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Mitochondrial Benzodiazepine Receptors Mediate Inhibition of Mitochondrial Respiratory Control

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SUMMARY

Drugs that bound to the peripheral-type or mitochondrial benzodiazepine receptors in rat kidney mitochondria produced several effects on mitochondrial respiration with succinate and malate/pyruvate as substrates. These drugs increased state IV and decreased state III respiration rates, which resulted in a significant decrease in the respiratory control ratio. ADP: O ratios were not affected. The receptor binding affinities of a set of 10 compounds (Ro5-4864, PK11195, diazepam, mesoporphyrin IX, flunitrazepam, deuteroporphyrin IX, dipyridamole, dibutylphthalate, cyclosporin A, and CL259,763) correlated over a concentration range of almost 4 orders of magnitude with their potencies at inhibiting respiratory control ($r = 0.95$). The anxiolytic benzodiazepine clonazepam had no effect on mitochondrial respiratory control and bound with negligible affinity to the receptor. The magnitude of the effect of Ro5-4865 on respiration increased in parallel with the density of mitochondrial benzodiazepine receptors in mitochondria from liver, kidney, and adrenal. These results suggest that ligand binding to mitochondrial benzodiazepine receptors results in inhibition of mitochondrial respiratory control. This effect may help to explain the pleiotropic effects of receptor ligands on intact cells.

Benzodiazepines produce many effects on nonneuronal tissues, including the alteration of cellular growth and differentiation (1–4), chemotactic and immune responsiveness (5, 6), steroidogenesis (7, 8), protooncogene expression (9), and the duration of the cardiac action potential (10). Furthermore, many nonneuronal tissues contain specific, saturable, pharmacologically distinct binding sites for these compounds, which are referred to as peripheral-type benzodiazepine receptors. These sites are clearly distinct from the benzodiazepine receptors located on neurons (11).

In radioligand binding experiments using [^{3}H]Ro5 or PK, these peripheral-type benzodiazepine receptors are identified by their distinctive pharmacological profile as follows: nanomolar affinity for the nonanxiolytic benzodiazepine Ro5 and the isoquinoline carboxamide PK; 10–100-fold lower affinity for the anxiolytic benzodiazepines DZ and FLU; and negligible affinity for the anxiolytic CLZ and the benzodiazepine antagonist Ro15-1788. This potency profile is basically the mirror image of that shown by neuronal benzodiazepine receptors (11). Many compounds in addition to benzodiazepines and PK bind to peripheral-type benzodiazepine receptors, including some naturally occurring porphyrins (12), dipyridamole (13), steroid derivatives (14), dihydropyridines (15), thiazides and related drugs (16), and a wide variety of other naturally occurring and synthetic drugs (17).

It has not yet been possible to establish whether peripheral-type benzodiazepine receptors have any functional significance. Attempts to correlate the cellular activities of receptor ligands with their receptor binding potencies have, in some cases, produced widely disparate results, in that large differences in binding potency were not reflected similarly in biological potency (3–6). However, some insight into this discrepancy is provided by reports that these sites are primarily intracellular and localized on mitochondria (19, 20). If the actions of these drugs were mediated through intracellular receptors, the presence of a plasma membrane barrier could help to explain the differences observed between biological and binding potencies.

Based on the evidence that these sites are localized to the mitochondrial outer membrane, peripheral-type benzodiazepine receptors have been recently designated as MBR (20). On this basis, it has also been hypothesized that they function as modulators of cellular energy metabolism (11). To investigate this possibility, we studied the effects of nine ligands for MBR on the respiration and oxidative phosphorylation of rat kidney mitochondria *in vitro* and determined the binding affinities of the drugs to MBR in these organelles. We report here that

ABBREVIATIONS: Ro5, Ro5-4864; PK, [^{3}H]PK11195; FLU, flunitrazepam; DZ, diazepam; CLZ, clonazepam; MBR, mitochondrial benzodiazepine receptors; MP, mesoporphyrin IX; DP, deuteroporphyrin IX; DBP, dibutylphthalate; DPY, dipyridamole; CyA, cyclosporin A; RCR, respiratory control ratio; DMSO, dimethyl sulfoxide; MOPS, 3-[*N*-morpholino]propenesulfonic acid; IB, isolation buffer; RAB, respiration assay buffer; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; VDAC, voltage-dependent anion channel.

these compounds (Ro5, PK, DZ, MP, FLU, DP, DBP, DPY, and CL259,763) inhibited mitochondrial respiratory control with potencies that correlated extremely well with their binding affinity for these receptors. CyA, which inhibited respiratory control in kidney mitochondria (21), was also identified as an MBR ligand in these experiments (17). Incorporating the data for CyA, the correlation between MBR binding and respiratory control inhibition extends over almost 4 orders of magnitude in concentration. In addition, we have investigated the effects of MBR ligands in rat adrenal and liver mitochondria. The results of our experiments suggest that MBR can modulate respiratory control in mammalian mitochondria. A preliminary report of some of these data has appeared previously (22).

Experimental Procedures

Materials. Benzodiazepines were provided by Hoffmann-LaRoche, (Nutley, NJ)and PK11195 was a gift from Rhone-Poulenc Sante (France). Porphyrins, DPY, DBP, succinate, malate, pyruvate, ADP, and MOPS were purchased from Sigma Chemical Company (St. Louis, MO). CyA was obtained from Sandoz (East Hanover, NJ). [^{3}H]Ro5 (77.9 Ci/mmol) was purchased from Dupont/New England Nuclear (Boston, MA).

