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Characterization of Porphyrin Interactions with Peripheral Type Benzodiazepine Receptors

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SUMMARY

We have examined organ and species variations in interactions of porphyrins with peripheral-type benzodiazepine receptors and explored structure-activity requirements for porphyrin-receptor interactions. Whereas potency of the benzodiazepine RO5-4864 varies several orders of magnitude in competing for receptors in different organs and species, effects of porphyrins and the isoquinoline carboxamide PK11195 are relatively constant. The structural requirements determining porphyrin affinity for benzodiazepine binding sites are fairly strict. The most potent porphyrins are those with prominent physiological functions.

Benzodiazepines are thought to exert their antianxiety and other central nervous effects via high affinity binding sites generally referred to as "central type" benzodiazepine receptors. A number of benzodiazepines also bind with high affinity to various peripheral tissues, with structure-activity relationships differing markedly from the central type receptor. This PBR displays nanomolar affinity for diazepam and interacts only weakly with some drugs that are potent at the central type receptor, such as clonazepam and RO15-1788, whereas certain benzodiazepines, such as RO5-4864, are several orders of magnitude more potent at peripheral than at central type receptors (1–4).

The physiological function of PBR has not been established, so that it is not clear whether the binding site is a functional receptor. However, PBR is routinely designated as a "receptor" site in many publications and, for the sake of simplicity, will be designated PBR here. Drugs with selective effects on PBR influence steroidogenesis (5, 6), cardiac function (7, 8), cell growth and differentiation (9–12), chemotaxis (13), protooncogene expression (14), and mitochondrial respiratory control (15). Autoradiographic studies show high densities of these sites discretely localized in endocrine tissues, such as the interstitium of the testes (16), the adrenal cortex (16), the corpus luteum of the ovary (17), and the posterior pituitary (16). Subcellular fractionation studies show that the PBR is highly enriched in the outer membranes of mitochondria (18).

If PBR has a physiological role, one might anticipate the existence of an endogenous modulator. Investigations monitoring interactions of tissue extracts with PBR have revealed that porphyrins are the predominant endogenous species with high affinity for these receptors (19). The major physiological porphyrins, protoporphyrin IX and heme, display K_i values for the receptor of 20–50 nM, whereas they are about 1000 times less potent at central type benzodiazepine receptors (19). In the present study we have characterized in some detail properties of the interactions of porphyrins with PBR.

Materials and Methods

Tissue preparation. Rats (Sprague-Dawley males, 150–175 g), guinea pigs (300–500 g), and rabbits (New Zealand White females; Hazelton, Denver, PA) were sacrificed by decapitation or CO_2 asphyxiation and various organs were dissected immediately. Bovine tissues were obtained from a local slaughterhouse immediately after death. Human placenta was obtained from the Johns Hopkins Hospital Pathology department within 1–2 hr of removal from patients. All tissues were processed similarly by homogenization in 20 volumes (w/v) of Tris·HCl (50 mM, pH 7.7 at 4°), using a Polytron (Brinkmann, Westbury, NY) at speed 8 for 30 sec, and centrifugation at 20,000 × *g* for 10 min. The resulting pellets were washed twice in 50 mM Tris·HCl and stored frozen until required, without any noticeable loss of binding activity.

Subcellular fractionation. Mitochondria were prepared essentially as described previously (20). Sprague-Dawley male rats (150–175 g) were decapitated and the kidneys were removed and minced at 4°. The minced tissue was rinsed twice with ice-cold isolation medium (2 mM HEPES, 70 mM sucrose, 0.22 M D-mannitol, 0.5 mg/ml bovine serum albumin, pH 7.4) and homogenized in 2 volumes (w/v) of isolation medium in a glass homogenizer by four slow up and down strokes with a loose-fitting Teflon pestle with multiple radial serrations. The homogenate was diluted 1:4 with ice-cold isolation medium and centrifuged for 10 min at 660 × *g* at 4°. The resulting pellet was

