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INHIBITION OF SUBSTRATE OXIDATION IN MITOCHONDRIA BY THE PERIPHERAL-TYPE BENZODIAZEPINE RECEPTOR LIGAND AHN 086

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Abstract—The effects, of the benzodiazepines RO5-4864, AHN 086, PK 11195 and clonazepam on respiration of mitochondria from heart, kidney, and liver were studied. ADP-stimulated respiration of heart mitochondria was the most sensitive to inhibition by AHN 086; clonazepam was not inhibitory. Several respiratory chain segment activities of submitochondrial particles were insensitive to AHN 086, except for NADH oxidase which was partially inhibited. However, in contrast to submitochondrial particles, the succinate–cytochrome *c* oxidoreductase activity in intact mitochondria was inhibited by AHN 086, suggesting an effect at the substrate transport level. Phosphate-induced, succinate-dependent swelling was also inhibited by AHN 086 it was not affected by clonazepam. Uncoupled ATP hydrolysis was partially inhibited by RO5-4864, AHN 086, and clonazepam. It is suggested that there is an unspecific inhibition of NADH oxidase and ATP hydrolysis by these benzodiazepines and a specific inhibition on oxidizable substrate transport by the peripheral-type benzodiazepine AHN 086.

Benzodiazepine recognition sites fall into two biochemically and pharmacologically distinct classes. The central benzodiazepine receptors (CBR) are located in the synaptosomal fractions of tissues derived from the neuronal crest [1, 2]. The anxiolytic, anticonvulsant, hypnotic and muscle-relaxant properties of the benzodiazepines and related compounds appear to be mediated by the CBR [2–5]. In contrast, the peripheral-type benzodiazepine receptors (PBR) are distributed widely throughout peripheral tissues as well as the central nervous system [6, 7]. The prototypic PBR ligands RO5-4864 (4′-chlorodiazepam, Ref. 6) and PK 11195 (an isoquinoline carboxamide, Ref. 8) bind to the PBR with nanomolar affinity, while showing micromolar affinity for the CBR.

Presently, the function of the PBR is unclear. The PBR have been involved in certain cellular functions such as the immune response of macrophages [9], the action potential, contractility and coronary flow in heart [10, 11], renal function [12], monocyte chemotaxis [13], depression of neuronal activity [14], and stimulation of cholesterol side chain cleavage in bovine adrenal mitochondria [15, 16]. The PBR have been found subcellularly in high concentrations in the mitochondrial fraction [17–20, but see 21], but the interaction between these PBR and the mitochondrial metabolism has not been established. Evidence has been presented recently that the PBR inhibit O_2 consumption of neuroblastoma cells [22], and isolated mitochondria [23], with potencies that correlate well with binding affinities [24]. The present study investigates the specific site of interaction of the PBR ligands RO5-4864, PK 11195 and AHN 086 (an acylated derivative of RO5-4864, Ref. 25) on oxidative phosphorylation in mitochondria from heart, kidney and liver. A preliminary report of this work was given at the 73rd Annual Meeting of the Federation of American Societies for Experimental Biology in New Orleans, LA [26].

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¶ Abbreviations: EGTA, ethylene glycolbis (aminoethylether) tetra - acetate; and MOPS, 3-(*N*-morpholino) propane sulfonic acid.

METHODS

Preparation of mitochondria. Heart mitochondria were isolated from male Sprague–Dawley rats, as described previously [27]. Liver and kidney mitochondria were isolated essentially as described elsewhere [28], except that the medium was modified slightly (250 mM sucrose, 10 mM HEPES–Tris, 1 mM EGTA¶–Tris, pH 7.4) and the concentration of the fatty acid free albumin for the incubation with the mitochondrial pellet was 1% (w/v). Submitochondrial particles were prepared from heart mitochondria (15 mg protein/mL) resuspended in 250 mM sucrose, 10 mM HEPES–Tris, 1 mM EGTA–Tris, 0.2% (w/v) fatty acid free albumin, pH 7.3, by sonication in the presence of 1 mM ATP + 15 mM $MgCl_2$ [29]. Mitochondrial protein was determined by a slight modification of the method described by Murphy and Kies [30]. The differential absorbance at 215–225 nm was taken to be 1.368 mg/cm with bovine serum albumin as standard [31].

