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Molecular Characterization and Mitochondrial Density of a Recognition Site for Peripheral-Type Benzodiazepine Ligands

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SUMMARY

In a previous report, mitochondria were proposed as a subcellular structure where recognition sites for peripheral benzodiazepine ligands are located in adrenal glands. The present study examines the subcellular distribution of specific binding sites for PK 11195 in eight tissues and compares the relative densities of these binding sites in mitochondrial-enriched fractions with the relative activities of two mitochondrial marker enzymes. In all eight tissues examined, PK 11195 binding sites were found to subfractionate in a manner nearly identical to that of the mitochondrial enzyme succinate dehydrogenase. The subcellular distribution patterns of specific PK 11195 binding sites were unrelated to the distribution patterns of marker enzymes for plasma membranes, lysosomes, or endoplasmic reticulum. Scatchard analyses of mitochondrial fractions from all eight tissues demonstrated a greater than 100-fold difference in the densities of PK 11195 binding sites, the extremes being 140 and 1 pmol/mg of protein in adrenal and brain tissues, respectively. There was no correlation between the relative density of PK 11195 binding sites and the specific activities of succinate dehydrogenase and cytochrome *c* oxidase. These results suggest that the density of peripheral-type benzodiazepine receptors in mitochondria is tissue dependent and apparently regulated independently of the mechanisms by which these two mitochondrial enzymes are expressed or function. The photoaffinity probe PK 14105 was used to photolabel the peripheral-type benzodiazepine binding sites of mitochondrial fractions prepared from the eight tissues. In all preparations, a 17,000-Da polypeptide is specifically labeled as determined by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. Thus, it appears that the protein recognition site for isoquinoline carboxamides of peripheral-type benzodiazepine receptor complexes is similar in all mitochondrial preparations.

PBZD recognition sites are present in most peripheral organs but they are also present in brain and spinal cord at levels that equal or even exceed that of the central-type benzodiazepine receptor complex (1–4). The density of these receptors varies quite considerably between various tissues (4–6). Significant progress has been made toward understanding the tissue and subcellular localization of PBZD recognition sites. It was determined by autoradiographic techniques that PBZD binding sites appear to colocalize with CCO activity, a mitochondrial marker (6). Additional studies utilizing rat adrenal glands demonstrated a close correspondence between the distribution of PBZD binding sites and the distribution of CCO in different subcellular fractions (7). It is therefore believed that mitochondria are the primary membrane organelles with which these binding sites are associated.

Thus far, little insight has been gained concerning the identification of the receptorial function with which these recognition sites are associated in mitochondria. Recently it has been suggested that PBZD binding sites may be part of the mitochondrial anion channel protein porin (8), and it was also demonstrated that PBZD recognition sites appear to interact with porphyrins (9). It has also been proposed that PBZD binding sites may function as a regulator of certain metabolic activities in mitochondria (5, 6).

In an attempt to begin to determine the molecular construction of PBZD receptors, a photoaffinity probe, PK 14105, has recently been synthesized (10). This probe exhibits a high affinity for PBZD binding sites. PK 14105 binding to membranes is specifically displaced by other compounds that also show high affinities for PBZD recognition sites, such as its isoquinoline carboxamide concatemer PK 11195 and the benzodiazepines Ro 5-4864 and diazepam. Upon UV irradiation PK 14105 covalently inserts into or near the binding domain for isoquinoline carboxamides. This has led to the identification of an 18-kDa protein from heart membranes that is specifically photolabeled.

In the present report, the subcellular fractionation of PBZD binding sites is examined in eight different tissues and compared with the fractionation of the mitochondrial enzyme SDH. To ensure that tissue-dependent levels of these binding sites are reflected in their abundances on mitochondrial structures, the densities of PBZD binding sites in mitochondrial-enriched fractions from these eight tissue sources are determined. The activities of two mitochondrial marker enzymes, SDH and

ABBREVIATIONS: PBZD, peripheral-type benzodiazepine; SDH, succinate dehydrogenase; CCO, cytochrome *c* oxidase.

CCO, were also measured in these fractions in an attempt to determine whether any correlation could be made between their relative activities and the relative densities of PBZD binding sites. In addition, [^{3}H]PK 14105 was used to examine whether there is molecular heterogeneity in the binding sites for isoquinoline carboxamides of mitochondrial fractions prepared from different tissues.

Materials and Methods

Isolation of mitochondria. Adult Sprague-Dawley rats were killed by decapitation and the brain (cerebrum and cerebellum), adrenal glands, kidneys, heart, lungs, liver, diaphragm, and testis were removed and placed in at least 4 volumes (w/v) of ice-cold 2 mM HEPES (pH 7.4), 0.32 M sucrose, henceforth referred to as homogenization buffer. Kidney, heart, lung, liver, and diaphragm tissues were first homogenized for 30 sec with a sonic homogenizer (SDT 1810, Tekmar Co., Cincinnati, OH) and then passed through 12 strokes with a tight-fitting Potter-Elvehjem tissue grinder. The remaining tissues were homogenized using only the Potter-Elvehjem tissue grinder. The homogenates were first centrifuged at 770 × *g* for 15 min and the resulting supernatants were recovered and centrifuged again at 5200 × *g* for 20 min. The pellet of this second centrifugation was resuspended in the same initial volume of homogenization buffer and centrifuged at 11,000 × *g* for 20 min. According to this described procedure, three subcellular fractions were prepared, including the 770 × *g* pellets, 5200 × g pellets, and 5200 × *g* supernatants. Because it was not essential to separate microsomal membranes from cytosolic components, the supernatant fractions in this study contained both of these compartments.

