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EFFECTS OF CHLORDIAZEPOXIDE AND DIAZEPAM ON RESPIRATION AND OXIDATIVE PHOSPHORYLATION IN RAT BRAIN MITOCHONDRIA*

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Abstract—The effects of chlordiazepoxide and diazepam on respiration and oxidative phosphorylation of succinate, pyruvate and alpha-ketoglutarate by rat brain mitochondria were investigated polarographically. Chlordiazepoxide and diazepam were each found to decrease respiration as evidenced from decreased state 3 and 2,4-dinitrophenol-stimulated state 4 oxygen uptake in the presence of the above three substrates. The decreases in state 3 and 2,4-dinitrophenol-stimulated state 4 rates were related to the added concentration of each drug. With respiratory control indices and ADP:O ratios as an index of oxidative phosphorylation, it was found that chlordiazepoxide and diazepam decreased oxidative phosphorylation. The decrease in oxidative phosphorylation may be due in part to an increase in adenosine triphosphatase activity as evidenced from the increased rates of oxygen uptake during state 4. To achieve a given effect on the above mitochondrial functions, it was found that the concentration *in vitro* of chlordiazepoxide had to be five to seven times greater than that of diazepam.

CHLORDIAZEPOXIDE hydrochloride (Librium) and diazepam (Valium) have been utilized extensively for relief of tension and anxiety for 10 and 7 years respectively.[1] However, the mechanism(s) by which these antianxiety agents, or "minor tranquilizers", produce their pharmacologic effects remains obscure. In fact, there is no information presently available concerning the comparative effects of chlordiazepoxide and diazepam on mitochondrial respiration and oxidative phosphorylation. It is possible that one mechanism of action of the above drugs may be that of decreased oxygen consumption and subsequent attenuation of subcellular function of the CNS. The present communication compares the effects of chlordiazepoxide† and diazepam† upon respiration and oxidative phosphorylation in rat brain mitochondria.

METHODS

All experiments were conducted with intact rat brain mitochondria obtained from unanesthetized Sprague–Dawley male rats, weighing between 250 and 350 g. The animals were maintained on Rockland Mouse/Rat Diet and water *ad lib.* After decapitation, the top of the cranial vault was removed by using scissors and ronguers. The cerebrum, cerebellum and brain stem were removed by enucleation. The brain

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was rinsed in a beaker containing ice-cold isolation medium,‡ blotted on filter paper, placed in a second beaker containing isolation medium, and immediately minced with fine tissue scissors. All isolation procedures were conducted at 0–4°. The minced suspension was homogenized in a Potter–Elvehjem homogenizer with a loose fitting Teflon pestle at approximately 1000 rev/min. Sufficient isolation medium was added to make a final homogenate suspension to correspond to 10 vol. %. This suspension was further homogenized at approximately 5000 rev/min, taking care to avoid foaming The homogenate was centrifuged at 900 *g* for 10 min. The supernatant was decanted and again centrifuged at 900 *g* for 10 min to remove nuclei and residue which had remained from the first step. The supernatant was then centrifuged at 12,000 *g* for 10 min to sediment the mitochondria. The mitochondrial pellet was washed twice and resuspended in isolation medium to approximately two-thirds the volume of the original homogenate. The suspension was again centrifuged at 12,000 *g* for 10 min. The pellet was rinsed with isolation medium and resuspended as before. This suspension was then centrifuged at 7000 *g* for 10 min. The mitochondrial pellet was rinsed as before and resuspended in isolation medium to correspond to 1 ml of mitochondrial suspension per 1 g of brain tissue, and contained between 16 and 20 mg protein per 1 ml of suspension. Protein content of the mitochondrial suspension was determined by the biuret method.[2]