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ABBREVIATIONS: PBR, peripheral-type benzodiazepine receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

rehomogenized and centrifuged for 10 min at 660 × *g* once more and the supernatants were combined and centrifuged for 15 min at 6800 × *g* at 4°. The pellet was then gently homogenized into a paste and resuspended in 0.5 volume of isolation medium (w/v) based on original wet weight. After centrifugation of this suspension for 15 min at 9770 × *g*, the pellet was homogenized again in 0.25 volume of isolation medium (w/v) based on original minced wet weight. After another centrifugation for 15 min at 9770 × *g*, the resulting mitochondrial pellet was suspended in Tris·HCl (50 mM, pH 7.7) and stored frozen at −70° until required, without any noticeable loss of binding activity over 2 months.

Radioligand binding assays. PBR binding was assayed using [^{3}H]PK11195 (90 Ci/mmol; NEN-DuPont, Boston, MA), isoquinoline carboxamide derivative, and the benzodiazepine [^{3}H]RO5-4864 (87 Ci/mmol; NEN-DuPont). Central type benzodiazepine receptors were assayed with 1 nM [^{3}H]flunitrazepam and rat cerebral cortical membranes. For binding assays, tissue fractions were diluted with 50 mM Tris·HCl buffer, pH 7.7 at 4°, to a concentration of 20–100 μg of protein/ml and incubated with radioligand at the desired concentration for 60 min in a total volume of 0.2 ml at 4°. Assays were terminated by the rapid addition of 3 ml of ice-cold 10 mM Tris·HCl buffer, pH 7.7, followed immediately by vacuum filtration through Schleicher and Schuell no. 32 glass fiber filters, presoaked in 0.3% polyethyleneimine. The filters were washed with 5 ml of ice-cold buffer, extracted in formula 963 scintillation cocktail (NEN-DuPont), and counted in a liquid scintillation counter at 50% efficiency. Nonspecific binding, assessed as binding of radioligand in the presence of the respective unlabeled ligand at 10 μM, was typically less than 10–15% of total binding for all ligands used. Protein concentration was determined using the bicinchoninic acid protein assay reagent (Pierce, Rockville, IL) with bovine serum albumin as standard.

Porphyrin preparation. All porphyrins examined were prepared fresh and used within 6 hr of preparation. Protoporphyrin IX and coproporphyrin isomers were dissolved in 1 ml of 0.1 M Tris base and diluted with 50 mM Tris-HCl (pH 7.7) providing 100 μM–1 mM stock solutions. Other porphyrins were dissolved in a small amount of 0.1 M NaOH and diluted using 50 mM Tris·HCl. Thin layer chromatography analysis of porphyrins (21) was conducted when stability or purity of a compound was in question. All procedures involving porphyrins were carried out in dim lighting.

Materials. Porphyrins were obtained from Porphyrin Products (Logan, UT). All other materials were provided by standard sources. Benzodiazepines [7-chloro-1,3-dihydro-1-methyl-5-(*p*-chlorophenyl)-2H-1,4-benzodiazepine-2-one (RO5-4864) and ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate (RO15-1788) were a kind gift of P. Sorter Hoffman LaRoche (Nutley, NJ), and 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK11195) was a gift of G. Le Fur (Pharmaku, Paris). Amino acid porphyrin conjugates were graciously provided by J. Baumer of Porphyrin Products.