Because in brain tissue the 5200 × *g* pellet is contaminated with synaptosomal membranes, an additional procedure described by Jones and Matus (11) involving hypotonic lysis and discontinuous sucrose gradient centrifugation was utilized to further enrich the mitochondria from brain preparations. The mitochondrial fraction was recovered as a pellet after sucrose gradient centrifugation whereas another membrane fraction containing synaptosomal plasma membranes and myelin was obtained by dilution of the material suspended within the gradient and recentrifugation, yielding an additional membrane pellet.

Protein determinations of all subcellular fractions were performed according to the method of Bradford (12). Samples were stored frozen at −70° until ready for use.

Measurement of PK 11195 and PK 14105 binding and photoaffinity labeling. Subcellular fractions were suspended in 50 mM Tris·HCl (pH 7.6) and incubated for 1 hr in a final volume of 200 μl at 0° with [^{3}H]PK 11195 or [^{3}H]PK 14105 (1–3 nM). Nonspecific binding was measured normally in the presence of nonradioactive 1 μM PK 11195. The membranes were then filtered under vacuum through 2.4-cm Whatman GF/C filters and washed with 15 ml of cold Tris buffer, and the filters were subjected to liquid scintillation counting. Photoaffinity labeling of mitochondrial membranes was performed in Tris buffer on ice for 1 hr with 3 nM [^{3}H]PK 14105, using the procedure described by Doble *et al.* (10).

Enzyme assays. All enzyme assays were performed at 37° and were ensured to be within a range in which the enzyme activity measured was proportional to the amount of protein used. When determination of inorganic phosphate was required, the procedure described by Chen *et al.* (13) was used.

SDH was measured by incubating 250-μg protein aliquots at 37° in 450 μl of 10 mM sodium phosphate (pH 7.4), 10 mM sodium succinate, 1 mM KCN. The reaction was initiated with the addition of 50 μl of 30 mM neotetrazolium chloride dissolved in dimethylsulfoxide. After 3–5 min the reaction was terminated with the addition of 1 ml of 0.1 M sodium formate (pH 3.5), 0.1 M formaldehyde, 2% Triton X-100 (v/v), and the absorbance was measured at 505 nm. Control assays were performed by omitting succinate in the reaction mixture.

CCO activity was determined by measuring the oxidation of ferrocytochrome *c* at 550 nm (14). The amount of mitochondrial protein varied between 4 and 80 μg, depending on the tissue source. The specific activity of CCO was determined from the slope of the first-order decay in absorbance.

The activity of 5′-nucleotidase was measured by incubation of 100 μg of protein in 500 μl of 50 mM glycine-NaOH (pH 9.1), 5 mM adenosine monophosphate, 10 mM $MgCl_2$ (15). After 30 min the reaction was stopped by the addition of 2.5 ml of 8% trichloroacetic acid, and the samples were placed in ice for 2 hr and then centrifuged at 1500 × *g* for 10 min. The amounts of inorganic phosphate in 1-ml aliquots of the supernatants were then quantitated. Blank control tubes, lacking either substrate or enzyme, were also processed and the quantities of phosphate measured in these controls were subtracted from the values of the experimental tubes.

Acid phosphatase was measured by incubation of 200 μg of protein in 500 μl of 100 mM Na acetate (pH 5.0), 100 mM β-glycerophosphate, 0.1% Triton X-100 for 30 min (16). Termination of the reaction, quantitation of liberated phosphate, and analogous control assays were performed as described for 5′-nucleotidase.

Rotenone-insensitive NADPH-cytochrome *c* reductase was measured by placing 200–900 μg of protein in 1.6 ml of 300 mM potassium phosphate (pH 7.7), 50 μM cytochrome *c*, 500 μM KCN, 10 μM rotenone. After addition of the enzyme, the basal rate of reduction of ferricytochrome *c* was monitored spectrophotometrically at 550 nm. The reaction was initiated by addition of 50 μl of 10 mM NADPH and the reduction of cytochrome *c* was measured. The endogenous rate of reduction of cytochrome *c* by NADPH alone was also determined in this manner. The specific activity of the enzyme was calculated by determining the total rate of reduction by the sample in the presence of NADPH and subtracting from it the rates of reduction due to NADPH alone and the basal rate of reduction.

Materials. [*N-methyl*-^{3}H]PK 11195 was purchased from New England Nuclear (Boston, MA). [*N-methyl*-^{3}H]PK 14105 was received from Dr. H. Van Hove (Commissariat a l'Energie Atomique, Gif-sur-Yvette Cedex, France). PK 11195 and PK 14105 were the gift of Dr. C. Gueremy (Pharmuka Laboratories, Groupe Rhone Poulenc Sante, Gennevilliers, France). Diazepam, clonazepam, and Ro 5-4864 were a gift from Hoffman-LaRoche (Nutley, NJ). Neotetrazolium chloride, adenosine monophosphate, β-glycerophosphate, NADPH, and cytochrome *c* were all obtained from Sigma Chemical Co. (St. Louis, MO).