Oxygen uptake by the mitochondrial preparation was measured at 30° with a Gilson recording polarograph utilizing Clark-type oxygen electrodes. The respiratory control medium used in the polarographic chamber of 3·2 ml of volume consisted of: mannitol, 0·3 M; EDTA, 0·1 mM; Tris buffer (pH 7·4), 10·0 mM; bovine serum albumin, 1 mg/ml; potassium chloride, 10·0 mM; phosphate buffer (pH 7·4), 1·5 mM; and either succinate, 6·5 mM; alpha-ketoglutarate, 13 mM; or pyruvate, 13 mM, with malate "sparker", 0·13 mM. Chlordiazepoxide was studied at four different concentrations (0·1, 0·5, 1·0 and 5·0 mM) and diazepam was studied at five different concentrations (0·1, 0·2, 0·3, 0·4 and 0·5 mM). While chlordiazepoxide is freely soluble, diazepam is virtually insoluble in most laboratory media. Thus, diazepam was utilized as supplied by the manufacturer for intramuscular or intravenous use. Control studies were conducted utilizing only the vehicle.* The pH of all experiments was continuously monitored with combination glass electrodes. The oxygen uptake was calculated from the change in oxygen tension measured polarographically. The oxygen uptake by the mitochondria in the chamber was calculated from the oxygen solubility of solutions equilibrated with room air at 30°, and the factor of 246 nmoles oxygen per ml of solution in the chamber was utilized for calculations. ADP : O ratios were calculated from the measured oxygen uptake and the known quantity of ADP added as described by Chance and Williams.[3] Similarly, the respiratory control ratio was calculated as defined by Chance and Williams by dividing the rate of oxygen uptake during state 3, after the addition of ADP, by the rate of oxygen uptake during state 4, a slower rate, after the conversion of the added ADP to ATP.[4] The respiratory control ratio is an index of the integrity of the mitochondria. Fresh, tightly coupled, well-prepared mitochondria have numerically high control ratios.

‡ The isolation medium contained: mannitol, 0·3 M; EDTA, 0·1 mM; Tris buffer (pH 7·4), 10·0 mM; bovine serum albumin (Pentex), 1 mg/ml; and ATP, 2·0 mM.

* The vehicle for the parenteral administration of diazepam consisted of: 40% propylene glycol, 10% alcohol, 5% sodium benzoate and bezoic acid as buffers and 1·5% benzyl alcohol as preservative.

RESULTS

The results in Tables 1–3 show the comparative effects of increasing concentration of chlordiazepoxide and diazepam on respiration (state 4, state 3 and 2,4-dinitrophenol-stimulated state 4 oxygen uptake) and oxidative phosphorylation (respiratory control indices and ADP : O ratios) of succinate, pyruvate and alpha-ketoglutarate respectively.

TABLE 1. COMPARATIVE EFFECTS OF CHLORDIAZEPOXIDE (CDZP), DIAZEPAM (DZP) AND DIAZEPAM VEHICLE (VEH) UPON RESPIRATION AND OXIDATIVE PHOSPHORYLATION WITH SUCCINATE AS SUBSTRATE*

Drug	Concn (mM)	Q_4†	Q_3†	RCI	ADP : O	Q_{DNP}†
None	—	12·1 ± 0·6	46·7 ± 2·4	4·1 ± 0·2	1·85 ± 0·07	33·8 ± 1·9
CDZP	0·1	12·8 ± 0·6‡	44·2 ± 2·2‡	4·1 ± 0·2‡	1·85 ± 0·06‡	32·4 ± 2·3‡
	0·5	14·2 ± 0·5	33·6 ± 0·7	3·0 ± 0·1	1·84 ± 0·05‡	24·5 ± 0·8
	1·0	14·7 ± 0·6	24·9 ± 0·5	2·1 ± 0·1	1·46 ± 0·05	16·9 ± 1·2
	5·0	15·2 ± 0·8	19·5 ± 1·2	1·4 ± 0·1	1·11 ± 0·04	16·9 ± 1·1
DZP	0·1	13·6 ± 0·5	39·0 ± 0·7	3·6 ± 0·2	1·77 ± 0·06‡	27·7 ± 0·7
	0·2	16·1 ± 0·2	31·7 ± 0·9	2·4 ± 0·1	1·78 ± 0·04‡	26·0 ± 0·9
	0·3	17·8 ± 0·6	28·0 ± 1·1	2·0 ± 0·1	1·59 ± 0·04	25·5 ± 1·0
	0·4	20·2 ± 0·4	25·8 ± 0·7	1·6 ± 0·1	1·28 ± 0·06	21·3 ± 0·3
	0·5	21·4 ± 0·3	23·5 ± 0·6	1·3 ± 0·1	1·16 ± 0·05	16·2 ± 0·6
VEH§	0·1	18·3 ± 1·5	47·9 ± 1·2‡	3·1 ± 0·2	1·77 ± 0·04‡	35·0 ± 0·5‡
	0·3	25·0 ± 1·6	47·3 ± 2·2‡	2·2 ± 0·2	1·59 ± 0·04	33·8 ± 0·8‡
	0·5	28·5 ± 1·8	44·9 ± 0·8‡	1·6 ± 0·1	1·46 ± 0·03	32·5 ± 0·4‡

* All data are expressed as mean values ±S.E. of five to nine experiments. RCI = respiratory control index.