Preparation of kidney mitochondria. Rat kidney mitochondria were prepared by a modification of a method used to prepare well coupled rat liver mitochondria (23). All procedures were performed at 0–4°. The IB contained 250 mM sucrose, 1 mM dipotassium EDTA, 1 mM EGTA, and 10 mM MOPS (pH 7.4). Decapsulated, whole rat kidneys were finely minced and rinsed well with IB. A 10% (w/v) homogenate was prepared in IB using a loose-fitting, longitudinally grooved Teflon pestle in a glass homogenizer. After one hand-pass of the pestle, the homogenate was centrifuged for 10 min at 600 × *g*. The supernatant was withdrawn by pipet and centrifuged at 1500 × *g* for 10 min. The supernatant was discarded, and the mitochondrial pellet was resuspended in IB (0.25 ml of IB/pair of kidneys), stored in glass on ice, and not washed before use. Rat liver and adrenal mitochondria were prepared as described above.

Mitochondrial [^{3}H]Ro5 binding. Binding of [^{3}H]Ro5 (2 nM final concentration) was conducted with mitochondria prepared as described above. Assays were conducted in RAB (see below) in triplicate for 10 min at 30.5° in a final volume of 0.2 ml. Plastic U-bottom, nonsterile 96-well plates were used. Experiments were terminated by rapid vacuum filtration through glass-fiber filters with a Cambridge PHD cell harvester (Cambridge Technology, Cambridge, MA). Filter strips were washed three times with RAB at 30.5°. Nonspecific [^{3}H]Ro5 binding was determined in the presence of 10 μM unlabeled Ro5. Each assay well contained 13–20 μg of mitochondrial protein. Drugs were dissolved in DMSO and diluted with buffer before testing. At concentrations <0.1% (v/v), DMSO had no effect on binding.

Mitochondrial respiration. RAB contained 60 mM sucrose, 1 mM dipotassium EDTA, 5 mM K_2HPO_4, 20 mM KCl, 2.5 mM $MgCl_2$, and 30 mM Tris (pH 7.4). Mitochondrial oxygen consumption was measured with a YSI Clark-type polarographic oxygen electrode system using standard techniques (24). Each assay (1 ml) contained 120–250 μg of mitochondrial protein. Respiration was measured at 30.5° with constant stirring. Drugs were added with the mitochondria before insertion of the oxygen electrode into the chamber. After 3–4 min, during which a stable baseline was attained, state IV was initiated by the addition of succinate (final concentration, 10 mM). Two to three minutes later, state III was initiated by the addition of ADP (final concentration, 150 μM).

Results

In searching for effects of MBR ligands on mitochondrial energy metabolism, we first examined the action of Ro5 on succinate-supported state IV and ADP-supported state III respiration (Fig. 1). At a concentration of 1 μM, Ro5 increased the rate of state IV and decreased the rate of state III respiration. A return to state IV (referred to here as state IV′) occurred upon exhaustion of the added ADP. The state IV′ rate was also increased by Ro5, but the magnitude of the increment was comparable to that observed in state IV. The opposite effects of Ro5 on the rates of states IV (+13%) and III (−15%) resulted in a 28% decline in the (RCR), which classically defines the degree of coupling between mitochondrial respiration and oxidative phosphorylation (23). In all cases, these changes were statistically significant (Table 1). There was no change in the ADP:O ratio, which expresses the efficiency of ADP phosphorylation (24). In contrast to Ro5, the anxiolytic benzodiazepine CLZ (at 1 μM) was without any of these effects. Although controls (addition of DMSO) were indistinguishable from CLZ additions, CLZ was always used in control experiments.

Ro5-4864, but not CLZ, had similar effects on malate and pyruvate-supported mitochondrial respiration, although with these substrates there was a small decline in the ADP:O ratio (Table 1).

The effects of PK, a nonbenzodiazepine ligand for MBR, on succinate-supported respiration were very similar to those obtained with Ro5. There was an increase and decrease in the rates of state IV and III, respectively, resulting in a significant decrease (20%) in the RCR. PK, like Ro5, had no effect on the ADP:O ratio with succinate as substrate (Table 2).

After observing these distinctive effects of Ro5 and PK, these drugs were tested for possible interactions with other known stimulants of mitochondrial respiration and for other effects on mitochondrial respiration. However, neither drug at 1 μM had any effect on the action of classical respiratory uncouplers like 2,4-dinitrophenol, carbonylcyanide *m*-chlorophenylhydrazone, or Ca^{2+}, all of which stimulate state IV respiration (25). Ro5 and PK did not behave like typical uncouplers either, inasmuch as, at final concentrations of ≤5 μM, neither drug had any stimulatory effect on state IV rates when added after state IV was established. Ro5 and PK at final concentrations of ≤5 μM did not affect the otherwise barely detectable rates of ADP-supported NADH oxidation (assayed in the absence of succinate) or myxothiazol-insensitive oxygen consumption that were carried out by these mitochondria.

Our studies with Ro5 and PK clearly indicated that the RCR was the most informative index of drug-induced respiratory perturbation. Thus, subsequent experiments designed to examine the effects of other MBR ligands on mitochondrial respiration emphasized this parameter. Like Ro5 and PK, the other MBR ligands tested increased the rate of state IV and decreased the rate of state III, resulting in lower RCR values. No significant effects on ADP:O ratios were noted. Concentration-response curves for the effects of Ro5, PK, and the other MBR ligands on mitochondrial RCR values are shown in Figs. 2 and 3. The IC_{50} values derived in these experiments are shown in Table 3. After Ro5, PK was the most potent inhibitor of respiratory control and was followed in potency by DPY, DZ, DP, MP, FLU, DBP, and CL259,763. All of the compounds tested had approximately equivalent efficacy in these experiments.