Results

Codistribution of PK 11195 and SDH by subcellular fractionation. To examine whether the binding sites for PK 11195 subfractionate with mitochondrial markers, the binding of [^{3}H]PK 11195 and SDH were measured in the membrane fractions obtained by differential centrifugation from eight tissue homogenates. These fractions include pellets obtained at 770 × *g* and 5200 × *g* and supernatants recovered at 5200 × *g*. It should be noted that the supernatant fractions contain microsomal and cytosolic components. In all tissue preparations the highest activity for PK 11195 binding, as well as SDH, was found in the 5200 × *g* pellet (Fig. 1). These membrane subfractions are therefore most enriched in mitochondria, regardless of the tissue source.

Examination of specific PK 11195 binding and SDH activities in the 770 ×*g* pellets, 5200 × *g* pellets, and 5200 × *g* supernatants of all tissues demonstrates that there exists a close correlation between the distribution of PK 11195 binding sites and the mitochondrial marker enzyme (Fig. 1). The relative ratios of PK 11195 binding and SDH do not vary more than 2-fold between the three membrane subfractions of nearly each tissue. These results suggest that in the tissues examined here the majority of PK 11195 binding sites are associated with mitochondrial membranes or some other membrane subfraction

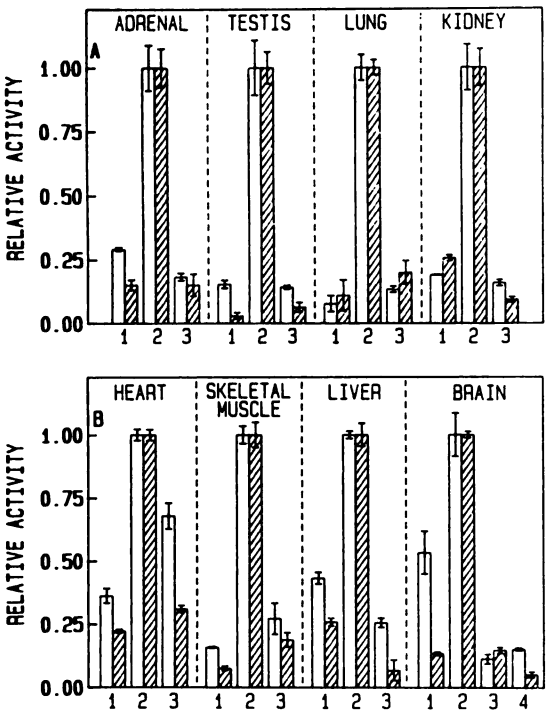

Fig. 1. Subcellular fractionation of PK 11195 binding sites and SDH. Specific binding of [^{3}H]PK 11195 at a concentration of 3 nM (□) and SDH (▨) were determined in the different subcellular fractions from each tissue. Binding and enzyme activities were determined as a function of protein content and are expressed relative to the values obtained with the mitochondrial fractions (5200 × *g* pellets) in each tissue. *Error bars* indicate the standard deviations of three determinations. The notation for the subcellular fractions is as follows: *1*, 770 × *g* pellet; *2*, 5200 × *g* pellet; *3*, 5200 × *g* supernatant; *4*, the synaptosomal plasma membrane and myelin fraction prepared from the 5200 × *g* pellet (see Materials and Methods).

that cosediments with mitochondria. In two cases there appears to be a significant deviation in the ratio of PK 11195 binding sites to SDH activity when comparing different subfractions of the same tissue. For example, the 770 × *g* membrane pellets of testis and brain exhibit a higher proportion of PK 11195 binding to SDH than those observed in the corresponding 5200 × *g* membrane pellets. It is possible that in these tissues a small subpopulation of PBZD binding sites may also be localized on other membranous structures, in addition to mitochondria.

The 5200 × *g* pellets are fractions enriched in mitochondria; however, it is likely that other membrane components cosediment in these pellets. To demonstrate that the distribution of PBZD binding sites is related to that of mitochondrial membranes and is not accounted for by the distribution of these other membranes, the relative activities of 5′-nucleotidase, acid phosphatase, and rotenone-insensitive NADPH-cytochrome *c* reductase were determined in the different subfractions of adrenal, kidney, liver, and brain subfractions. These represent marker enzymes for plasma membranes, lysosomes, and endoplasmic reticulum, respectively, membranes that are likely to contaminate mitochondrial fractions. The relative distributions of these enzymes do not correlate with the distribution of PK 11195 binding sites (Fig. 2), supporting the suggestion that PBZD binding sites are largely localized on mitochondrial structures.

Analysis of PK 11195 binding site densities with the specific activities of two mitochondrial marker enzymes. In order to examine the tissue-dependent variation in density of PBZD binding sites in the mitochondrial fractions, Scatchard analyses of PK 11195 binding to mitochondrial membrane preparations from the eight different tissues was performed. Table 1 lists the dissociation constants and B_{max} for specific PK 11195 binding sites observed for the different mitochondrial fractions. The K_D determined for all tissues was nearly constant (approximately 1 nM) and only one class of binding sites was detected within the tested concentration range for PK 11195. The B_{max} was found to exhibit great variation, in that mitochondrial fractions from adrenal glands contained the highest density of binding sites (140 pmol/mg of protein) followed by a succession of tissues showing progressively lower densities, of which preparations from brain demonstrated the lowest density (1 pmol/mg of protein).