Oxygen consumption. Mitochondria (1 to 1.5 mg protein/mL for heart and kidney; 2.6 to 3.6 mg

protein/mL for liver) were incubated in 2 mL of air-saturated standard medium that contained 120 mM KCl, 25 mM potassium-MOPS, 0.5 mM EGTA–Tris, 5 mM potassium-phosphate, 10 mM NaCl, 1 mM $MgCl_2$, pH 7.2, at 25°. O_2 uptake was recorded by means of an oxygen electrode. The solubility of O_2 in equilibrium with air was taken to be 508 ng atoms/mL at 25°.

Measurement of the respiratory chain segment activities. Submitochondrial particles (0.2 to 0.4 mg protein/mL) were incubated in 2 mL of a medium that was comprised of: 120 mM KCl, 25 mM potassium-MOPS 5 mM $MgCl_2$, 5 mM potassium-phosphate, 0.5 m EDTA–Tris, pH 7.2, at 25°. The activity of the NADH–CoQ oxidoreductase (site 1) was measured following the rate of reduction of ferricyanide (1.5 mM) from the wavelength difference 440–490 nm in an Aminco DW-2 spectrophotometer in the presence of 0.8 μM antimycin and 5 mM azide. The reaction was started by the addition of 1 mM NADH. The activity of the NADH–cytochrome *c* oxidoreductase (sites 1 + 2) was measured as described for the site 1 assay except that antimycin was omitted from the incubation medium. The activity of the succinate–cytochrome *c* oxidoreductase (site 2) was measured as described for the site 1 assay except that 2.5 μM rotenone was included and antimycin was omitted. The reaction was started by addition of 20 mM succinate. The activities of the NADH (sites 1 + 2 + 3) and succinate oxidase (sites 2 + 3) were measured by the rate of O_2 uptake as described above in the presence of 1 mM NADH or 20 mM succinate + 2.5 μM rotenone, respectively. The assays of site 2 and succinate oxidase activities were performed with phosphate-activated submitochondrial particles to abolish the strong inhibition by oxaloacetate on succinate dehydrogenase [32]: submitochondrial particles were diluted 10-fold with 0.1 M potassium-phosphate, pH 7.0, and incubated in an Erlenmeyer flask under orbital agitation (150 rpm) at 34°. After 30 min the suspension was diluted 3-fold, centrifuged at 105,000 *g*, and resuspended in 0.1 M potassium-phosphate. Following this treatment the succinate oxidase activity was increased by 5–10 times.

The activity of the cytochrome *c* oxidase (site 3) was measured by a polarographic method [33]: submitochondrial particles (0.4 to 0.5 mg protein/mL) were incubated in 2 mL of an air-saturated medium comprised of: 25 mM Tris–acetate, 7 mM ascorbate, 5 μM cytochrome *c*, 0.5 μM antimycin, pH 7.4, at 25°. After 1 min 1 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) was added, and 1 min later the cytochrome *c* oxidase activity was started by adding 1.4 mM TMPD (tetramethylphenylenediamine). Under these conditions the O_2 uptake induced by TMPD was inhibited 95–98% by 5 mM azide. Addition of 0.05% (v/v) Triton X-100 did not increase the TMPD-induced O_2 uptake.

Determination of ATP hydrolysis. This was done as described previously [34] except that mitochondria were incubated for 3 min before addition of 3 mM ATP + 2 μM CCCP (carbonyl cyanide *m*-chlorophenylhydrazone).

Determination of mitochondrial swelling. Mitochondria were incubated in 2.5 mL of a medium comprised of 100 mM NH_4-succinate, 10 mM HEPES–Tris, 0.5 mM EGTA–Tris, 2 μM rotenone, pH 7.4, at 25° and the change in light scattering at 520 nm was recorded in a spectrophotometer. The decrease in light scattering induced by 2 mM potassium-phosphate was considered as an indicator of the extent of succinate transport [35]. A decrease in absorbance of 0.03 to 0.05 was observed for 0.6 to 0.8 mg protein/mL as induced by inorganic phosphate.

Presentation of data and statistical analysis. The values shown in this work represent means ± standard deviation (SD) with the number of preparations assayed indicated in parentheses. A Student's *t*-test was used to determine significant differences.

Chemicals. AHN 086 was synthesized as described previously [36] and dissolved in ethanol. RO5-4864 HCl was synthesized by A. H. N. Clonazepam was a gift from Dr. P. Skolnick, NIDDK, NIH, Bethesda, MD. TMPD, CCCP, FCCP, rotenone and antimycin were from the Sigma Chemical Co.