It is interesting to observe that the effects of the putative MBR antagonist PK (11) were identical to those produced by Ro5, except that PK was less potent than Ro5. The fact that

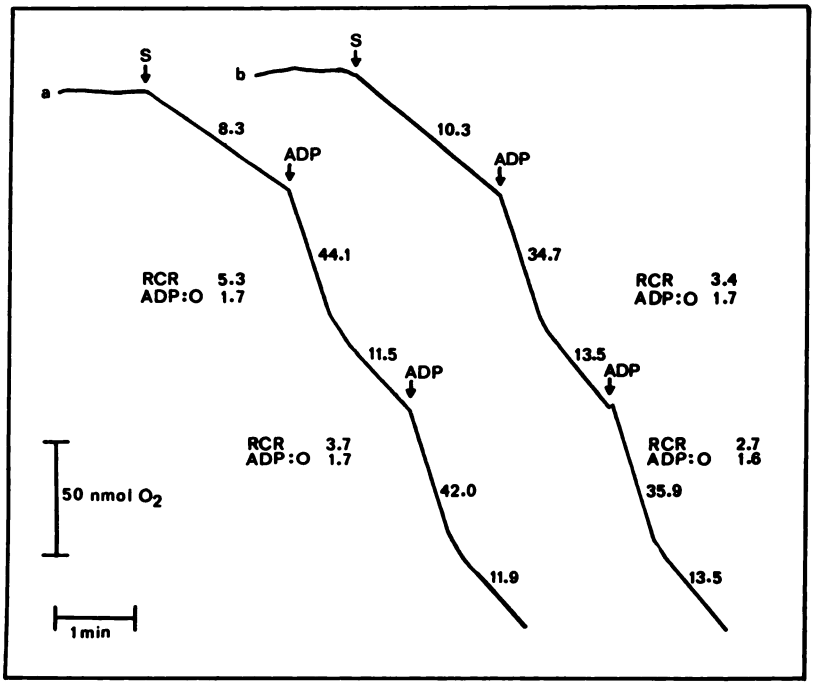

Fig. 1. The effect of Ro5 on succinate-supported state IV and ADP-supported state III mitochondrial respiration. *a*, control curve (1 μM clonazepam added); *b*, 1 μM Ro5 added. *S*, succinate added; *ADP*, ADP added; and ADP:O = ADP:O ratio. Rat kidney mitochondria were prepared and oxygen consumption was measured as described in Experimental Procedures. Drugs were added with the mitochondria 3–4 min before the addition of succinate. The *traces* depict oxygen consumed per unit time as indicated by the *scale bars*. *Numbers* next to the traces represent nAtom of O consumed/min/mg of protein. These tracings are representative of those obtained during these studies; they were obtained with 294 μg of mitochondrial protein/assay.

TABLE 1

Effects of Ro5-4864 on mitochondrial respiration

Values with succinate are means ± standard errors from 16 experiments with four separate preparations of mitochondria. The values with pyruvate/malate are means ± standard errors from seven experiments with two separate preparations of mitochondria and were obtained in the presence of 5 mM malonate. Ro5-4864 was added with the mitochondria to a final concentration of 1 uM; control = clonazepam at 1 μM. Clonazepam had no effect and was equivalent to no addition or addition of an appropriate dilution of DMSO. ADP:O = ADP:O ratio.

Respiration parameter	Drug	Respiration	
		Succinate	Malate/Pyruvate
		natom of O/min/mg of protein	
State IV	Control	17.93 ± 0.44	11.80 ± 0.40
	Ro5-4864	20.24 ± 0.93[a]	12.50 ± 0.40[a]
State III	Control	58.05 ± 1.40	30.30 ± 0.80
	Ro5-4864	46.73 ± 1.96[b]	27.40 ± 0.90[c]
State IV′	Control	22.15 ± 0.65	15.50 ± 0.40
	Ro5-4864	25.50 ± 0.81[d]	16.70 ± 0.30[c]
RCR	Control	3.27 ± 0.13	2.58 ± 0.09
	Ro5-4864	2.35 ± 0.13[b]	2.19 ± 0.12[c]
ADP:O	Control	1.63 ± 0.04	2.02 ± 0.04
	Ro5-4864	1.55 ± 0.05	1.85 ± 0.05[c]

[a] $p < 0.05$ (two-tailed Student's *t* test).
[b] $p < 0.001$.
[c] $p < 0.025$.
[d] $p < 0.005$.

TABLE 2

Effects of PK on mitochondrial respiration

Values were obtained with succinate as the substrate and are means ± standard errors from four or five separate experiments. PK was added with the mitochondria to a final concentration of 1 μM; control = clonazepam at 1 μM. Clonazepam had no effect and was equivalent to no addition or addition of an appropriate dilution of DMSO. ADP:O = ADP:O ratio.

Respiration parameter	Drug	Respiration
		natom of O/min/mg of protein
State IV	Control	16.12 ± 0.61
	PK	17.75 ± 0.20[a]
State III	Control	62.95 ± 2.65
	PK	56.11 ± 1.43[a]
State IV′	Control	21.12 ± 0.71
	PK	22.45 ± 0.65
RCR	Control	3.92 ± 0.04
	PK	3.16 ± 0.06[b]
ADP:O	Control	1.82 ± 0.03
	PK	1.91 ± 0.09

[a] $p < 0.05$ (two-tailed Student's *t* test).
[b] $p < 0.001$.

