Diazepam increases membrane fluidity of rat hippocampus synaptosomes

Tiziana Mennini, Angelo Ceci, Silvio Caccia, Silvio Garattini, Pietro Masturzo and Mario Salmona*

Istituto di Ricerche Farmacologiche 'Mario Negri', Via Eritrea 62, 20157 Milan, Italy

Received 5 June 1984

Diazepam in vitro produced a concentration-dependent increase of membrane fluidity in crude synaptic membranes from rat hippocampus, but not cerebellum. Similar effects were obtained with higher concentrations of Ro 15-1788 and PK 11195, while zopiclone was completely inactive. In vivo acute treatment with diazepam and Ro 15-1788 gave results similar to those in vitro. The specific benzodiazepine antagonist also significantly increased membrane fluidity and was not able to reverse diazepam's effect. The data are discussed in terms of a possible role of protein kinase inhibition by the drugs not mediated by the 'central' or 'peripheral' type of benzodiazepine receptors.

Hippocampus *Cerebellum* *Membrane fluidity* *Benzodiazepine receptor* *Diazepam* *Ro 15-1788* *PK 11195* *Zopiclone*

1. INTRODUCTION

It was recently reported that occupancy of benzodiazepine (BDZ) binding sites in cultured C_6 astrocytoma cells by this group of drugs enhanced [^{3}H]methyl moiety incorporation into phosphatidylcholine [1]. Although BDZ-binding sites on astrocytoma cells have different properties from those in the brain (i.e., peripheral type BDZ receptors) this was the first biochemical event reported as a consequence of receptor occupancy [2].

The synthesis and translocation of methylated phospholipids were reported to change the number of [^{3}H]diazepam-binding sites, unmasking the high-affinity component and raising the apparent B_{max} values for the low-affinity component of [^{3}H]GABA receptors [3]. We therefore decided to check whether in vivo occupancy of brain BDZ receptors by pharmacologically relevant doses of diazepam affected membrane fluidity.

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

Male rats (Sprague-Dawley CD-COBS, 200 g, Charles River Italy, Calco, Como) were given 10 mg/kg i.p. of diazepam (Hoffmann-La Roche, Basel) or vehicle then 1 min later 30 mg/kg p.o. Ro 15-1788 (Hoffmann-La Roche) or vehicle; they were killed 15 min after diazepam. Drugs were suspended in 0.5% carboxymethylcellulose. Synaptosomes were obtained from brain areas as in [4]. The P_2B fractions (interface between 0.8 and 1.2 M sucrose) were diluted with 0.05 M phosphate buffered-saline (PBS; pH 7.4), centrifuged at 17000 × *g* for 10 min and then resuspended in PBS using an Ultra-Turrax (TP 18-10) at medium setting for 20 s to give a final protein concentration of about 0.25 mg/ml [5].

For in vitro experiments, crude synaptic membranes were obtained from P_2B fractions of untreated rats by resuspension in ice-cold water for 15 min, homogenization with the Ultra-Turrax for 30 s, and centrifugation at 8000 × *g* for 20 min. The supernatants and buffy coats were carefully

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00145793/84/$3.00

removed, and centrifuged at 48000 × *g* for 20 min. The final pellets were resuspended in PBS as described above.

Membrane microviscosity was determined by the fluorescence polarization technique as in [6] using 1,6-diphenylhexatriene (DPH) (Aldrich) as fluorescent probe. Membrane preparations (2 ml) were incubated with a final DPH concentration of 10^{-6} M for 30 min at 4°C. The fluorescence polarization value, *P*, was determined at 37°C with an Elscint MV-1 instrument (Haifa, Israel).

For in vitro experiments, diazepam and Ro 15-1788 were dissolved as their hydrochlorides in PBS (pH 7) at a final concentration of 10^{-4} M. PK 11195 (Pharmuka Laboratories, Gennevilliers, France) was dissolved in PBS–EtOH (50:50, v/v) at a concentration of 0.1 M. Zopiclone (Rhône-Poulenc, Vitry-sur-Seine, France) was dissolved as its hydrochloride in PBS (pH 7) or in PBS–dimethylformamide (50:50, v/v) at 10^{-3} M.