Results

General properties of porphyrin effects on peripheral benzodiazepine sites. Earlier, we showed that porphyrins are substantially more potent in competing for the binding of [^{3}H]PK11195 and [^{3}H]RO5-4864, selective ligands for PBR, than for the binding of [^{3}H]RO15-1788, a centrally selective ligand (19). Although [^{3}H]PK11195 and [^{3}H]RO5-4864 are both used to identify the PBR, recent studies of species variations in drug interactions with PBR suggest that the receptor recognition site for benzodiazepines displays much less conservation than the recognition site for isoquinolines (22). To ascertain whether the recognition site for porphyrins is conserved among different species, we have evaluated the influences of protoporphyrin IX, RO5-4864, and PK11195 at peripheral type receptors in various species (Table 1). In confirmation of the findings of Awad and Gavish (22), observe that RO5-4864 is several orders of magnitude less potent in competing for PBR in bovine, rabbit, and human tissues than in rat or guinea pig tissues. By contrast, the isoquinoline carboxamide PK11195 displays similar potency in all tissues of all species examined. Protoporphyrin IX behaves more like PK11195, with similar potencies in all tissue preparations examined.

Because of their lipophilicity, porphyrins tend to adhere to tissue membranes. Such effects might influence receptor binding interactions. Accordingly, we evaluated the extent to which infinite dilution of rat kidney mitochondria for varying periods of time affects the inhibition of ligand binding by protoporphyrin IX (Fig. 1). Inhibition of binding is slowly reversible. Thirty minutes after dilution, the extent of inhibition of [^{3}H]PK11195 binding by protoporphyrin IX is not changed. After 60 min, binding has recovered to about 45% of initial levels. Even after 2 hr, binding has returned only to about 85% of control values.

Equilibrium and kinetic properties of porphyrin effects on PBR binding. To explore the nature of the interactions of porphyrins with PBR, we evaluated the saturation of [^{3}H]PK11195 binding to rat kidney mitochondria in the presence or absence of 100 nM protoporphyrin IX (Fig. 2). In the absence of porphyrins, [^{3}H]PK11195 binding is monophasic in a Scatchard analysis, with a K_D of 3 nM and a B_{max} of 13 pmol/mg of protein. In the presence of protoporphyrin IX, the Scatchard plot remains linear with no change in the B_{max} but with a substantial increase in K_D, suggesting competitive inhibition. Similar results are obtained using [^{3}H]RO5-4864 as a ligand (data not shown).

Although the Scatchard analysis suggests competitive interactions between porphyrins and [^{3}H]PK11195 at the receptor, these experiments do not rule out the possibility of negatively cooperative interactions. Accordingly, we examined the dissociation of [^{3}H]PK11195 from rat kidney mitochondria with dissociation initiated by infinite dilution or by 1 μM concentrations of RO5-4864, PK11195, or protoporphyrin IX (Fig. 3).

TABLE 1

[^{3}H]PK11195 displacement in membranes from tissues of various species

Specific binding of 1 nM [^{3}H]PK11195 was detected in various tissue of rats, guinea pigs, rabbits, cows, and humans as described in Methods and Materials. Tissue membranes were incubated for 60 min at 4° with at least 10 different concentrations of inhibitor as described. Nonspecific binding was <10% of the total binding in all preparations tested. Data are mean ± standard error values from three separate experiments.

Species/Tissue	IC_{50}		
	PK11195	RO54864	Protophorphyrin IX
		nM	
Rat			
Brain	1.5 ± 0.1	8.0 ± 0.5	25 ± 7.1
Heart	2.0 ± 0.2	10 ± 1.2	22 ± 10.1
Kidney	1.5 ± 0.4	6.0 ± 0.7	27 ± 5.6
Guinea Pig			
Brain	2.5 ± 0.7	10 ± 1.5	30 ± 10
Rabbit			
Kidney	2.1 ± 0.3	483 ± 62	14 ± 5.4
Bovine			
Cerebral Cortex	1.7 ± 0.4	800 ± 141	26 ± 14.3
Heart	2.0 ± 0.4	960 ± 230	17 ± 2.5
Human			
Placenta	3.1 ± 1.5	262 ± 98	30 ± 3.1