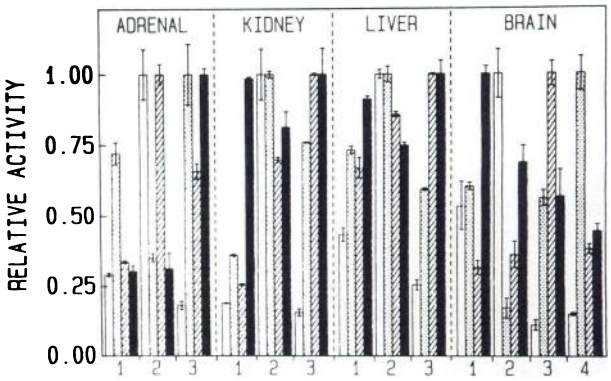

Fig. 2. Subcellular fractionation of PK 11195 binding sites and three additional marker enzymes. Specific binding of [^{3}H]PK 11195 (□) and activities of 5′-nucleotidase (□) acid phosphatase (▨) and rotenone-insensitive NADPH-cytochrome *c* reductase (■) were quantitated as a function of protein content. Results are expressed relative to the highest specific activity determined from each tissue with the standard deviations of three determinations illustrated. The notation for subcellular fractions is listed in the legend to Fig. 1.

Additional studies not shown here demonstrate that quantitatively the same density of binding sites for the benzodiazepine [^{3}H]Ro 5-4864 is observed in these mitochondrial fractions. Furthermore, nonradioactive Ro 5-4864 at submicromolar concentrations completely inhibits specific [^{3}H]PK 11195 binding to these membranes whereas benzodiazepines such as clonazepam, which are much more selective for central-type benzodiazepine binding sites, in this same concentration range do not inhibit [^{3}H]PK 11195 binding.

To examine whether these differences could be correlated with tissue-dependent variations found in different mitochondrial markers, the PK 11195 binding studies were complemented with a determination of the specific activities of two constitutive mitochondrial marker enzymes, SDH and CCO. A comparison of the PK 11195 binding site density and mitochondrial enzymatic activity in the eight mitochondrial fractions is shown in Table 1. It was found that the specific activities of SDH and CCO from the different mitochondrial preparations also showed considerable variation. This is not unexpected, inasmuch as it has been reported that the levels for many mitochondrial enzymes vary from tissue to tissue (17).

The relationship between specific PK 11195 binding and SDH and CCO activities of the eight mitochondrial fractions are shown as double-log plots in Fig. 3. The correlation coefficient of the log of SDH versus the log of CCO is 0.878, indicating that there is some, albeit relatively weak, correlation in the

TABLE 1

Analysis of specific [^{3}H]PK 11195 binding, SDH, and CCO activities in different mitochondrial fractions

Composite results of three experiments are given listing the mean and standard deviation for each value. Scatchard analyses of [^{3}H]PK 11195 binding were performed at concentrations of 0.1–30 nM. The relative activities of SDH and CCO were measured as described in Materials and Methods.

Tissue	[^{3}H]PK 11195 Binding		SDH	CCO
	K_d	B_{max}		
	nM	*pmol/mg of protein*	*units/mg of protein*	*units/mg of protein*
Adrenal	1.3 ± 0.2	140 ± 4	0.31 ± .056	0.31 ± .080
Testis	1.1 ± 0.1	35 ± 9.5	0.79 ± .16	4.1 ± .72
Lung	1.2 ± 0.1	32 ± 12	0.35 ± .048	1.4 ± .30
Kidney	0.8 ± 0.4	13 ± 1.4	0.90 ± .14	4.3 ± .32
Heart	0.9 ± 0.3	12 ± 5.8	2.4 ± .34	6.2 ± .020
Skeletal muscle	0.8 ± 0.1	3.1 ± 0.8	1.2 ± .23	6.2 ± .24
Liver	0.8 ± 0.3	1.2 ± 0.3	0.32 ± .086	1.2 ± .33
Brain	1.3 ± 0.8	0.95 ± 0.36	1.6 ± .20	12 ± 2.7