† Rates are expressed as nanoatoms oxygen/min/mg of protein.

‡ No significant change compared to control data ($P > 0·05$).

§ Volume equal to that present in the same concentration of DZP.

TABLE 2. COMPARATIVE EFFECTS OF CHLORDIAZEPOXIDE (CDZP), DIAZEPAM (DZP), AND DIAZEPAM VEHICLE (VEH) UPON RESPIRATION AND OXIDATIVE PHOSPHORYLATION WITH PYRUVATE AS SUBSTRATE*

Drug	Concn (mM)	Q_4†	Q_3†	RCI	ADP : O	Q_{DNP}†
None	—	5·0 ± 0·9	33·2 ± 0·9	7·2 ± 0·6	2·78 ± 0·09	21·8 ± 0·7
CDZP	0·1	4·3 ± 0·4‡	28·1 ± 0·9	6·7 ± 0·5‡	2·61 ± 0·12‡	20·0 ± 0·7
	0·5	6·6 ± 0·5	23·3 ± 0·8	4·4 ± 0·1	2·64 ± 0·05‡	18·4 ± 0·7
	1·0	7·6 ± 0·4	16·4 ± 0·6	2·9 ± 0·2	2·22 ± 0·03	19·6 ± 0·8
	5·0	10·6 ± 0·1	15·9 ± 0·4	2·0 ± 0·1	1·52 ± 0·06	22·0 ± 0·7‡
DZP	0·1	7·1 ± 0·4	23·6 ± 0·8	4·2 ± 0·3	2·76 ± 0·15‡	17·7 ± 0·7
	0·2	8·6 ± 0·5	21·2 ± 0·6	3·2 ± 0·2	2·66 ± 0·04‡	17·4 ± 0·5
	0·3	10·6 ± 0·4	19·9 ± 0·4	2·2 ± 0·1	2·23 ± 0·06	17·2 ± 0·7
	0·4	11·5 ± 0·3	16·7 ± 0·4	1·9 ± 0·1	1·90 ± 0·11	15·0 ± 0·5
	0·5	11·8 ± 0·3	14·2 ± 0·2	1·4 ± 0·1	1·74 ± 0·13	13·4 ± 0·6
VEH§	0·1	4·9 ± 0·4‡	26·3 ± 1·1	8·4 ± 1·1‡	2·67 ± 0·04‡	21·0 ± 0·1‡
	0·3	8·5 ± 0·4	26·1 ± 1·3	4·0 ± 0·1	2·51 ± 0·03	21·7 ± 0·7‡
	0·5	11·2 ± 1·1	24·8 ± 0·7	2·6 ± 0·1	2·29 ± 0·02	21·6 ± 0·8‡

* All data are expressed as mean values ±S.E. of five to nine experiments. RCI = respiratory control index.

† Rates are expressed as nanoatoms oxygen/min/mg of protein.

‡ No significant change compared to control data ($P > 0·05$).

§ Volume equal to that present in the same concentration of DZP .

Both drugs were found to decrease respiration as evidenced by dose-dependent diminution of state 3 rates and 2,4-DNP-stimulated state 4 rates. The increased state 4 respiration is suggestive of drug-stimulated adenosine triphosphatase (ATPase) activity. It is of interest to note that 0·5 mM chlordiazepoxide and 0·1 mM diazepam decreased state 3 rates and 2,4-DNP-stimulated state 4 rates without evoking any significant change in ADP : O ratios in the presence of all three substrates. At concentrations $\geqslant$1·0 mM chlordiazepoxide and concentrations $\geqslant$0·4 mM diazepam, oxidative phosphorylation was significantly decreased as evidence from decreased ADP : O ratios and respiratory control indices.

TABLE 3. COMPARATIVE EFFECTS OF CHLORDIAZEPOXIDE (CDZP), DIAZEPAM (DZP) AND DIAZEPAM VEHICLE (VEH) UPON RESPIRATION AND OXIDATIVE PHOSPHORYLATION WITH ALPHA-KETOGLUTARATE AS SUBSTRATE*