The in vitro membrane-fluidizing effect of the drugs was determined as follows: to 3 ml of DPH-labelled membrane preparation 0.050–0.100 ml drug solutions were added to obtain final concentrations ranging from 10^{-5} to 10^{-12} M. The *P* value was first recorded before the addition of the drug, then every 2 min until no further membrane microviscosity change was detected. The data for all the determinations are in relation to this time. A blank of the solutions used for dissolving the drugs was always run to estimate any change in microviscosity due to the solvent effect. The possible spectral interaction between DPH and the drugs was verified by checking the absence of interfering absorbances at DPH emission wavelength.

3. RESULTS

Fig.1 reports the data in vitro after addition of 10^{-5}–10^{-10} M diazepam (A) or Ro 15-1788 (B) to hippocampal or cerebellum crude synaptic membranes. The data are expressed as the ratio of the membrane microviscosity 20 min after adding the drug (i.e., η_D) and the basal value (i.e., η_o) *vs* the antilogarithm of drug concentration. At 10^{-5} M diazepam increased the microviscosity by 10% and at 10^{-10} M the figure was still ~2%. On the other hand, this compound had little effect on cerebellum crude synaptic membranes. In this case

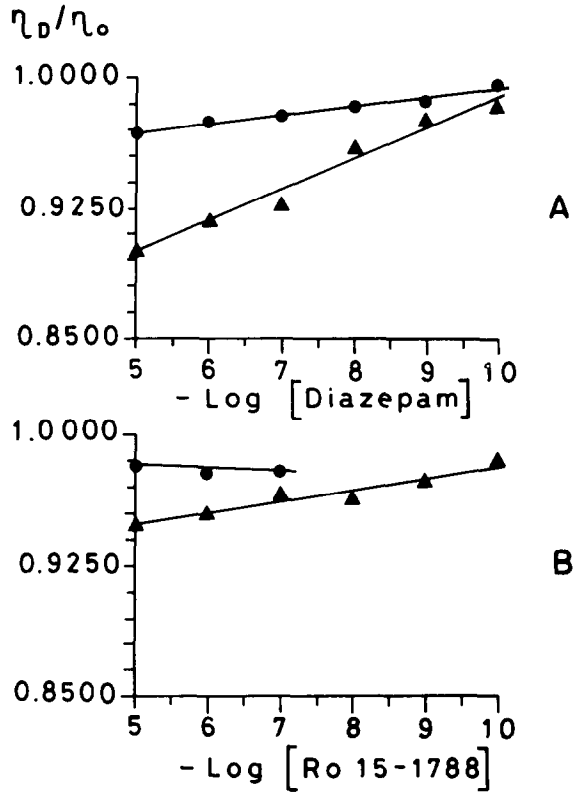

Fig.1. Dose-response curves of in vitro fluidizing effect of diazepam (A) and Ro 15-1788 (B) on hippocampus (▲—▲) and cerebellum (●—●) synaptic membranes. η_D, microviscosity 20 min after addition of the drug; η_o, basal value. Each point is the mean of 4 different experiments.

at 10^{-5} M the change amounted only to 3.2%. Panel B shows the findings with Ro 15-1788. This compound had much less effect than diazepam in hippocampus, the maximum effect, at 10^{-5} M, being about 5% of basal value. No effect at all was observed with cerebellum synaptosomal membranes.

The in vitro effects of two other compounds (PK 11195 and zopiclone) were tested at 10^{-5} M on hippocampal crude synaptic membranes. PK 11195 gave an η_D/η_o value of 0.95, thus causing 5% fluidization, whereas zopiclone was completely ineffective (not reported).

