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INTERACTIONS OF LIPIDS WITH PERIPHERAL-TYPE BENZODIAZEPINE RECEPTORS

KEVIN BEAUMONT,* ROMAN SKOWRONSKI, DUKE A. VAUGHN and DARRELL D. FANESTIL

Department of Medicine, Division of Nephrology, University of California, San Diego, La Jolla, CA 92093, U.S.A.

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Abstract—Peripheral-type benzodiazepine receptors (PBRs) are present at high densities in the rat kidney distal tubule. [^{3}H]RO 5-4864 binding to PBRs in kidney membranes is inhibited by several unidentified low molecular weight hydrophobic compounds in urine and serum. We tested representative hydrophobic compounds from several lipid classes for ability to inhibit binding to rat kidney PBRs of two high affinity ligands, [^{3}H]RO 5-4864 and [^{3}H]PK 11195. Unsaturated fatty acids and alcohols inhibited [^{3}H]RO 5-4864 binding with half-maximal inhibition occurring at 3×10^{-6} M to 10^{-4} M. Inhibitory potency increased with the degree of unsaturation. Phospholipids inhibited [^{3}H]RO 5-4864 in the same concentration range, with inhibitory potency in this case dependent both upon an unsaturated fatty acid moiety and upon the polar head group. Phosphatidylethanolamine was the most potent phospholipid tested ($IC_{50} = 2 \times 10^{-6}$ M), whereas phosphatidylcholine was not inhibitory. Although phospholipids inhibited both [^{3}H]RO 5-4864 and [^{3}H]PK 11195 binding equally, unsaturated fatty acids had a much greater inhibitory effect upon [^{3}H]RO 5-4864 than upon [^{3}H]PK 11195 binding. Similar effects were obtained with digitonin-solubilized PBRs. These data demonstrate that in our experiments PBR binding was inhibited by specific lipids and that binding of proposed agonist (RO 5-4864) and antagonist (PK 11195) ligands was differentially affected by unsaturated fatty acids.

Benzodiazepines (BZDs) are widely used for their anxiolytic, anticonvulsant, muscle relaxant, and sedative properties. These properties are mediated by central-type benzodiazepine receptors (CBRs) in the brain, which bind the BZDs with a specificity paralleling their ability to produce behavioral effects [1, 2]. Benzodiazepines also bind with high affinity to another class of sites first identified in peripheral tissues, such as the kidney [3], and therefore are designated as peripheral-type benzodiazepine receptors (PBRs).

Though their distribution is widespread throughout the organism, PBRs are localized within tissues to specific sites, such as the distal nephron in the kidney [4, 5], the cortex in the adrenal gland [6, 7], and the choroid plexus, ependyma and afferent olfactory nerves in the brain [8, 9]. Anholt *et al.* [10] have shown that the distribution of PBRs determined autoradiographically in neonatal rats parallels the distribution of cytochrome *c* oxidase activity, suggesting a potential role in modulation of oxidative energy metabolism. The mitochondrial localization of PBRs in the adrenal cortex [11], brain [12] and kidney [13] would support such a role.

Benzodiazepines that have high affinity for PBRs increase phospholipid methylation in rat glioma cells [14], alter the rate of repolarization of guinea pig papillary muscle [15], inhibit mouse thymoma proliferation [16], elicit humoral immune responses in rats [17], and stimulate human monocyte chemotaxis [18]. Benzodiazepines also induce differentiated functions in mouse melanoma cells [19] and Friend erythroleukemia cells [20] and inhibit neurite outgrowth and induce ornithine decarboxylase activity in rat PC 12 cells [21]. However, Morgan *et al.* [21] have demonstrated that the pharmacological characteristics of some of these actions of benzodiazepines, such as induction of cellular differentiation, are not consistent with mediation by PBRs. The mechanisms by which benzodiazepines produce these diverse effects, and which of these are indeed mediated by PBRs, have not been determined.

PBRs have been measured by the binding of the benzodiazepine [^{3}H]RO 5-4864 [22, 23] and of [^{3}H]PK 11195, an isoquinoline derivative [24]. PK 11195 was initially proposed to be an antagonist of PBRs because of the thermodynamic characteristics of its binding, which differed from those of RO 5-4864 in being entropy-driven rather than enthalpy-driven [24]. Physiological studies have provided some support for the proposed antagonist nature of PK 11195, since PK 11195 blocks effects of RO 5-4864 on repolarization of guinea pig papillary muscle [15] and Ca^{2+} flux in a pituitary cell line [25, 26].

Urine and serum contain competitive inhibitors of [^{3}H]RO 5-4864 binding to PBRs [27]. This inhibitory activity is relatively hydrophobic and is much more potent at inhibiting PBRs than CBRs or several other receptors. Methanol extracts of several rat tissues also contain high and low molecular weight inhibitors of PBRs [28]. While further characterizing the inhibitory material present in urine and serum, we found that certain lipids inhibit PBRs with relatively high potency. We report here upon the relative ability of lipids of different classes to inhibit PBRs and upon

* Correspondence: K. Beaumont, Ph.D., Department of Medicine, M-023B, University of California, San Diego, La Jolla, CA 92093.

the finding that unsaturated fatty acids and alcohols differentially affect the binding of putative agonist and antagonist PBR ligands.