RESULTS AND DISCUSSION

The rate of state 3 respiration in heart mitochondria oxidizing succinate was inhibited strongly by 25 μM AHN 086, whereas RO5-4864 showed a slight inhibitory effect and clonazepam was ineffective in heart mitochondria oxidizing succinate (Table 1). State 3 respiration of kidney and liver mitochondria with succinate as substrate was inhibited only slightly by RO5-4864 or AHN 086. State 3 respiration with glutamate + malate as substrates was also slightly sensitive to AHN 086 and RO5-4864. Removal of Mg^{2+} gave a similar degree of inhibition by the three benzodiazepines assayed in heart (data not shown). The basal rate of respiration (state 4) was not modified significantly by a 25 μM concentration of the three benzodiazepines assayed in this work: 110 ± 9% (N = 14) of control for 25 μM AHN 086. The respiratory control ratio (RCR) was diminished significantly by AHN 086 and RO5-4864 in mitochondria oxidizing succinate + rotenone: this value was 4.1 ± 0.6 (N = 12) for control heart mitochondria, 2.0 ± 0.4 (N = 12) in the presence of 25 μM AHN 086 ($P < 0.001$), and 3.2 ± 0.5 (N = 9) in the presence of 25 μM RO5-4864 ($P < 0.01$). The RCR value was 5.2 ± 0.7 (N = 5) for control kidney mitochondria, 3.9 ± 0.5 (N = 4) in the presence of 25 μM AHN 086 ($P < 0.01$), and 4.4 ± 0.6 (N = 5) in the presence of 25 μM RO5-4864 ($P < 0.05$). The ADP/O ratios were 1.9 ± 0.1 (N = 8) and 1.7 ± 0.2 (N = 5) for control heart and kidney mitochondria, respectively; this parameter was not modified substantially by 25 μM AHN 086 or 25 μM RO5-4864 in either type of mitochondria.

The rate of state 3 respiration in heart mitochondria oxidizing NAD-dependent substrates (palmitoyl-carnitine + malate, pyruvate + malate or α-ketoglutarate) was inhibited only 16–26% by 25 μM AHN 086 or RO5-4864. An inhibition of 20–30% of state 3 respiration by 0.25 to 1 μM RO5-4864 in kidney mitochondria oxidizing pyruvate was

Table 1. Effects of benzodiazepines on the ADP-stimulated (state 3) respiration of mitochondria from different organs

	% Inhibition					
	Succ + Rot			Glu + Mal		
Organ	+ RO5-4864	+ AHN 086	+ Clonazepam	+ RO5-4864	+ AHN 086	+ Clonazepam
Heart	22 ± 5* (5)	66 ± 9† (9)	7 ± 4 (3)	28 ± 7 (3)	29 ± 3 (4)	26 (2)
Kidney	18 ± 5 (3)	22 ± 5 (4)	9 ± 8 (4)	1 ± 12 (3)	2 ± 14 (5)	3 ± 14 (4)
Liver	9 (2)	10 ± 12 (3)	8 (2)	16 (2)	7 (2)	10 (2)

Mitochondria were incubated in 2 mL of standard medium with the indicated oxidizable substrates: 20 mM succinate + 2.5 μM rotenone (Succ + Rot) or 5 mM glutamate + 5 mM malate (Glu + Mal). The benzodiazepines RO5-4864, AHN 086 and clonazepam were added at a final concentration of 25 μM. ADP (1 mM) was added after 2.5 min of incubation. The control rates of state 3 respiration with succinate + rotenone were 263 + 19 (N = 10), 248 ± 26 (N = 4), and 127 ± 8 (N = 3) ng atoms O_2/mg protein/min; and with glutamate + malate the rates were 190 ± 14 (N = 4), 124 ± 37 (N = 4), and 96 ± 5 (N = 3) ng atoms O_2/mg protein/min for heart, kidney, and liver mitochondria, respectively. The values in the legend and in the table are means ± SD, except where only two experiments were performed.

* $P < 0.001$, RO5-4864 vs clonazepam.

† $P < 0.001$, AHN 086 vs clonazepam.