Drug	Concn (mM)	Q_4†	Q_3†	RCI	ADP : O	Q_{DNP}†
None	—	4·6 ± 0·6	31·1 ± 0·8	7·0 ± 0·3	2·69 ± 0·12	28·6 ± 0·8
CDZP	0·1	4·9 ± 0·3‡	28·1 ± 1·3‡	7·3 ± 0·3‡	2·63 ± 0·06‡	27·2 ± 1·6‡
	0·5	6·6 ± 0·4	26·6 ± 1·0	4·3 ± 0·3	2·64 ± 0·08‡	26·7 ± 0·6
	1·0	7·8 ± 0·2	20·1 ± 0·5	3·0 ± 0·3	2·27 ± 0·07	24·2 ± 1·1
	5·0	11·2 ± 0·3	18·2 ± 0·7	2·1 ± 0·1	1·68 ± 0·11	18·8 ± 0·8
DZP	0·1	7·0 ± 0·5	23·2 ± 1·1	4·3 ± 0·4	2·52 ± 0·07‡	24·6 ± 0·7
	0·2	8·2 ± 0·4	20·6 ± 0·9	3·8 ± 0·2	2·50 ± 0·06‡	22·6 ± 0·7
	0·3	9·2 ± 0·4	19·5 ± 0·9	2·6 ± 0·2	2·32 ± 0·03	20·6 ± 0·5
	0·4	11·2 ± 0·3	17·6 ± 0·6	1·8 ± 0·1	2·17 ± 0·05	18·7 ± 0·7
	0·5	13·0 ± 0·1	16·1 ± 0·6	1·4 ± 0·1	1·60 ± 0·01	16·7 ± 0·6
VEH§	0·1	6·5 ± 0·2	30·7 ± 0·6‡	6·3 ± 0·3‡	2·60 ± 0·08‡	33·1 ± 1·1‡
	0·3	10·3 ± 0·4	30·9 ± 0·6‡	3·7 ± 0·3	2·40 ± 0·04	33·1 ± 0·8‡
	0·5	13·6 ± 0·4	29·3 ± 0·5‡	2·5 ± 0·1	2·21 ± 0·05	33·1 ± 1·1‡

* All data are expressed as mean values ±S.E. of five to nine experiments. RCI = respiratory control index.
† Rates are expressed as nanoatoms oxygen/min/mg of protein.
‡ No significant change compared to control data ($P > 0·05$).
§ Volume equal to that present in the same concentration of DZP.

Data obtained from control studies (Tables 1–3) indicated that diazepam vehicle caused significant depression of oxidative phosphorylation as evidenced from decreased ADP:O ratios and respiratory control indices. However, state 3 and 2,4-DNP-stimulated state 4 rates were not significantly altered with succinate and alpha-ketoglutarate as substrates.

Diazepam was found to produce effects on the above mitochondrial functions at concentrations five to seven times lower than those of chlordiazepoxide.

DISCUSSION

Racker[5] has defined four categories of uncouplers and inhibitors of oxidative phosphorylation and respiration. Based on these definitions, the results of the present investigation would tend to place chlordiazepoxide and diazepam in the second category of inhibitory uncouplers. "Inhibitory" in regard to diminutions of state 3 rates and 2,4-DNP-stimulated state 4 rates of oxygen uptake, and "uncouplers" in regard to diminutions of ADP:O ratios and respiratory control indices. The vehicle

for diazepam would appear to be a true uncoupler of oxidative phosphorylation in that it produces changes in mitochondrial function similar to those produced by 2,4-DNP (i.e. stimulation of state 4 rates, diminution of ADP:O ratios and respiratory control indices with no significant changes in state 3 rates).

Another interpretation of the data might be drawn from the finding that chlordiazepoxide reportedly forms a monolayer on Ringer's solution at a concentration approximately ten times that calculated to ensue from a therapeutic dose.[6] Thus, at the concentrations utilized in this study, chlordiazepoxide, as well as diazepam, might evoke effects on respiration and oxidative phosphorylation by the formation of a permeability-blocking monolayer on the mitochondrial membrane. This hypothesis would be in agreement with the observation that both diazepam and chlordiazepoxide diminished the 2,4-DNP-stimulated oxygen uptake in a dose-dependent fashion. In addition, a permeability-blocking monolayer might also account for the observation that the vehicle-stimulated state 4 oxygen uptake was significantly less in the presence of diazepam.

It should also be noted that, with low concentrations of chlordiazepoxide and diazepam, mitochondrial respiration was decreased as much as 29 per cent, while oxidative phosphorylation was not significantly decreased. The control studies with and without vehicle showed no such effects on mitochondrial respiration. Therefore, another hypothesis can be suggested for the mechanism of action for chlordiazepoxide and diazepam, which may be associated with a decreased oxygen consumption and subsequent attenuation of subcellular function of the central nervous system. Further investigations are presently in process concerning sites and mechanisms of action of these and other tranquilizers.

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