Vol. 161, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1989 Pages 295-299

EFFECT OF DIAZEPAM ON THE CARNITINE TRANSLOCATION IN RAT HEART MITOCHONDRIA

L. Sartorelli, G. Mantovani and M. Ciman

Dipartimento di Chimica Biologica e
Centro per lo studio della fisiologia mitocondriale del CNR
Via F. Marzolo 3 - 35131 PADOVA, ITALY

Received April 10, 1989

Diazepam acts as an inhibitor of the carnitine translocation through the mitochondrial inner membrane. Diazepam needs however to be added during the phase of exchange. If added during the loading phase and washed during the usual washing the diazepam still found in the mitochondrial fraction is not sufficient to exert any inhibition. Kinetic studies indicate a non-competitive inhibition and a complex carnitine-diazepam -translocase is likely to be formed.

Carnitine is translocated through the mitochondrial (1,2) and the heart sarcolemma membranes (3) in an exchange diffusion process. The two translocations have in common certain substrate but differ in some feature and in the physiological purpose.

During the search of compounds affecting the sarcolemmal translocation of carnitine it has been found that diazepam is an effective and interesting inhibitor. The same compound was also tested on the heart mitochondria translocation of carnitine. Diazepam had been tested because of its interference in the CNS with GABA, whose chemical formula shows similarity to that of carnitine. GABA itself is not a substrate nor an inhibitor of the mitochondrial translocase (5).

Diazepam is widely used as an anxiolitic and anticonvulsant drug. It has been found that the benzodiazepines receptor in the CNS is associated with the GABA receptor anion channel complex. Binding sites for diazepam are also abundant in the peripheral tissues. The binding sites of the latter are often associated with the mitochondrial outer membrane (4).

Many inhibitors have been described for the mitochondrial translocation of carnitine. Pande and coworkers found that thiol reagents, as mersalyl and N-ethylmaleimide are inhibitory (5). Other inhibitors are sulfobetaine, hydroxycinnamate etc. (6,7). The inhibitors studied were either irreversible or competitive, while diazepam apparently behaves as a reversible non-competitive inhibitor.

0006-291X/89 $1.50

Materials and Methods

Wistar albino rats (200-250 g) were used for all the experiments.

L-carnitine and L-acetylcarnitine were gift from Sigma Tau corporation (Rome, Italy). All other reagents were market available reagent grade compounds. Diazepam was dissolved in dioxane. Blanks carried throughout the experiments show that the amount of dioxane used does not have any effect.

Rat heart mitochondria were prepared according to Linden Mayer et al.(8), and mitoplasts following the procedure of Sottocasa et al. (9). The preparations were checked with electron microscopy according the technique of Sorgato et al.(10), and the mitoplasts found deprived of most of the outer membrane. The mitochondrial and mitoplast protein were determined by the Bio-Rad protein essay according to Bradford (11).

The uptake of diazepam was performed by incubating mitochondria (3 mg/ml) in a medium containing 180 mM KCl, 5 mM Hepes, 0.5 % BSA buffered at pH 7.4 with Tris, at 0°C, at different times and diazepam concentrations as described in tables and figures.

The carnitine(in)/acetylcarnitine(out) exchange was performed by a modification of the procedures of Pande (1) and Ramsay and Tubbs(2). Heart mitochondria or mitoplasts (3 mg of protein/ml) were first preincubated in the above described medium with 50 μM ^{3}H-carnitine (0.4 MBq/μmol) for 20 min at 0°C . At this point the maximum loading was achieved and lasted for at least 2 hrs. Other additions or different conditions are described in tables and figures. Mitochondria, or mitoplasts, (usually 0.3 ml) were then washed by centrifugation, resuspension and recentrifugation in microcentrifuge at 7000·g per 1 min. The exchange was initiated by quickly resuspending (with an Eppendorff pipet) the precipitate in 0.3 ml of Krebs-Ringer medium containing acetylcarnitine and incubated for 1 min at 20°C with continuous stirring. At the close of the minute 0.1 ml layered over silicon-phthalate (45:55, v:v) and 0.2 ml as such were centrifuged simultaneously at 12000 g per 1 min. The precipitate from the layered 0.1 ml and 0.1 ml of the supernatant of the other tube were utilized for radioactivity counting. Team work was necessary and allowed a fair utilization of the method described. The fraction of exchange was calculated from the CPM found in the precipitate divided by the sum CPM of the precipitate plus CPM of the supernatant corrected for the 'unspecific' release fraction (see Sartorelli et al. 12) .