PK has full intrinsic activity suggests that is not an MBR antagonist in this system. However, PK could partially block the ability of Ro5 to decrease respiratory control, *and vice versa*, in a manner consistent with the ability of these drugs to competitively inhibit the binding of each other to the MBR (17).

To facilitate direct comparisons of activity potencies with binding affinities, we examined the ability of these drugs to displace [^{3}H]Ro5 from MBR in kidney mitochondria under conditions duplicating those used in the respiration studies (Table 3). The details of these binding experiments and their analyses are provided in the accompanying manuscript (17).

The binding experiments showed that Ro5 and PK had high affinity for these sites (IC_{50} = 28 and 48 nM, respectively), whereas CLZ and Ro15-1788 had very low affinity ($IC_{50} > 19$ μM). DPY, DZ, DP, MP, and FLU had intermediate affinities ranging from 170 to 560 nM whereas CyA, DBP, and CL259,763 had affinities in the micromolar range (Table 3). The results indicated that the rank order of binding affinity for these 10 compounds was similar to that of their activity potency. When the IC_{50} values for binding were plotted against the IC_{50} values for inhibiting the RCR, a correlation coefficient of 0.95 was obtained (Fig. 4). This correlation reflects the fact that the potencies of these compounds at stimulating state IV and inhibiting state III individually were similar to their potencies at inhibiting the RCR. Concentration-response curves for Ro5, PK, DZ, and FLU modulation of states IV and III are shown in Fig. 5, A and B. The respective EC_{50} values (means ± standard errors, three experiments) for stimulation of state IV were 8 ± 3 (Ro5), 85 ± 8 (PK), 300 ± 23 (DZ), and 600 ± 19 nM (FLU). The respective IC_{50} values (means ± standard errors, three experiments) for inhibition of state III were 18 ± 6 (Ro5),

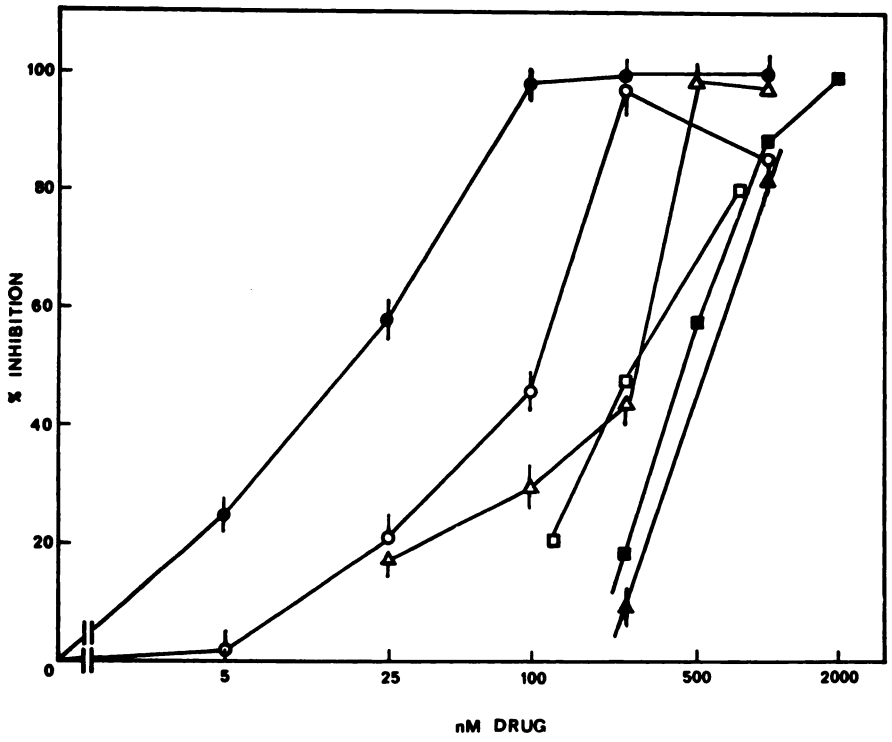

Fig. 2. The effect of MBR ligands on the respiratory control ratio in rat kidney mitochondria. Results are means ± standard errors from two to five determinations at each concentration of Ro5 (●), PK (○), DZ (△), DP (□), MP (■), and FLU (▲). Each drug and CLZ was evaluated with a separate preparation of mitochondria, and the effect elicited by 250 nM Ro5 in each experiment was taken as 100% and used to calculate the values presented. The IC_{50} values derived from these data are shown in Table 3.

