for the gift of analytical reagents. This study was supported by a grant from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina).

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