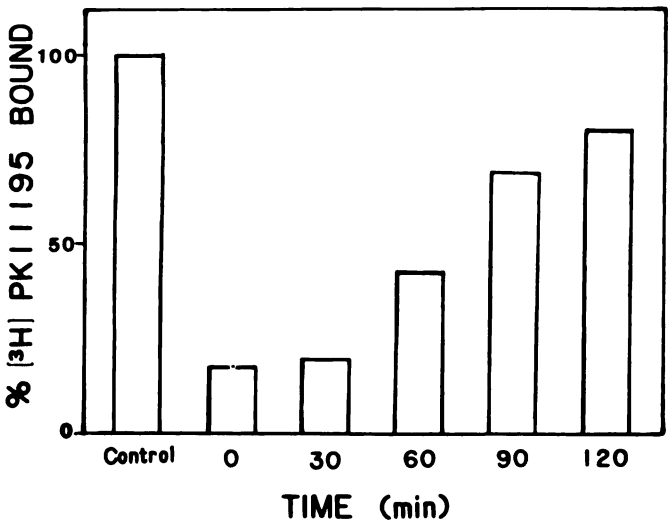

Fig. 1. Dissociation of protoporphyrin IX from rat kidney mitochondrial membranes. Rat kidney mitochondrial membranes were incubated for 1 hr with 1 μM protoporphyrin IX. The reaction mixtures were diluted 1000 times with 50 mM Tris·HCl buffer (pH 8.0) and the membranes were pelleted at different times at 30,000 × *g* for 10 min. The pellets were resuspended and analyzed for specific binding of 1 nM [^{3}H]PK11195 as described in Methods and Materials. The times given in the figure refer to the time between dilution of the reaction mixture and pelleting of the membranes. This experiment was repeated twice with similar results. Control binding remained constant over the duration of the experiment.

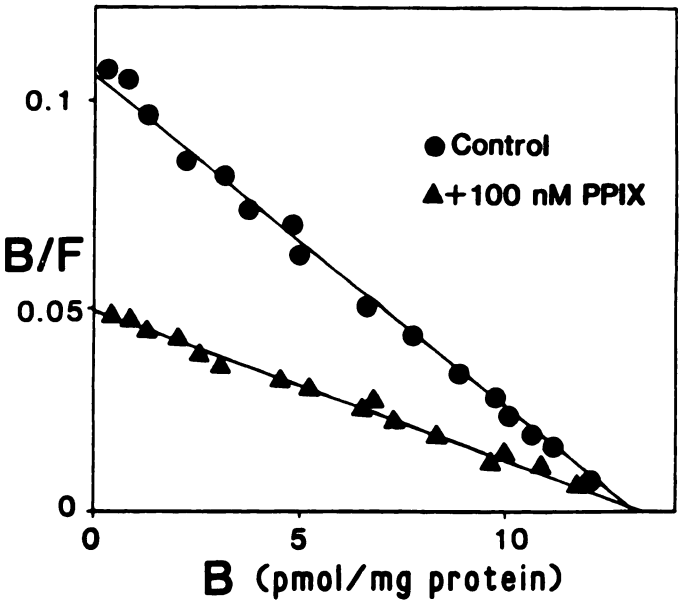

Fig. 2. Scatchard analysis of [^{3}H]PK11195 binding to rat kidney mitochondria in the presence and absence of protoporphyrin IX (*PPIX*). Rat kidney mitochondria were incubated for 90 min in the presence of 0.05–30 nM [^{3}H]PK11195 with and without the addition of 100 nM protoporphyrin IX as described in Methods and Materials. Nonspecific binding was less than 10% of total binding.

The pattern and rate of dissociation appear essentially the same under all experimental conditions. Dissociation is monophasic, with a $t_{1/2}$ for dissociation of about 60 min. These observations provide no support for cooperative interactions between protoporphyrin IX, PK11195, or RO5-4864 and the receptor.

Structure-activity analysis of porphyrin interactions with PBR. In our earlier study we observed substantial differences in potencies at PBR with the limited number of porphyrins examined. In the present study we have explored in considerable detail the influence of different porphyrins.