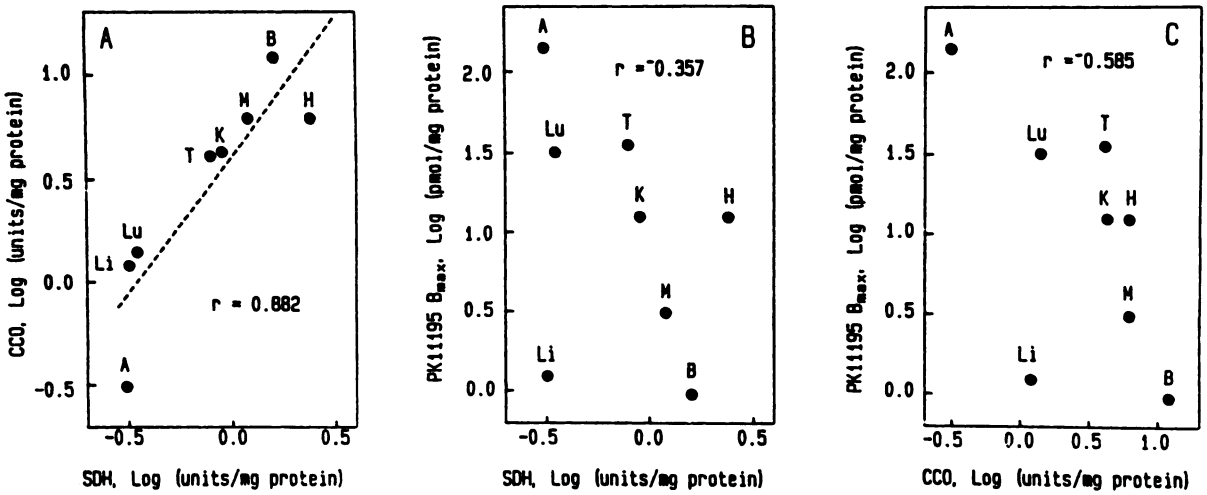

Fig. 3. Relationships between densities of [^{3}H]PK 11195 binding sites and activities of SDH and CCO in different mitochondrial fractions. The averaged results of three separate experiments are plotted on logarithmic scales. The correlation coefficient corresponding to each relationship is given inside the figures. The different mitochondrial preparations are listed as follows: *A*, adrenal glands; *B*, brain; *H*, heart; *K*, kidney; *Li*, liver; *Lu*, lung; *M*, skeletal muscle; and *T*, testis.

activities of these two enzymes. In contrast, correlation coefficients of the log of PK 11195 binding versus the log of SDH and CCO were −0.357 and −0.585, respectively, demonstrating that there is no apparent relationship between the abundance of PK 11195 binding sites and the activities of either enzyme.

Photoaffinity labeling of peripheral benzodiazepine binding sites with PK 14105. The isoquinoline carboxamide PK 14105 is very similar in structure to PK 11195 and also enhibits high specificity for PBZD receptors. This compound has been used to photolabel benzodiazepine binding sites located on cardiac membranes (10). In further characterizing and comparing the PBZD binding sites from different tissues, [^{3}H] PK 14105 was used to examine its binding parameters to the different mitochondrial preparations. At submaximal concentrations the levels of specific [^{3}H]PK 14105 binding to the mitochondrial membranes paralleled that of [^{3}H]PK 11195 binding (Table 2). In mitochondria from all eight tissues, specific PK 14105 binding was completely inhibited by PK 11195, suggesting that both ligands bind to common receptor sites.

Irradiation of membranes with UV light promotes covalent attachment of [^{3}H]PK 14105 to the membranes, which cannot be displaced by an excess of PK 11195. The relative quantity of specific [^{3}H]PK 14105 photoincorporation into the different mitochondrial preparations exhibited the same relation in comparison with specific [^{3}H]PK 11195 and [^{3}H]PK 14105 binding under nonirradiating conditions within the limits of experimental variability for the photolabeling procedure (Table 2). Typically 33–68% of the specific [^{3}H]PK 14105 binding in the

TABLE 2

Comparison of specific [^{3}H]PK 14105 binding with specific [^{3}H]PK 11195 binding

Results of a single representative experiment are given showing the quantities of specific binding in pmol/mg of protein. Nonspecific binding was measured in the presence of 1 μM nonradioactive PK 11195. Binding to mitochondrial fractions were performed as described in Materials and Methods. Irreversible binding to irradiated membranes included an additional incubation at 20° for 15 min with 1 μM nonradioactive PK 11195 before filtration.

Tissue	Specific binding		
	[^{3}H]PK 11195 (3 nM) nonirradiated	[^{3}H]PK 14105 (2 nM) nonirradiated	[^{3}H]PK 14105 (3 nM) irradiated
	pmol/mg of protein		
Adrenal	33	10	5.0
Testis	6.1	4.4	0.54
Lung	6.2	4.0	2.2
Kidney	6.6	4.4	1.5
Heart	5.8	2.4	0.86
Skeletal muscle	3.6	1.2	0.25
Liver	1.2	0.84	1.1
Brain	0.57	0.33	0.13