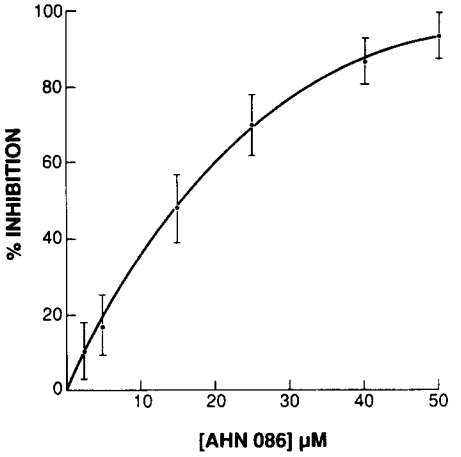

Fig. 1. Inhibition of state 3 respiration by AHN 086 in heart mitochondria. Mitochondria were incubated with the indicated concentrations of AHN 086 and with succinate + rotenone as substrate. After 2.5 min, 1 mM ADP was added to initiate state 3 respiration. Values are the statistical means ± SD of four different mitochondrial preparations. The points were joined by a line calculated by non-linear regression analysis according to the equation of a hyperbolic curve.

Table 2. Effects of benzodiazepines on the uncoupled respiration of heart mitochondria

	% Inhibition
+ RO5-4864	40 ± 19* (4)
+ AHN 086	63 ± 7† (4)
+ Clonazepam	20 ± 8 (3)

Heart mitochondria were incubated as described in the legend of Table 1 with succinate (+ rotenone) as substrate, except that 1 μM FCCP was added instead of ADP after 2.5 min of incubation. The control rate of uncoupled respiration was 214 ± 14 (N = 4) ng atoms O_2/mg protein/min. Values are means ± SD.

* $P < 0.05$, RO5-4864 vs clonazepam.

† $P < 0.01$, AHN 086 vs clonazepam.

reported recently by Hirsch *et al.* [23]. In the present study the inhibition of state 3 respiration induced by 1 μM RO5-4864 in heart mitochondria oxidizing succinate or glutamate + malate was 12 ± 2% (N = 3) and 5% (N = 2), respectively; in kidney mitochondria RO5-4864 at a concentration range of 0.1 to 1 μM was ineffective.

The data of Table 1 taken as a whole suggest a specific inhibition on the succinate oxidation pathway and a rather unspecific inhibition on the NAD-dependent substrate oxidation pathway by the peripheral-type benzodiazepine AHN 086 in heart mitochondria. This is supported by the effect of the central-type benzodiazepine clonazepam on state 3 respiration of heart mitochondria: significant inhibition with glutamate + malate, but ineffective with succinate (see Table 1).

Figure 1 shows a curve of AHN 086 concentration versus percentage inhibition of the rate of state 3 respiration of heart mitochondria oxidizing succinate. A near-complete inhibition of state 3 respiration was accomplished with 50 μM AHN 086; 50% inhibition was attained with 19 μM AHN 086 as derived from a non-linear regression analysis for a concentration range of heart mitochondria of 1 to 1.5 mg protein/mL.

Uncoupled respiration of heart mitochondria oxidizing succinate was also inhibited by RO5-4864 and AHN 086 (Table 1), but in this case clonazepam showed a slight inhibitory effect.

To obtain insight about specific sites of interaction of peripheral-type benzodiazepines in the mitochondrial oxidative machinery, the effects of RO5-4864, AHN 086 and clonazepam on different mitochondrial activities were assayed. Table 3 shows the results obtained for submitochondrial particles,

Table 3. Effects of benzodiazepines (25 μM) on the activity of several segments of the respiratory chain in submitochondrial particles

Segment	% Inhibition		
	+ RO5-4864	+ AHN 086	+ Clonazepam
NADH–CoQ oxidoreductase (site 1)	0	4 (2)	0
NADH–cytochrome oxidoreductase (sites 1 + 2)	4	12 (2)	0
Succinate–cytochrome *c* oxidoreductase (site 2)	5 (2)	9 ± 9 (4)	3 (2)
NADH oxidase (sites 1 + 2 + 3)	18 ± 9 (3)	22 ± 10 (5)	9 ± 5 (3)
Succinate oxidase (sites 2 + 3)	11 ± 8 (3)	8 ± 4 (7)	11 (2)
Cytochrome oxidase (site 3)		3 (2)	

The control activities were as follows: 8.4 (N = 2) μmol ferricyanide reduced/mg protein/min for site 1; 9.3 (N = 2) μmol ferricyanide reduced/mg protein/min for sites 1 and 2; 403 ± 42 (N = 4) nmol ferricyanide reduced/mg protein/min for site 2; 1503 ± 135 (N = 5) ng atoms O_2/mg protein/min for NADH oxidase; 481 ± 82 (N = 7) ng atoms O_2/mg protein/min for succinate oxidase; and 1574 (N = 2) ng atoms O_2/mg protein/min for cytochrome oxidase. The values in the legend and in the table are means ± SD, except where 1–2 experiments were made.