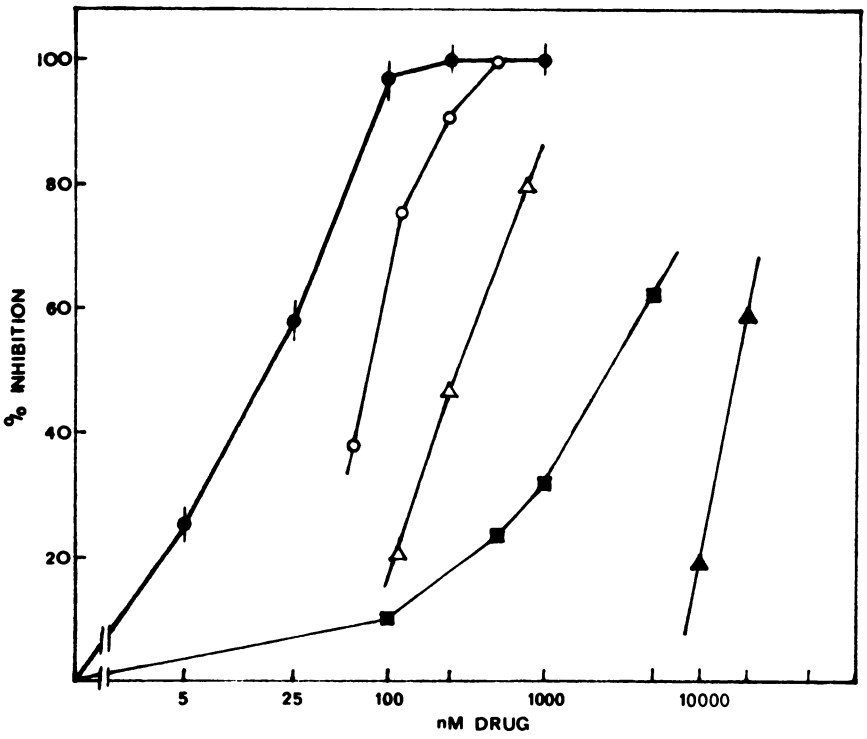

Fig. 3. The effects of additional MBR ligands on the RCR in rat kidney mitochondria. Results are means of two determinations at each concentration of DPY (○), DBP (■), and CL259,763 (▲). Concentration-response curves for Ro5 (●) and DP (△), shown in Fig. 2, are also included for reference. Details of these experiments are outlined in the legend to Fig. 2 and in Experimental Procedures. The IC_{50} values derived from these data are shown in Table 3.

76 ± 4 (PK), 140 ± 11 (DZ), and 340 ± 12 nM (FLU). A correlation coefficient of 0.97 was obtained when the EC_{50} for stimulation of state IV by Ro5, PK, DZ, and FLU was plotted versus their binding affinity (Fig. 6). Similarly, if the IC_{50} for inhibiting the rate of state III was plotted versus the binding IC_{50}, a correlation coefficient of 0.94 resulted (Fig. 6). These results suggest that drug modulation of kidney mitochondrial respiration, oxidative phosphorylation, and respiratory control resulted from drug binding to the MBR.

Because rat tissues vary greatly in their MBR content (11, 26), we wondered whether Ro5 would alter any of the mitochondrial respiration characteristics in other tissues and whether this ability correlated with the number of MBR present. Thus, mitochondria from rat liver and adrenal were prepared and evaluated in respiration experiments. Liver was chosen because it contains low numbers of receptors, whereas adrenal contains the highest levels of receptors in the rat (11, 26). There was no consistent effect of Ro5 at a final concentration of 1 μM on the rate of state IV respiration of liver mitochondria (0 ± 2% stimulation; three experiments). In contrast,

TABLE 3

Inhibition of mitochondrial [^{3}H]Ro5 binding and respiratory control by drugs and porphyrins

Results are means ± standard errors, where indicated, from three separate experiments. Other values are means of two separate experiments, which varied by about 15%. Intact rat kidney mitochondria were prepared and binding of 2 nM [^{3}H]Ro5 was performed as described in Experimental Procedures. Six to eight concentrations of each drug were evaluated in each binding experiment. Mitochondrial respiration was studied and drug effects on respiratory control were determined as described in Experimental Procedures. The dose-response curves for the drug effects on respiratory control are shown in Figs. 2 and 3.

Drug	IC_{50}	
	[^{3}H]Ro5 binding	Respiratory control
	nM	
Ro5	28 ± 1	17 ± 5
PK	48 ± 4	90 ± 7
DPY	170	72
DZ	358 ± 61	370 ± 17
DP	370	285
MP	500	450
FLU	523 ± 8	500 ± 20
CyA	3000	12000[a]
DBP	4700 ± 760	2600
CL259,763	17000	17500
CLZ	19000 ± 2021	inactive
Ro15-1788	>100000	inactive

[a] This result was obtained from Ref. 21.

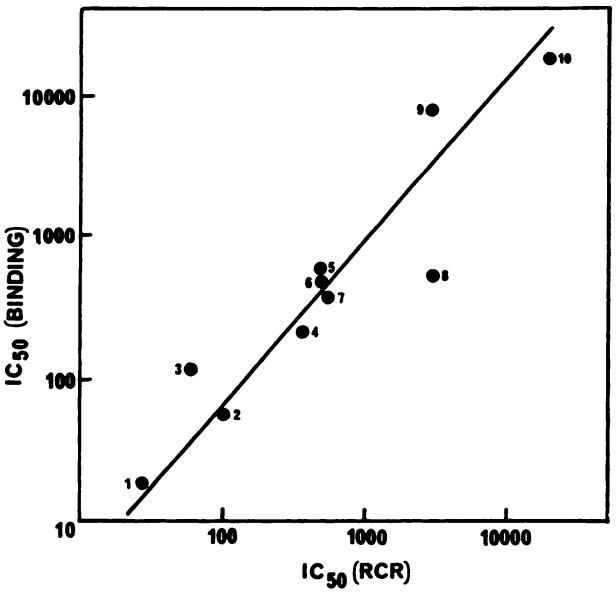

Fig. 4. Correlation between MBR binding and inhibition of the respiratory control ratio. Data used to derive this figure are found in Table 3 and Figs. 2 and 3. The correlation coefficient (0.95) was computed by unweighted linear regression analysis. The ordinate represents the mean IC_{50} values of the drugs at displacing [^{3}H]Ro5 from mitochondria. The *abscissa* represents the mean IC_{50} values for drug inhibition of the respiratory control ratio. **1** = Ro5, **2** = PK, **3** = DPY, **4** = DZ, **5** = MP, **6** = FLU, **7** = DP, **8** = DBP, **9** = CyA, **10** = CL259,763.

this drug stimulated state IV respiration in adrenal mitochondria by 30 ± 9% (three experiments), which is about double that observed in kidney (13 ± 5%; 16 experiments; Table 1). The effects of Ro5 on the state III rate and the RCR in adrenal mitochondria were similar to those obtained in kidney.