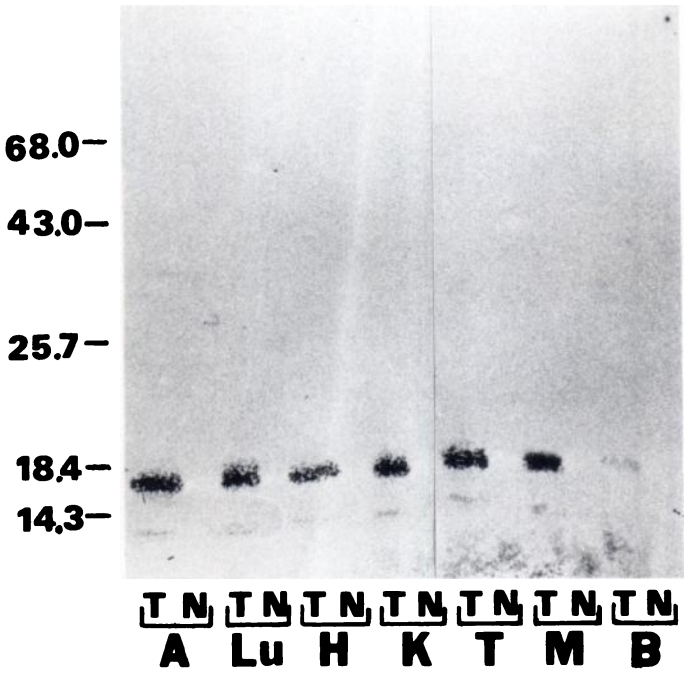

Fig. 4. Identification of the photolabeled PK 14105 binding site in different mitochondrial preparations. Mitochondrial fractions (25–100 μg of protein) were photolabeled with [^{3}H]PK 14105 as described in Materials and Methods. The membranes were pelleted by centrifugation at 40,000 × *g* and incubated 15 min on ice in 50 mM Tris·HCl (pH 7.6) containing 1 μM PK 11195. The membranes were again harvested by centrifugation and solubilized in 160 μl of 12.5 mM Tris·HCl (pH 6.8), 3 mM dithiothreitol, 20% glycerol, 2% sodium dodecyl sulfate, 0.005% bromophenol blue. The samples were then electrophoresed by the method of Laemlli (18) on 3-mm thick 12% polyacrylamide gels and transferred to a nitrocellulose membrane (19). The membrane was sprayed with En^{3}Hance (New England Nuclear) and autoradiographed for 5 days. The notation for different tissues is given in the legend to Fig. 3. Approximately 15,000 cpm of specifically bound radioactivity was applied (*T*). An equivalent amount of protein was also applied from duplicate samples irradiated under identical conditions but also including 1 μM PK 11195 (*N*). Positions of molecular weight markers (Bethesda Research Laboratories, Gaithersburg, MD) are indicated with their respective sizes given in kilodaltons.

dark is irreversible after irradiation. This would suggest that the specific binding sites for [^{3}H]PK 14105 binding are covalently modified after irradiation. Liver mitochondrial fractions exhibit higher [^{3}H]PK 14105 binding upon irradiation when compared with binding under nonirradiating conditions. This anomalous result can be accounted for by the difficulty in measuring specific PK 14105 binding of these membranes because of the relatively low number of binding sites and high nonspecific binding detected during irradiation.

Electrophoretic analysis of photolabeled PK 14105 binding site. Mitochondrial fractions from the eight tissue sources were irradiated in the presence of [^{3}H]PK 14105 at a concentration of 3 nM. Nonradioactive PK 11195 was added at concentrations of 1 μM to control samples to determine sites that are photolabeled nonspecifically. The photolabeled membranes were then subjected to electrophoresis in 12% polyacrylamide gels, transferred to nitrocellulose, and autoradiographed. With all mitochondrial preparations, one polypeptide with an apparent molecular weight of 17,000 Da was specifically photolabeled. We have found it particularly difficult to detect a specifically labeled component from liver mitochondria; however, much longer exposures reveal qualitatively the same results as mitochondrial fractions from the other seven tissues. Similarly, mitochondrial fractions from brain yielded a less intense band when compared with the other tissues, with the 17,000-Da band being the only component specifically labeled.

This 17,000-Da protein is undoubtedly the same component reported by Doble *et al.* (10), who had photolabeled cardiac membranes with [^{3}H]PK 14105. Thus, the binding site for isoquinoline carboxamides in mitochondrial fractions prepared from various tissues appears to consist of a common polypeptide constituent. In addition to the 17,000-Da component, another minor, specifically labeled, band, which migrated near the leading front, was found in some cases. This probably represents some degradation products of the 17,000 polypeptide.

Discussion

PBZD binding sites have been suggested, at least in adrenal glands, to be primarily associated with mitochondria. The data presented in this paper suggest that a mitochondrial localization of these binding sites is also apparent in several other tissues. Subcellular fractionation of eight different organs demonstrated a close correspondence between the level of PK 11195 binding sites and the level of SDH activity in different subcellular fractions. These findings confirm the results of Anholt *et al.* (7), who had studied the subcellular distribution of PBZD binding sites in adrenal glands.

The association of PBZD binding sites with some other membrane structures that cosediment with mitochondria cannot be excluded; however, this possibility is unlikely based on two criteria examined in these studies. First, marker enzymes for plasma membranes, lysosomes, and endoplasmic reticulum, membrane components that are likely to contaminate the mitochondrial fractions, do not parallel the subcellular distribution of PBZD binding sites. Second, the subcellular distribution of PK 11195 binding sites exhibits a pattern very close to that of the distribution of SDH in all eight tissues examined. If another membrane structure contained the PBZD binding sites, it would be quite unlikely that this membrane would exhibit a subcellular distribution with exactly the same pattern as that of mitochondria in all eight tissues.

From these and previous studies there is strong evidence to suggest that mitochondria are primary sites at which PBZD binding sites are located. It is possible, however, that other membrane structures may also contain PBZD binding sites but in considerably lower densities, such that their existence is masked by the mitochondrial population. This may be the case as was found for the subcellular distribution of PK 11195 binding sites in brain and testis, where the 770 × *g* pellets of these preparations contain significantly higher levels of PK 11195 binding sites than would be expected based on the mitochondrial contamination of these fractions as determined by measuring SDH. Other groups reported relatively high levels of [^{3}H]Ro 5-4864 binding in the "nuclear" pellets of brain in comparison with the "mitochondrial" pellets (4, 20). This might indicate at least a second membrane structure containing PBZD binding sites in brain and testis.