Table 1 lists the findings in hippocampal synaptosomes after in vivo treatments. Diazepam significantly increases membrane fluidity, by 6%. This effect appeared to be confined to specific

Table 1

Membrane lipid microviscosity in rat hippocampus synaptosomes

Treatment	Microviscosity (P) at 37°C
Vehicle + vehicle	2.7325 ± 0.0519 (8)
Diazepam + vehicle	2.5856 ± 0.0036[a] (9)
Vehicle + Ro 15-1788	2.4967 ± 0.0505[b] (5)
Diazepam + Ro 15-1788	2.4706 ± 0.0499[b] (6)

Data are the mean ± SE; number of animals in brackets. [a] $p < 0.05$; [b] $p < 0.01$ different from controls; Dunnett's test. F for interaction (ANOVA), not statistically significant

membrane domains, since it was not detected in crude P_2 fraction or in whole homogenate (not shown).

Diazepam seemed to show regional specificity since fluidity was unmodified in synaptosomes from cerebellum (controls 2.65 ± 0.040 η *vs* diazepam 2.74 ± 0.01). The levels of diazepam were 0.45 ± 0.1 μg/g in hippocampus and 0.51 ± 0.1 μg/g in cerebellum. Ro 15-1788 per se increased membrane fluidity in hippocampal synaptosomes and the two drugs showed no statistically significant interaction when administered together.

4. DISCUSSION

This paper reports for the first time the in vivo modulation of membrane fluidity after a single active dose of diazepam, reported to give 90% occupancy of brain receptors in vivo [7]; this effect showed regional specificity since it was observed in hippocampus but not cerebellum of treated rats. The differences between the two areas cannot be ascribed to different levels of diazepam, since we found uniform regional drug distribution after in vivo treatment and, when added in vitro to crude synaptic membrane preparations, diazepam produced a dose-dependent effect in hippocampus but not in cerebellum. The lack of fluidizing effect in diazepam in cerebellum may be due to the presence of only one type of BDZ receptor protein [8,9] or to different membrane constituents.

The enhancement of membrane fluidity by diazepam does not seem to be related to the 'neuronal type' of benzodiazepine receptors, since it was shared in vitro by the potent benzodiazepine antagonist Ro 15-1788 and by PK 11195, a specific ligand for the peripheral type of receptors [10]. Moreover zopiclone, a non-benzodiazepine ligand for the neuronal type of benzodiazepine receptors [11], had no effect on in vitro crude synaptic membrane preparation. Our data also indicate that the enhancement of membrane fluidity by diazepam does not correlate with its pharmacological activity, since Ro 15-1788, given in vivo at a dose which completely antagonizes the pharmacological effects of diazepam [12], per se increased membrane fluidity in hippocampal synaptosomes and was not able to reverse diazepam's fluidizing effects.

Moreover, the enhancement of membrane fluidity observed in our conditions does not seem to be mediated by the peripheral type of BDZ receptors present in the brain, since these sites are more concentrated in cerebellum than in hippocampus [13] and PK 11195, which binds these sites with higher affinity than diazepam [10], was considerably less active than the benzodiazepine in enhancing membrane fluidity, its effect being comparable to that of Ro 15-1788. In any case the concentrations of the tested drugs active in vitro on membrane fluidity are higher than their affinity constants for nanomolar receptors. A micromolar benzodiazepine receptor, which also binds phenytoin, has been described [14] and seems to correlate with the ability of anticonvulsants to inhibit Ca^{2+}-calmodulin kinase activity [15].

Protein phosphorylation activity has been reported to be passively modulated by in vitro or in vivo changes in membrane fluidity [16]. Studies are in progress in our laboratory to check whether the increase in fluidity of hippocampal crude synaptic membranes is related to the inhibition of protein kinase linked to a generalized anticonvulsant receptor, through which the drugs under study modulate neuronal excitability.

ACKNOWLEDGEMENTS

Ro 15-1788 was a kind gift from Dr W. Haefely, Hoffmann-La Roche, Basel. PK 11195 was generously donated by Dr G. Le Fur, Pharmuka Laboratories, Gennevilliers, France.

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