Porphyrin biosynthesis proceeds by the fusion of two molecules of δ-aminolevulinic acid to form the pyrrole porphobilinogen, with four pyrroles than cyclized to form a tetrapyrrole ring. The initial tetrapyrrole, designated uroporphyrinogen (the reduced form of the corresponding porphyrin), contains eight charged carboxylates, which are sequentially lost through the formation of coproporphyrinogen to the final product protoporphyrin IX, which contains only two carboxyl groups. The extent of carboxylation markedly influences affinity for PBR (Fig. 4; Table 2). δ-Aminolevulinic acid, porphobilinogen, and uroporphyrin fail to influence PBR even at 1 mM concentration. Coproporphyrin III, formed from uroporphyrin and the immediate precursor of protoporphyrin IX, displays an IC_{50} of 2–4 μM, whereas coproporphyrin I is inactive at 100 μM. The transformation of two propionyl groups to vinyl groups in the conversion of coproporphyrin III to protoporphyrin IX results in an increase of several 100-fold in potency.

Metal substitution also influences affinity for PBR. Replacing the iron of hemin with zinc results in an 8–10-fold reduction in potency whereas tin and cobalt replacement produces derivatives about 100 times less potent than the zinc-containing species.

To ascertain whether the varying effects of metals on potency could derive from any substituent in the pyrrolic nitrogen, we evaluated *N*-methyl-protoporphyrin IX, which can be formed *in vivo* under certain experimental conditions (23). This compound is only modestly less potent than hemin.

Because of the apparent importance of the vinyl group at positions 2 and 4 of protoporphyrin IX in determining effects on PBR, we evaluated a variety of porphyrins with other substituents in this position (Fig. 4; Table 3). Mesoporphyrin IX and deuteroporphyrin IX are naturally occurring porphyrins that, respectively, possess ethyl and hydrogen substitutions at positions 2 and 4. These relatively hydrophobic substitutions preserve affinity for the receptor, inasmuch as these two compounds are one third to one half as potent as protoporphyrin IX. Hematoporphyrin IX, with hydroxyethyl groups of positions 2 and 4, is not naturally occurring and is about 8% as potent as protoporphyrin IX. Apparently, the polarity of the hydroxyl addition to the ethyl group causes hematoporphyrin IX to lose potency in comparison with mesoporphyrin IX, in which the two ethyl groups are unmodified. Hydroxyethyl vinyl deuteroporphyrin IX, a derivative which possesses one vinyl and one hydroxyethyl group at the 2- and 4-positions, is intermediate in potency between hematoporphyrin IX and mesoporphyrin IX. Vinyl hydroxymethyl deuteroporphyrin IX differs from hydroxyethyl vinyl deuteroporphyrin IX in that a more polar hydroxymethyl substituent occurs at position 4 instead of the less polar hydroxyethyl. Vinyl hydroxymethyl deuteroporphyrin IX is somewhat less potent than hematoporphyrin and 3–10 times less potent than HVD. Incorporation of polar sulfonates or bisglycol groups at positions 2 and 4 abolishes affinity for the receptor.

Porphyrin derivatives find clinical utility in the treatment of porphyria and various cancers (24–26), but their poor water solubility hinders efficacy. To improve water solubility, derivatives of protoporphyrin IX have been prepared with amino acids at the 6- and 7-positions, which normally contain the