Discussion

The major finding of this study is that mitochondrial function is modulated by ligands that bind to MBR found on these

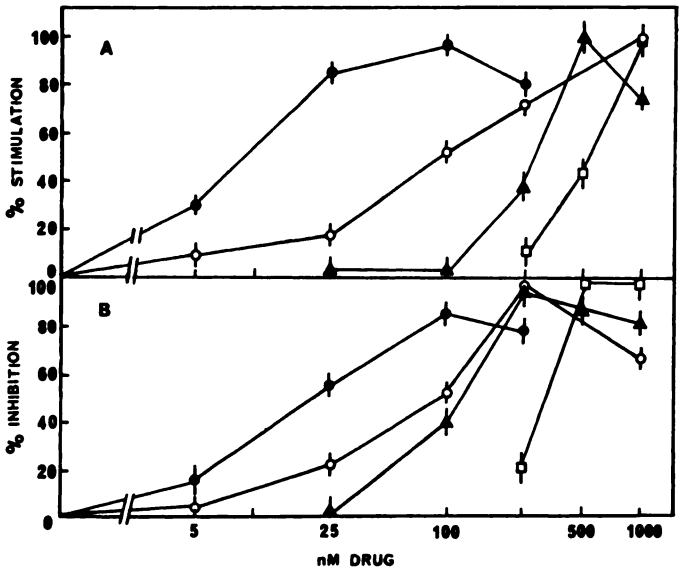

Fig. 5. The effects of MBR ligands on state IV and III respiration rates in rat kidney mitochondria. Results are means ± standard error from three separate determinations at each concentration of Ro5 (●), PK (○), DZ (△), and FLU (□). Details of these experiments are outlined in the legend to Fig. 2 and in Experimental Procedures. The EC_{50} values (state IV stimulation) and IC_{50} values (state III inhibition) are given in the text.

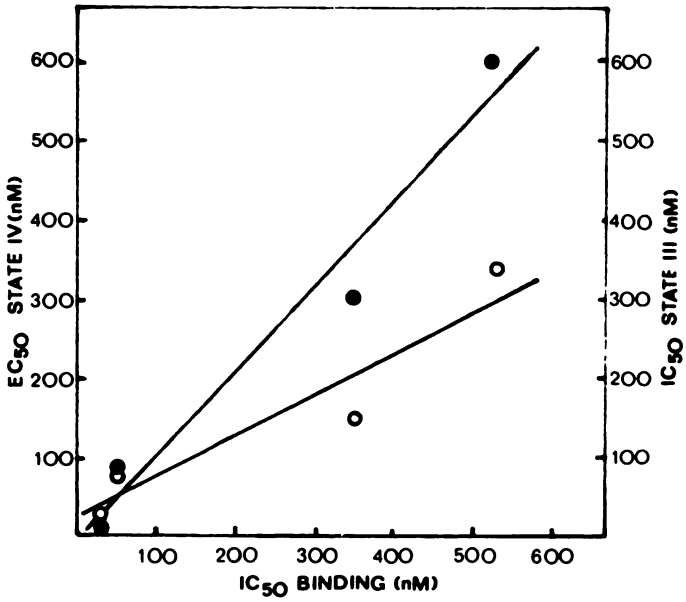

Fig. 6. Correlation between MBR binding and stimulation of state IV respiration and inhibition of state III respiration. The binding data used (*abscissa*) is listed in Table 3. The EC_{50} values for stimulation of state IV (*left ordinate*) are given in the text. The correlation coefficient for the *line* shown was 0.97 (●). The IC_{50} values for inhibition of state III (*right ordinate*) are also given in the text. The correlation coefficient for the *line* shown was 0.94 (○). For both sets of data, only the means are depicted in the figure. The correlation coefficients were computed by unweighted linear regression analysis.

organelles. Ten compounds, including three benzodiazepines and seven nonbenzodiazepines, bound to these sites and inhibited mitochondrial respiratory control, as predicted by their receptor binding affinities. Thus, one of the essential requirements for classification of MBR as functional receptors has been satisfied (27). Also, these results provide direct support for the idea that MBR modulate cellular energy metabolism

(11, 19). Additional evidence for classifying the MBR as a receptor is our finding that the magnitude of the effect of Ro5 on state IV respiration increases in parallel with the increasing density of receptors for this drug in different mitochondria (11, 26). Taken together, these results may help to explain the pleiotropic actions of MBR ligands on a variety of cell types (11).

Our data suggest that the MBR modulate mitochondrial respiratory control in a complex manner. Ligands for these receptors increase the rate of substrate oxidation and decrease the rate of oxidative phosphorylation, thereby decreasing the RCR. This occurs in the absence of significant changes in the ADP:O ratio. This activity profile is distinctive, is common to both benzodiazepines and nonbenzodiazepines, and does not resemble that of classical uncouplers, ionophores, electron transport inhibitors, or drugs that inhibit adenine nucleotide or substrate uptake (25, 28). Our results also indicate that PK is not an MBR antagonist in mitochondria as it is in other systems (11) because PK has intrinsic potency and full efficacy in our experiments.