Although it had been demonstrated that the density of PBZD binding sites varies greatly between many tissues (2–6), before the present report no one had studied the density of PBZD recognition sites on mitochondrial fractions prepared from different tissues. These studies demonstrate that mitochondrial fractions prepared from different tissues also exhibited a wide

range in the density of binding sites for the PBZD ligand PK 11195. It was found that mitochondrial fractions of adrenal glands possessed the highest levels of these sites whereas those from the brain were the lowest, over 100-fold less than that detected in adrenal preparations.

The dissociation constant of PK 11195 for the different mitochondrial fractions was nearly equal, and only one class of binding sites was observed by Scatchard analysis within the concentration range of 0.1–30 nM PK 11195. The binding of PK 11195 to the mitochondrial fractions was completely inhibited by submicromolar concentrations of Ro 5-4864. Furthermore, [^{3}H]Ro 5-4864 showed a parallel pattern in the density of binding sites between different mitochondrial fractions from different tissues, when compared with the densities of [^{3}H]PK 11195 binding sites. These observations demonstrate that all of the mitochondrial fractions examined exhibit pharmacological properties reported for PBZD binding sites.

To determine whether tissue-specific differences in PK 11195 binding site density were related to differences found in the specific activities of mitochondrial marker enzymes, we also measured the activities of SDH and CCO. It was found that both SDH and CCO activities varied between the different tissues, but in a manner unrelated to PK 11195 binding.

The relative activities of SDH and CCO varied somewhat independently of each other, a phenomenon observed earlier, implying that these enzymes have no special functional relationship with each other (17). The ratio of these enzymes varied at most 7-fold among the eight tissues examined. The correlation coefficient between the log of SDH activity and the log of CCO activity is 0.882, indicating that although the proportion of these two enzymes fluctuates there is a general trend whereby high SDH activity is associated with a relatively high CCO activity. The activities of these two mitochondrial enzymes probably reflect the metabolic activity of the tissue.

In contrast to this observation, the B_{max} of specific [^{3}H]PK 11195 binding appeared unrelated to the activities of either of the two enzymes. The correlation coefficient of the log of PK 11195 binding to the log of SDH and CCO activities was −0.357 and −0.585, respectively. It is noteworthy that mitochondria of the adrenal gland exhibit ratios of PK 11195 binding site density to SDH and CCO activities that range from 5- to greater than 1000-fold higher than the other mitochondrial preparation.

The extremely variable nature of PBZD binding site densities in relation to these mitochondrial markers implies that, if these binding sites are in fact primarily localized on mitochondria, the levels in which they are present are governed by the tissue. Thus, it is apparent that mitochondrial fractions from some tissues can be very rich in these binding sites (140 pmol/mg of protein in adrenals) whereas mitochondrial fractions from other tissues possess a much lower abundance (1 pmol/mg of protein in brain). Because this work used mitochondrial fractions prepared from complex tissues, it is likely that a diverse population of mitochondria arising from the different cell types of each tissue was recovered. Therefore, the possibility exists that mitochondria from some cell types may contain no or extremely low levels of PBZD binding sites. Interestingly, in a previous study, binding sites for PBZDs were not detected in membranes from the adrenal medulla (21). This is in agreement with autoradiographic studies showing the localization of PBZD binding sites in the adrenal cortex (1, 7) suggesting that the density of these binding sites in mitochondria of the adrenal cortex is still higher than the density determined in these studies using entire adrenal glands.

The reason for such differences regarding the mitochondrial density of these recognition sites is not known. These observations suggest that this recognition site may participate in an activity that varies in a manner independent of the activities of SDH and CCO and that may be implemented to greatly varying degrees in different mitochondrial populations. Therefore, the role of PBZD receptors may be a more specialized function to suit the physiological requirements of the host cell, rather than a generalized activity characteristic of all mitochondria.

With the use of the photoaffinity ligand PK 14105, it was determined that among mitochondria isolated from eight different tissues, the molecular composition of the binding site for isoquinoline carboxamides associated with PBZD recognition complex is similar, if not identical. These sites consist of a 17,000-Da polypeptide that is specifically, covalently labeled by [^{3}H]PK 14105 under UV irradiation. Thus, this molecule is common to all tissues possessing mitochondrial PBZD receptors.

The evidence that a 17,000-Da polypeptide is operative in the high affinity binding of drugs of the isoquinoline carboxamide class is reminiscent of the report (10) that a molecule of 18,000 Da is involved in the PK 14105 binding to cardiac membranes. Whether this polypeptide is a proteolytic fragment of a larger protein or a subunit of a macromolecular complex remains to be established.

Further corroboration that the 17-kDa polypeptide is part of PBZD recognition sites is given by the demonstration that the relative density of specific binding sites for PK 11195 and PK 14105 exhibits the same variation when mitochondrial preparations of different tissues are compared. After photoincorporation of [^{3}H]PK 14105 into mitochondrial membranes, the density of specific irreversibly bound ligand correlates with that of PK 11195 binding. This congruous tissue-dependent variation in these three parameters offers additional support that PK 14105 specifically labels PBZD binding sites.