Table 4. Effects of benzodiazepines on the phosphate-induced, succinate-dependent swelling of heart mitochondria

	% Inhibition
+ RO5-4864	6 ± 6 (3)
+ AHN 086	43 ± 16* (6)
+ Clonazepam	0 ± 0 (3)

Values are means ± SD.
* $P < 0.005$, AHN 086 vs clonazepam.

where a substrate transport reaction is not required. It is apparent that the sites involved in succinate oxidation were rather insensitive to AHN 086, whereas NADH oxidase activity exhibited some degree of inhibition by AHN 086 and RO5-4864. This last finding would explain by itself the inhibition of state 3 respiration with glutamate + malate by these benzodiazepines (see Table 1).

The activity of succinate–cytochrome *c* oxidoreductase in intact heart mitochondria was inhibited 65 ± 18% (N = 4) by 25 μM AHN 086, 28 ± 6% (N = 3) by RO5-4864, and 12% (N = 2) by clonazepam. Interestingly, this activity required succinate transport in mitochondria. Table 4 shows the effect of these benzodiazepines on the extent of phosphate-induced, succinate-dependent mitochondrial swelling. The rationale behind these experiments is to measure succinate transport in exchange with phosphate: phosphate enters into mitochondria in exchange with OH at least 10 times faster than succinate transport [37]. Ammonium succinate is used because ammonia permeates mitochondria and binds proton dissociated from phosphate, allowing the net uptake of ammonium succinate and swelling. As expected, AHN 086 showed a significant degree of inhibition on the phosphate-induced mitochondrial swelling, whereas clonazepam was again ineffective (Table 4).

Incubation of 50 μM PK 11195, an antagonist of some RO5-4864 functional effects [10, 12, 13, 38] but also a partial PBR agonist [11, 22, 23, 39], together with 25 μM AHN 086 did not prevent the inhibitory effect of AHN 086 on phosphate-induced succinate-dependent swelling or state 3 respiration of heart mitochondria in the presence of succinate (data not shown). PK 11195 (25–50 μM) alone had no effect on phosphate-induced swelling and only induced a slight diminution (19% inhibition, N = 2) of state 3 respiration in heart mitochondria. As RO5-4864 (see Table 4) and PK 11195 did not inhibit succinate-dependent swelling, the data suggest that the specific inhibition of the succinate transport reaction by AHN 086 is not associated with PBR in mitochondria.

Finally, the effects of AHN 086, RO5-4864, and clonazepam on the uncoupled ATP hydrolysis of heart mitochondria were assayed. A significant inhibition was observed which was not specific for the peripheral-type benzodiazepines as clonazepam showed a similar inhibitory effect: 43 ± 11% (N = 13), 36 ± 9% (N = 7) and 33 ± 14% inhibition (N = 12) for 25 μM AHN 086, RO5-4864 and clonazepam, respectively.

In conclusion the peripheral-type benzodiazepine AHN 086 appears to exert a specific inhibition on succinate transport in heart mitochondria which can account for the inhibition of state 3 respiration with succinate as substrate. It has been reported that AHN 086 can acylate PBR as well as several other proteins [40]; interestingly some alkylating drugs such as mersalyl also can inhibit the mitochondrial succinate transport [37]. However, there are also less specific inhibitory effects by AHN 086 on NADH oxidase and uncoupled ATP hydrolysis activities, which may account for the inhibition of state 3 respiration with glutamate + malate as substrate. Since the inhibitory range of AHN 086 concentrations was approximately 3 orders of magnitude higher than the reported affinity of PBR for AHN 086 [25], and because there was a lack of effect of RO5-4864 and PK 11195 on succinate-dependent swelling, it would seem that mitochondrial PBR are not involved in the inhibition of state 3 respiration and succinate transport by AHN 086. However, the affinity of

mitochondrial PBR for these benzodiazepines may be different in the experimental conditions of this study (25°, 2.5-min incubation, isotonic medium, pH 7.2). Until an answer to this question is known, an involvement of PBR in the effects of AHN 086 cannot be ruled out.

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