Although the molecular mechanism by which MBR ligands decrease respiratory control has not been identified, our data suggest that they do not function like classical uncouplers. We observed no uncoupler-like respiratory stimulation with Ro5 or PK added after establishment of state IV and, in contrast to uncouplers (29), these drugs did not significantly decrease the ADP:O ratio. Also, Ro5, PK, DZ, and FLU had no typical uncoupler-like ability to dissipate the inner mitochondrial membrane potential during states IV and III.[1] The fact that Ro5 failed to increase the rate of NADH-supported ADP phosphorylation also suggests that this drug does not nonspecifically violate the proton permeability barrier presented by the inner mitochondrial membrane (30). Taken together, these results imply that, unlike typical uncouplers, the locus of action of MBR ligands is not the inner mitochondrial membrane. This is borne out by reports that the MBR is located on the outer mitochondrial membrane in various rat tissues (11, 17). These results indicate that a drug receptor on the outer mitochondrial membrane can influence inner membrane activities.

An important issue concerning the MBR is the question of whether or not an endogenous ligand(s) for these receptors exists. Based on their binding to the MBR and their presence in MBR-rich tissues, porphyrins, have been proposed as endogenous receptor ligands (12). Thus, it is intriguing that the two porphyrins tested in the present experiments (DP and MP) produced the same type and magnitude of respiration modulation as the synthetic ligands. These results support the proposal that porphyrins are endogenous ligands for the MBR. However, there are also reports that DZ, which binds to MBR and inhibits respiratory control, is found in a variety of natural sources and in rat tissues, along with its *N*-desmethyl derivative (31). In fact, the propensity of many structurally diverse natural products to bind to the MBR (17) suggests that other active endogenous ligands could also exist.

The identity and possible physiological function of the MBR should also be discussed. Recently, on the basis of circumstantial evidence, it was suggested that this receptor might be identical to the integral mitochondrial outer membrane protein called VDAC or "porin," which functions as a voltage-dependent anion channel (11, 32). As such, the receptor could modulate the bidirectional flow of mitochondrial substrates and products that takes place during normal respiration and oxidative phosphorylation. It is possible to interpret the present data from the point of view that the MBR modulates respiratory rates from its outer membrane location indirectly, like VDAC would, by limiting and/or facilitating exchange of substrates and products. However, previous work indicates that there is a very high density of VDAC molecules in the mitochondrial outer membrane (i.e., 4×10^{11} molecules/cm^2) (33), whereas there is tremendous variation in the density of MBR in mammalian tissues and mitochondria (11, 26). Unless one speculates that a subtype of VDAC molecules are MBRs whereas the rest of these channels are not, it appears unlikely that VDAC and the MBR are identical. This is further supported by recent data indicating that apparent MBR subunits are smaller than those found in VDAC (34–36).

In conclusion, we now have the ability to monitor the actions of known or suspected MBR ligands by measuring their potencies at inhibiting mitochondrial respiratory control. This ability in conjunction with the radioligand binding assay provides many opportunities to discover new active MBR ligands. These data also provide a useful framework for future experiments concerning both the identity and the mechanism of action of the MBR. In the accompanying paper, we present a detailed analysis of ligand binding to the MBR (17).

[1] C. F. Beyer, unpublished data.

References

1. Wang, J. K. T., J. I. Morgan, and S. Spector. Benzodiazepines that bind at peripheral sites inhibit cell proliferation. *Proc. Natl. Acad. Sci. USA* **81**:753–756 (1984).
2. Wang, J. K. T., J. I. Morgan, and S. Spector. Differentiation of Friend erythroleukemia cells induced by benzodiazepines. *Proc. Natl. Acad. Sci. USA* **81**:3770–3772 (1984).
3. Matthew, E., J. D. Laskin, E. A. Zimmerman, I. B. Weinstein, K. C. Hsu, and D. L. Engelhardt. Benzodiazepines have high-affinity binding sites and induce melanogenesis in B16/C3 melanoma cells. *Proc. Natl. Acad. Sci. USA* **78**:3935–3939 (1981).
4. Morgan, J. I., M. D. Johnson, J. K. T. Wang, K. H. Sonnenfeld, and S. Spector. Peripheral-type benzodiazepines influence ornithine decarboxylase levels and neurite outgrowth in PC12 cells. *Proc. Natl. Acad. Sci. USA* **82**:5223–5226 (1985).
5. Ruff, M. R., C. B. Pert, R. J. Weber, L. M. Wahl, S. M. Wahl, and S. M. Paul. Benzodiazepine receptor-mediated chemotaxis of human monocytes. *Science (Wash. D. C.)* **229**:1281–1283 (1985).
6. Hirsch, J. D., C. F. Beyer, and C. A. Mullen. Benzodiazepines inhibit cell-mediated cytotoxicity. *Soc. Neurosci. Abstr.* **12**:338 (1986).
7. Ritta, M. N., M. B. Campos, and R. S. Calandra. Effect of GABA and benzodiazepines on testicular androgen production. *Life Sci.* **40**:791–798 (1987).
8. Wilkinson, M., W. H. Moger, and D. Grovestine. Chronic treatment with Valium (diazepam) fails to affect the reproductive system of the male rat. *Life Sci.* **27**:2285–2291 (1980).
9. Curran, T., and J. I. Morgan. Superinduction of c-fos by nerve growth factor in the presence of peripherally-active benzodiazepines. *Science (Wash. D. C.)* **229**:1265–1268 (1985).
10. Mestre, M., T. Carriot, C. Belin, A. Uzan, C. Renault, M. C. Dubroeucq, C. Gueremy, and G. Le Fur. Electrophysiological and pharmacological characterization of peripheral benzodiazepine receptors in a guinea pig heart preparation. *Life Sci.* **35**:953–962 (1984).
11. Anholt, R. R. H. Mitochondrial benzodiazepine receptors as potential modulators of intermediary metabolism. *Trends Pharmacol. Sci.* **12**:506–511 (1986).
12. Verma, A., J. S. Nye, and S. H. Snyder. Porphyrins are endogenous ligands for the mitochondrial (peripheral-type) benzodiazepine receptor. *Proc. Natl. Acad. Sci. USA* **84**:2256–2260 (1987).
13. Davies, L. P., and V. Huston. Peripheral benzodiazepine sites in heart and their interaction with dipyridamole. *Eur. J. Pharmacol.* **73**:209–211 (1981).
14. Deckert, J., and P. J. Marangos. Hormonal interactions with benzodiazepine binding sites *in vitro*. *Life Sci.* **39**:675–683 (1986).
15. Cantor, E. H., A. Kenessey, G. Semenuk, and S. Spector. Interaction of calcium channel blockers with non-neuronal benzodiazepine binding sites. *Proc. Natl. Acad. Sci. USA* **81**:1549–1552 (1984).
16. Lukeman, D. S., and D. D. Fanestil. Interactions of diuretics with the