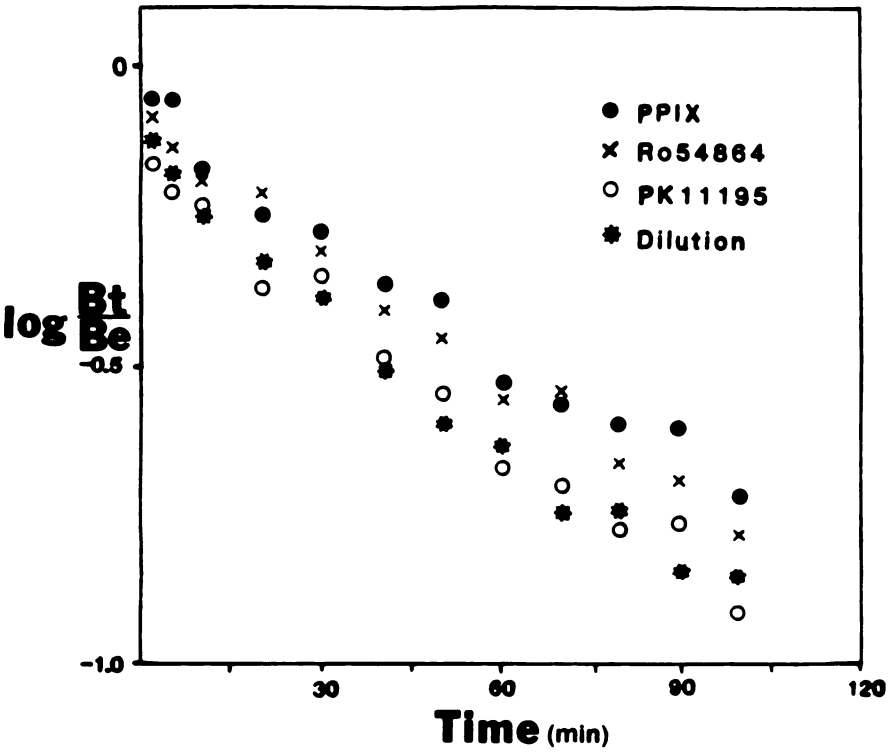

Fig. 3. Dissociation of [^{3}H]PK11195 from rat kidney mitochondrial membranes. Rat kidney mitochondria were incubated with 1 nM [^{3}H]PK11195 for 60 min, at which time dissociation was initiated by the addition of 1 μM PK11195, 1 μM RO5-4864, 1 μM protoporphyrin IX, or infinite dilution of the reaction mixture with 50 mM Tris·HCl buffer (pH 8.0. B_e, amount of binding at equilibrium; B_t, amount of binding at time t after dilution.

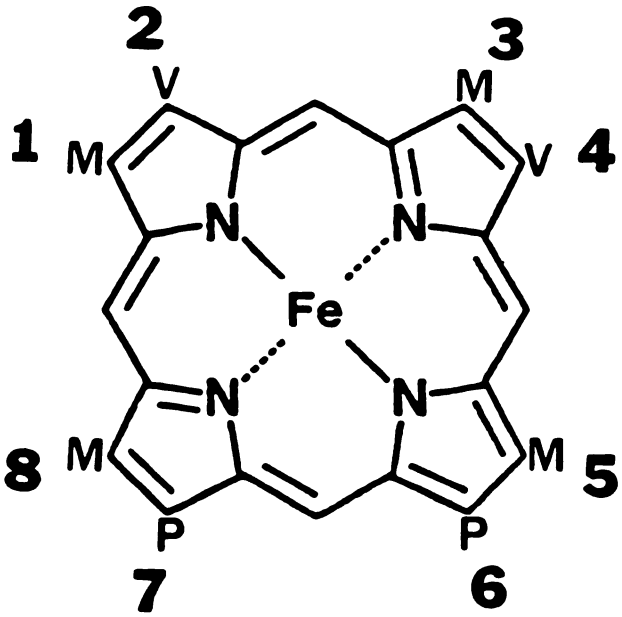

Fig. 4. The structure of heme. The standard numbering scheme for positions on the porphyrin ring is indicated and is to be used with Tables 2 and 3. Metalloporphyrins such as the one depicted have a metal positioned within the tetrapyrrolic structure. *M*, methyl; *P*, propionyl; *V*, vinyl; *Fe*, iron.

charged propionate groups (Fig. 4; Table 3). None of these derivatives are as potent as protoporphyrin IX, but several of them display substantial affinity. The most potent derivative possesses aspartates at position 6 and 7, whereas derivatives with ε-aminocaproate, serine, and taurine are one fourth to one half as potent as the aspartate derivative. Glycine, alanine, and glutamate derivatives are progressively less active.