Results and Discussion

Diazepam inhibits the carnitine exchange against both acetylcarnitine and palmitoylcarnitine as it is shown in Table I. Inhibition is obtained with concentration of diazepam lower than those of other inhibitors (5, 6,7) except Mersalyl (5).

Since in the literature (4) are reported diazepam binding sites in the outer mitochondrial membrane while carnitine translocase is thought to be linked to the inner membrane, inhibition experiments have been carried out with mitoplasts, which are in good part deprived of outer membrane. The results in Table II show that the extent of inhibition is exactly the same with mitochondria and with mitoplasts. This may indicate that the diazepam bound is not responsible for the inhibition. Stronger evidence is given by the fact that the concentration needed for the inhibition is much higher than the concentration required to saturate the outer membrane

TABLE I. EFFECT OF DIAZEPAM ON THE CARNITINE/ACETYLCARNITINE EXCHANGE IN RAT HEART MITOCHONDRIA

Counter ion	Conc µM	Exchange fraction	+ diazepam 200 µM	+ diazepam 100 µM	% Inhib.
Acetylcarnitine	100	0.922	0.059		94
"	"	"		0.886	4
"	50	0.908		0.705	25
"	10	0.698		0.331	52
Palmitoylcarnitine	50	0.882		0.736	16

Mitochondria (3 mg of protein/ml) were preloaded with 50 µM carnitine for 30 min at 0°C.

binding sites (7) and that diazepam needs to be added during the exchange phase. If diazepam is added during the loading of carnitine and then the mitochondria washed, as usually done, before the exchange, the amount that remains (20 nmol/3 mg mitochondrial protein) after washing is not effective as inhibitor. These results, togheter with the kinetic studies reported below, support the view that the inhibition is due to a diazepam loosely bound to a membrane site where carnitine translocase is located, be it the inner membrane or contact points between inner and outer membrane (13) and that the diazepam tightly bound to the sites described in the literature (14) is not involved.

In Fig 1 a Lineweaver and Burk plot of the diazepam inhibition of the exchange carnitine(in)/acetylcarnitine(out) is shown. The inhibition appears to be non-competitive. The Km found by us is lower than the one

TABLE II. COMPARISON BETWEEN THE CARNITINE/CARNITINE EXCHANGE OF MITOCHONDRIA AND MITOPLASTS

	Exchange fraction	+ diazepam 100 µM	% Inhib
Mitochondria	0.863	0.332	62
Mitoplasts	0.664	0.283	57

Mitochondria and mitoplasts (3 mg of protein/ml) were preloaded with 50 µM ^{3}H-carnitine for 30 min at 0°C. The exchange was carried out at 20°C for 1 min with 20 µM carnitine.

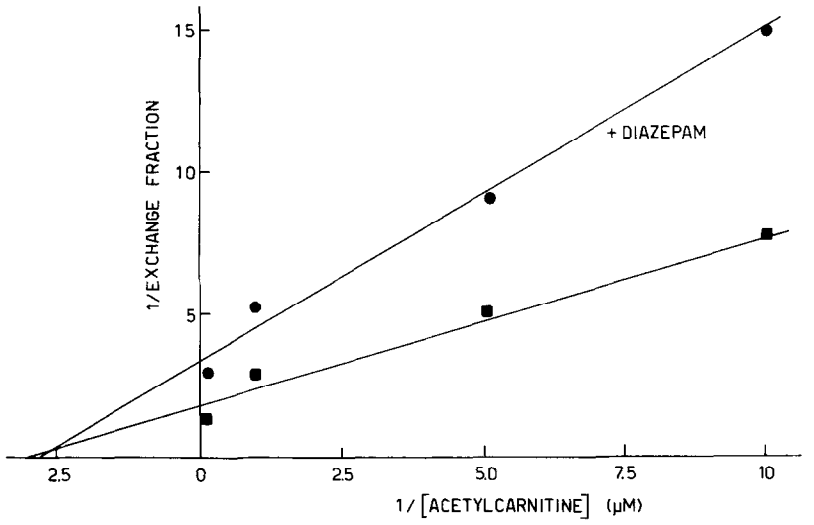

Figure 1. Lineweaver and Burk plot for the inhibition of diazepam on carnitine acetylcarnitine exchange. Mitochondria (3 mg of protein/ml) were preloaded with carnitine 50 μM at 0°C for 30 min. Exchange was carried out at 20°C for 1 min.

found by Murthy & Pande (6). This may be due to the differences in the method used : for instance the temperature in our case was 20°C that is higher than previously reported.