peripheral-type benzodiazepine receptor in rat kidney. *J. Pharmacol. Exp. Ther.* **241**:950–955 (1987).
17. Hirsch, J. D., C. F. Beyer, L. Malkowitz, C. C. Loullis, and A. J. Blume. Characterization of ligand binding to mitochondrial benzodiazepine receptors. *Mol. Pharmacol.* **34**:164–172 (1988).
18. Deleted in proof.
19. Hirsch, J. D. Pharmacological and physiological properties of benzodiazepine binding sites in rodent brown adipose tissue. *Comp. Biochem. Physiol. C. Comp. Pharmacol.* **77**:339–343 (1984).
20. Anholt, R. R. H., P. L. Pedersen, E. B. DeSouza, and S. H. Snyder. The peripheral-type benzodiazepine receptor: localization to the mitochondrial outer membrane. *J. Biol. Chem.* **261**:576–583 (1986).
21. Jung, K., and M. Pergande. Influence of cyclosporin A on the respiration of isolated rat kidney mitochondria. *FEBS Lett.* **183**:167–169 (1985).
22. Hirsch, J. D., C. F. Beyer, L. Malkowitz, C. C. Loullis, B. Beer, and A. J. Blume. A functional analysis of mitochondrial benzodiazepine receptors. *FASEB J.* **2**:A619 (1988).
23. Trumpower, B. L., and J. G. Haggerty. Inhibition of electron transfer in the cytochrome bc1 segment of the mitochondrial respiratory chain by a synthetic analogue of ubiquinone. *J. Bioenerg. Biomembr.* **12**:151–164 (1980).
24. Estabrook, R. W. Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. *Methods Enzymol.* **10**:41–47 (1967).
25. Heytler, P. G. Uncouplers of oxidative phosphorylation, in *Inhibitors of Mitochondrial Functions* (M. Erecinska and D. Wilson, eds.). Pergamon Press, New York, 199–210 (1981).
26. Antkiewicz-Michaluk, L., K. E. Krueger, A. Guidotti, and E. Costa. Characterization and density of the peripheral benzodiazepine binding sites in mitochondria from different organs. *Soc. Neurosci. Abstr.* **13**:1291 (1987).
27. Cuatracasas, P., and M. Hollenberg. Membrane receptors and hormone action. *Adv. Protein Chem.* **30**:251–445 (1976).
28. Tyler, D. D., and C. M. Sutton. Respiratory enzyme systems in mitochondrial membranes, in *Membrane Structure and Function* (E. E. Bittar, ed.), Vol. 5. John Wiley, New York, 33–115 (1984).
29. Terada, H. The interactions of highly active uncouplers with mitochondria. *Biochim. Biophys. Acta* **639**:225–242 (1981).
30. Rickwood, D., D. T. Wilson, and V. M. Darley-Usmar. Isolation and characterization of intact mitochondria, in *Mitochondria, a Practical Approach.* (V. M. Darley-Usmar, D. Rickwood, and M. T. Wilson, eds.). IRL Press, Oxford, 1–16 (1987).
31. Wildmann, J., H. Mohler, W. Vetter, U. Ranalder, K. Schmidt, and R. Maurer. Diazepam and *N*-desmethyldiazepam are found in rat brain and adrenal and may be of plant origin. *J. Neural Transm.* **70**:383–398 (1987).
32. Snyder, S. H., A. Verma, and R. R. Trifiletti. The peripheral-type benzodiazepine receptor: a protein of mitochondrial outer membranes utilizing porphyrins as endogenous ligands. *FASEB J.* **1**:282–288 (1987).
33. Freitag, H., W. Neupert, and R. Benz. Purification and characterization of a pore protein of the outer mitochondrial membrane from *Neurospora crassa*. *Eur. J. Biochem.* **123**:629–636 (1982).
34. Doble, A., O. Ferris, M. C. Burgevin, J. Menager, A. Uzan, M. C. Dubroeucq, C. Renault, C. Gueremy, and G. Le Fur. Photoaffinity labeling of peripheral-type benzodiazepine binding sites. *Mol. Pharmacol.* **31**:42–49 (1987).
35. Skowronski, R., D. D. Fanestil, and K. Beumont. Photoaffinity labeling of peripheral-type benzodiazepine receptors in rat kidney mitochondria with [^{3}H]PK14105. *Eur. J. Pharmacol.* **148**:182–193 (1988).
36. Beyer, C. F., J. D. Hirsch, C. C. Loullis, L. Malkowitz, B. Beer, and A. J. Blume. Photoaffinity labeling and partial purification of the mitochondrial benzodiazepine receptor. *FASEB J.* **2**:A619 (1988).

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