The physiological degradation of porphyrins commences with the cleavage of the porphyrin ring between the 2- and 3-positions, giving rise to biliverdin. Reduction of the 4-5 double bond of biliverdin gives rise to bilirubin. Biliverdin displays affinity for PBR in the low micromolar range, whereas bilirubin is 100-fold less potent. Mesobilirubin and stercobilin, fecal metabolites of bilirubin, display affinities between those of biliverdin and bilirubin.

Discussion

The major finding of this study is that porphyrin interactions with PBR are selective as well as potent. The pattern of interactions observed here is consistent with a physiological association of porphyrins and PBR. In a wide variety of species and tissues, affinities of porphyrins and the isoquinoline carboxamide PK11195 are relatively conserved, whereas affinities of the benzodiazepine RO5-4864 vary over several orders of magnitude. Thus, the benzodiazepine recognition site of PBR is an inconstant feature of the receptor, whereas conservation of the porphyrin recognition site implies a physiological role. The results also suggest that PK11195 and porphyrins interact with the same site. This accords with our earlier observations that porphyrins are the major endogenous substances detected that interact potently with the receptor (19). Moreover, endogenous concentrations of porphyrins are quite ample to provide substantial occupancy of the receptor under normal conditions (27). Fluctuations in porphyrin levels are known to noncovalently modulate enzymatic activity of tryptophan pyrrolase (28), guanylate cyclase (29), and glutathione-*S*-transferase (30) and could similarly regulate PBR activity.

The involvement of porphyrins with several mitochondrial proteins, and the role of mitochondria in porphyrinogenesis also coincide with the mitochondrial localization of PBR. Recently RO5-4864 and PK11195 were both shown to potently decrease respiratory control in isolated mitochondria (15). This

TABLE 2

Structural requirements of porphyrin precursors and metalloporphyrins for inhibition of [^{3}H]R05-4864 and [^{3}H]PK11195 binding

The specific binding of [^{3}H]PK11195 and [^{3}H]R05-4864 to rat kidney mitochondria was assayed as described in Methods and Materials. Porphyrins were prepared fresh as described and incubated with membranes in dim lighting in the presence of ^{3}H-ligand. Nonspecific binding was <10% for both ligands. K_i values are means of at least three independent determinations, which varied less than 20%. The positions indicated refer to Fig. 5. A, acetyl; P, propionyl; M, methyl; V, vinyl.

Compound	Position								Metal	K_i	
	1	2	3	4	5	6	7	8		[^{3}H]PK11195	[^{3}H]R05-4864
										nM	
δ-Amino levulinic acid										>1 mM	>1 mM
Porphobilinogen										>0 mM	>1 mM
Uroporphyrin I	A	P	A	P	A	P	A	P		>100,000	>100,000
Uroporphyrin II	A	P	A	P	A	P	P	A		>100,000	>100,000
Heptaporphyrin I	A	P	A	P	A	P	M	P		>100,000	>100,000
Hexaporphyrin I	A	P	M	P	A	P	M	P		24,000	40,000
Pentaporphyrin I	M	P	M	P	A	P	M	P		>100,000	>100,000
Coproporphyrin I	M	P	M	P	M	P	M	P		100,000	100,000
Coproporphyrin III	M	P	M	P	M	P	P	M		2,500	3,400
Protoporphyrin IX	M	V	M	V	M	P	P	M		20	15
Hemin	M	V	M	V	M	P	P	M	Iron	50	65
Zn-Protoporphyrin IX	M	V	M	V	M	P	P	M	Zinc	400	500
Sn-Protoporphyrin IX	M	V	M	V	M	P	P	M	Tin	3,267	6,000
Co-Protoporphyrin IX	M	V	M	V	M	P	P	M	Cobalt	3,700	5,500
N-methyl Protoporphyrin IX	M	V	M	V	M	P	P	M	Methyl	95	150