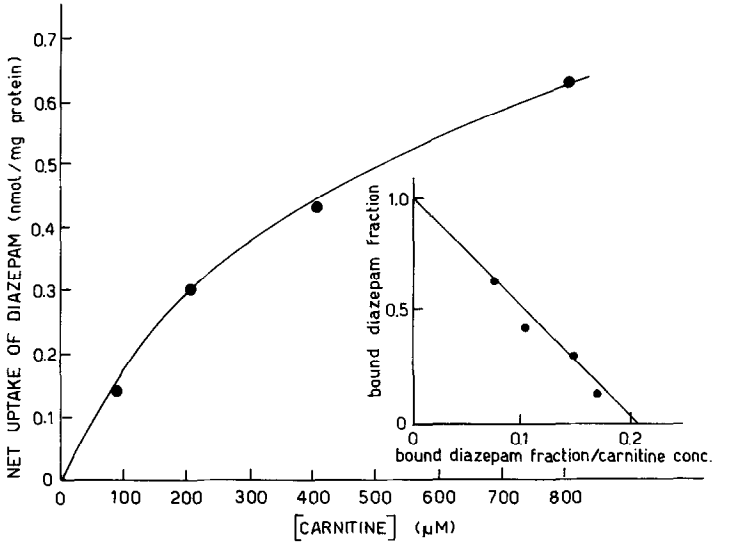

Figure 2. Net uptake of ^{3}H-diazepam in presence of various concentration of carnitine. Mitochondria (3 mg of protein/ml) were preincubated with various concentration of carnitine for 20 min at 0°C. 250 μM ^{3}H-diazepam was added and left for 30 second under stirring. Mitochondria were then centrifuged over silicone-phtalate. Blank was the amount of diazepam uptaken in asence of carnitine.
Inset. Scatchard plot-like graph for the net uptake of ^{3}H-diazepam. The data are from figure 2. The maximum uptake was calculated from the intercept on x axis of the double reciprocal plot.

The presence of carnitine, after a preincubation, enhances the uptake of ^{3}H-diazepam. Figure 2 shows that by increasing the concentration of carnitine the amount of uptaken diazepam increases following a saturation curve. Tentatively a Scatchard plot-like graph (Fig 2-inset) of ^{3}H-diazepam net uptake fraction *vs* the ratio of net uptake fraction over carnitine concentration was drawn and the single slope may indicate that interaction sites are 1 per molecule. From the present results the formation of a complex of the type E+S+I $\rightleftharpoons$ ESI, i.e. translocase-carnitine-diazepam, can be hypothesized.

The main point of this paper is the evidence that diazepam can be an interesting tool in the study of the carnitine translocase. As for any pharmacological activity of diazepam related to the translocation of carnitine, no inference can be put forward at this stage.

References

1) Pande Shri V. (1975) Proceedings National Academy of Sciences USA 72, 883-887
2) Ramsay R.R. & Tubbs P.K. (1975) FEBS Letters 54, 21-25
3) Sartorelli L., Ciman M., Rizzoli V. & Siliprandi N. (1982) Italian Journal of Biochemistry 31, 261-268
4) Anholt Robert R.H. (1986) Trends Pharmacological Sciences 7, 506-511.
5) Pande S.V., Parvin R. (1976) Journal Biological Chemistry 251, 6683-6691
6) Murthy M.S.R. & Pande S.V. (1984) Journal Biological Chemistry 259, 9082-9089
7) Beneking M., Oellerich M., Haeckler R., Binder L. (1987) Journal Clinical Chemistry & Clinical Biochemistry, 25, 467-471.
8) Lindenmayer G.E., Sordhal L.A. & Schwartz A. (1968) Circulation Researches 23, 439-450
9) Sottocasa G.L., Kuylenstierna B., Ernstern L. & Bergstrand A. (1967) Methods in Enzymology 10, 448-463
10) Sorgato Catia M., Keller B.V. & Stuhmer W. (1987) Nature 330, 498-500
11) Bradford M. (1976) Analytical Biochemistry 72, 248-254
12) Sartorelli L., Ciman M., Mantovani G. & Siliprandi N. (1985) Italian Journal of Biochemistry 34, 282-287
13) Brdiczka D., Knoll G., Riesinger I., Weiler U., Klug G., Benz R. & Krause J. In: Brautbar N, ed. Myocardial and skeletal muscle energetics. Plenum Press, New York, pp 55-69
14) Antkiewicz-Michaluk L., Guidotti A. & Krueger K.E. (1988) Molecular Pharmacology 34, 272-278.