Another study using the benzodiazepine [^{3}H]flunitrazepam as a photoaffinity probe has reported the specific labeling of a 35-kDa protein from mitochondrial preparations (8). The most apparent explanation for the differences observed between using PK 14105 and flunitrazepam is that different proteins are labeled by each probe. There is evidence to suggest that the binding sites for benzodiazepines may be distinct from, although allosterically coupled to, the binding sites for isoquinoline carboxamides (22). This can account for the discrepancies observed in the photolabeled components. An alternative possibility is that the 17-kDa polypeptide labeled by PK 14105 is a fragment derived from the 35-kDa protein labeled by flunitrazepam.

The results presented in this paper give some clues concerning the function of PBZD receptors of mitochondria. Because a molecular diversity in the binding sites for PK 14105 was not observed among the eight tissues examined, these recognition sites are likely to regulate common biochemical pathways in different tissues. The magnitude of variability in densities of these recognition sites and their apparent lack of correlation with SDH and CCO activities in a variety of tissues suggest that they may not be an ubiquitous component of every mito-

chondrion. Moreover, this would tacitly imply that the activities in which the benzodiazepine recognition sites are involved are likely to be differentially regulated in a tissue-dependent manner.

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References

1. De Souza, E. B., R. R. H. Anholt, K. M. M. Murphy, S. H. Snyder, and M. S. Kuhar. Peripheral-type benzodiazepine receptors in endocrine organs: autoradiographic localization in rat pituitary, adrenal and testis. *Endocrinology* **116**:567–573 (1985).
2. Villiger, J. W. Characterization of peripheral-type benzodiazepine recognition sites in the rat spinal cord. *Neuropharmacology* **24**:95–98 (1985).
3. Awad, M., and M. Gavish. Binding of [^{3}H]Ro 5-4864 and [^{3}H]PK 11195 to cerebral cortex and peripheral tissues of various species: species differences and heterogeneity in peripheral benzodiazepine binding sites. *J. Neurochem.* **49**:1407–1414 (1987).
4. Shoemaker, H., R. G. Boles, W. D. Horst, and H. I. Yamamura. Specific high-affinity binding sites for [^{3}H]Ro 5-4864 in rat brain and kidney. *J. Pharmacol. Exp. Ther.* **225**:61–69 (1983).
5. Anholt, R. R. H. Mitochondrial benzodiazepine receptors as potential modulators of intermediary metabolism. *Trends Pharmacol. Sci.* **7**:506–511 (1986).
6. Anholt, R. R. H., E. B. DeSouza, M. L. Oster-Granite, and S. H. Snyder. Peripheral-type benzodiazepine receptors: autoradiographic localization in whole-body sections of neonatal rats. *J. Pharmacol. Exp. Ther.* **233**:517–526 (1985).
7. Anholt, R. R. H., P. L. Pedersen, E. B. DeSouza, and S. H. Snyder. The peripheral-type benzodiazepine receptor. *J. Biol. Chem.* **261**:576–583 (1986).
8. 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).
9. 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).
10. 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).
11. Jones, D. H., and A. I. Matus. Isolation of synaptic plasma membrane from brain by combined flotation-sedimentation density gradient centrifugation. *Biochim. Biophys. Acta* **356**:276–287 (1974).
12. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
13. Chen, P., S. Toribara, and H. Warner. Microdetermination of phosphorus. *Anal. Chem.* **28**:1756–1758 (1956).
14. Wharton, D. C., and A. Tzagoloff. Cytochrome oxidase of beef heart mitochondria. *Methods Enzymol.* **10**:245–250 (1967).
15. Aronson, N. N., and O. Touster. Isolation of rat liver plasma membrane fragments in isotonic sucrose. *Methods Enzymol.* **31**:90–102 (1974).
16. Trouet, A. Isolation of modified liver lysosomes. *Methods Enzymol.* **31**:323–329 (1974).
17. Munn, E. A. *The Structure of Mitochondria.* Academic Press, New York (1974).
18. Laemlli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
19. Towbin, H. T., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–5354 (1979).
20. Marangos, P. J., J. Patel, J. P. Boulenger, and R. Clark-Rosenberg. Characterization of peripheral-type benzodiazepine binding sites in brain using [^{3}H] Ro 5-4864. *Mol. Pharmacol.* **22**:26–32 (1982).
21. Kataoka, Y., Y. Gutman, A. Guidotti, P. Panula, J. Wroblewski, D. Cosenza-Murphy, J. Y. Wu, and E. Costa. Intrinsic GABAergic system of adrenal chromaffin cells. *Proc. Natl. Acad. Sci. USA* **81**:3218–3222 (1984).
22. Benavides, J., F. Begassat, T. Phan, C. Tur, A. Uzan, C. Renault, M. C. Dubroeucq, C. Gueremy, and G. Le Fur. Histidine modification with diethylpyrocarbonate induces a decrease in the binding of an antagonist, PK 11195, but not of an agonist, Ro 5-4864, of the peripheral benzodiazepine receptors. *Life Sci.* **35**:1249–1256 (1984).

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