TABLE 3

Inhibition of [^{3}H]PK11195 and [^{3}H]R05-4864 binding by substituted porphyrins

The binding of [^{3}H]PK11195 and [^{3}H]R05-4864 was assayed as described in Methods and Meterials. Porphyrins were prepared fresh as described. K_i values are means of at least three independent determinations, which varied less than 20%. A, acetyl; P, propionic; M, methyl; E, ethyl; H, hydrogen; V, vinyl; OHM, hydroxymethyl; OHE, hydroxyethyl; SO_3, sulfonate; BG, bisglycol; ACA, aminocaprylate. Amino acid constituents are denoted by their three letter code. 2,4-(4,2)Hydroxyethyl-vinyl-deuteroporphyrin IX (HVD) is an isomer mixture. Tetrapyrrole metabolites follow a different position-number scheme than porphyrins.

Compound	Position								K_i	
	1	2	3	4	5	6	7	8	[^{3}H]PK11195	[^{3}H]R05-4864
									nM	
Protoporphyrin IX	M	V	M	V	M	P	P	M	20	15
Mesoporphyrin IX	M	E	M	E	M	P	P	M	46	49
Deuteroporphyrin IX	M	H	M	H	M	P	P	M	59	46
2-Vinyl,4-hydroxymethyl deuteroporphyrin IX	M	V	M	OHM	M	P	P	M	900	620
2,4(4,2)-Hydroxyethyl vinyl deuteroporphyrin IX	M	OHE(V)	M	V(OHE)	M	P	P	M	90	210
Deuteroporphyrin IX disulfonate	M	SO_3	M	SO_3	M	P	P	M	>100,000	>100,000
Deuteroporphyrin IX bisglycol	M	BG	M	BG	M	P	P	M	>100,000	>100,000
Diaspartyl deuteroporphyrin IX	M	V	M	V	M	Asp	Asp	M	545	575
Di-ε-Amino capryl protoporphyrin IX	M	V	M	V	M	ACA	ACA	M	1,000	4,000
Diserinyl protoporphyrin IX	M	V	M	V	M	Ser	Ser	M	2,000	5,500
Ditauryl protoporphyrin IX	M	V	M	V	M	Taur	Taur	M	1,000	6,000
Diglycyl protoporphyrin IX	M	V	M	V	M	Gly	Gly	M	3,200	6,000
Diglutamyl protoporphyrin IX	M	V	M	V	M	Glu	Glu	M	7,600	20,000
Dialanyl protoporphyrin IX	M	V	M	V	M	Ala	Ala	M	14,000	5,000
Biliverdin									3,150	1,500
Bilirubin									100,000	100,000
Mesobilirubin									24,500	30,000
Stercobilin									19,000	22,000

effect was also seen with mesoporphyrin IX and deuteroporphyrin IX but not with clonazepam, and the relative potencies of these agents reflected their affinities for PBR (15). Regulation of mitochondrial function by PBR ligands may underlie the wide spectrum of actions produced by benzodiazepines and porphyrins (5–15, 28–32).

Structure-activity analysis further supports a physiological interaction of porphyrins with PBR. The most potent are dicarboxylic porphyrins, with major roles in normal physiology. Structure-activity relationships of porphyrins for regulation of guanylate cyclase (29), protein kinase and glutathione-*S*-transferase activity (30), neurotoxicity *in vitro* (32), and photodynamic destruction of cultured tumor cells[1] (25) resemble the structure-activity relationships observed here.

Acknowledgments

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[1] Unpublished observations.

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