MATERIALS AND METHODS

The binding of [^{3}H]RO 5-4864 [22, 23] and [^{3}H]PK 11195 [24] to rat kidney membranes was measured by filtration assay. Whole kidneys (capsules removed) from male Sprague–Dawley rats were homogenized with a Polytron in cold 50 mM sodium HEPES* buffer and washed three times by centrifuging (20 min at 40,000 *g*) and resuspending in fresh buffer. Membranes from 2 mg original wet weight of kidney were incubated in duplicate tubes for 1 hr at 4° with 0.75 nM [^{3}H]RO 5-4864 or 0.5 nM [^{3}H]PK 11195 and drugs or lipids. Incubations contained 1% ethanol used to solubilize lipids. Lipids were dissolved in ethanol, used immediately, and then stored under a nitrogen atmosphere at −20° for retesting. Nonspecific binding was determined in the presence of 10^{-5} M diazepam. Following incubation, membranes were filtered through glass fiber filters, washed with 15 ml of cold saline, and assayed for radioactivity by liquid scintillation spectrometry. [^{3}H]Flunitrazepam binding to rat brain membranes was measured as previously described [27, 29].

PBRs were solubilized from rat kidney mitochondria with digitonin [30, 31]. Mitochondria from kidney cortex and outer medulla were prepared and suspended in cold 0.25 M sucrose, 5 mM Tris Cl (pH 7.4) by the method of Johnson and Lardy [32]. An equal volume of a freshly prepared 1% digitonin solution in isolation buffer was added rapidly to isolated mitochondria at a protein concentration of 10 mg/ml. The suspension was incubated for 60 min at 4° on an end-over-end shaker and then centrifuged for 30 min at 150,000 *g*. The supernatant fraction containing solubilized receptors was used for binding assays following dilution in 50 mM Tris Cl, pH 7.4, buffer to reduce digitonin to a concentration (less than 0.03%) that did not inhibit binding. Following incubation of solubilized receptors with radiolabeled ligands and drugs under the same conditions used for membrane receptors, solutions were filtered through 0.3% polyethyleneimine-treated glass fiber filters, which were then washed three times with 5 ml of cold phosphate-buffered saline (PBS) and assayed by liquid scintillation spectrometry.

[^{3}H]RO 5-4864 (74.9 Ci/mmol), [^{3}H]PK 11195 (85 Ci/mmol) and [^{3}H]flunitrazepam (92.3 Ci/mmol) were from New England Nuclear, (Boston, MA). Unlabeled RO 5-4864 and diazepam were donated by Hoffmann-La Roche (Nutley, NJ). Unlabeled PK 11195 was donated by Pharmuka (Gennevilliers, France). Lipids were from Sigma (St. Louis, MO). All pure phospholipids are the L-α-isomers.

RESULTS

Several classes of lipids were tested for their abilities to inhibit [^{3}H]RO 5-4864 binding to kidney membranes. Saturated fatty acids of intermediate chain length (C14–C18) were weakly inhibitory at concentrations of 10^{-4} M (Table 1). Saturated fatty acids with shorter or longer chain lengths produced little or no inhibition. Unsaturated fatty acids were considerably more potent inhibitors of [^{3}H]RO 5-4864 binding. Inhibitory potency increased with increasing number of unsaturated bonds. Docosohexaenoic acid, arachidonic acid, and octadecatetraenoic acid inhibited [^{3}H]RO 5-4864 binding by more than 50% at 10^{-5} M. Unsaturated fatty acids with *cis*-configurations were somewhat more potent inhibitors than their *trans*-isomers (Table 1). Unsaturated fatty alcohols tested were nearly as potent (linoleyl alcohol) or approximately equal in potency (docosohexaenol) to the corresponding unsaturated fatty acids (Table 1). The analogous C18 fatty alcohol (stearyl) and alkane (octadecane) were not inhibitory (Table 1), indicating that inhibition by fatty alcohols was also related to the presence of unsaturated bonds. Cholesterol produced less inhibition than unsaturated fatty acids and alcohols (Table 1).

Several mono-, di- and triglycerides and phospholipids containing C16 to C20 fatty acyl groups were assayed for their inhibitory potencies (Table 2). Monoglycerides had an inhibitory potency equal to or greater than their fatty acid component. Mono-, di- and triglycerides containing only saturated fatty acyl groups were not inhibitory. Diglycerides and triglycerides were considerably less potent than their

Table 1. Inhibition of [^{3}H]RO 5-4864 binding to kidney membranes by fatty acids, alcohols, and cholesterol

Compound	% Inhibition	
	10^{-5} M	10^{-4} M
Caproic (C6-0)		0
Caprylic (C8-0)		3.2
Capric (C10-0)		4.4
Myristic (C14-0)		31.2
Palmitic (C16-0)		29.2
Stearic (C18-0)		21.9
Arachidic (C20-0)		11.0
Behenic (C22-0)		14.8
Lignoceric (C24-0)		1.8
Palmitelaidic (C16-1, *trans*-9)	34.3	76.1
Palmitoleic (C16-1, *cis*-9)	34.0	89.9
Elaidic (C18-1, *trans*-9)	11.4	42.3
Oleic (C18-1, *cis*-9)	38.5	90.2
Petroselinic (C18-1, *cis*-6)	18.7	64.4
Linoleic (C18-2, *cis*-9,12)	46.5	94.9
Linolenic (C18-3)	40.9	92.3
Octadecatetraenoic (C18-4)	70.0	100.0
Arachidonic (C20-4)	52.1	97.9
Erucic (C22-1, *cis*-13)	19.2	52.6
Docosohexaenoic (C22-6)	68.5	98.5
Nervonic (C24-1, *cis*-15)	4.0	7.5
Octadecane	0.9	5.2
Stearyl alcohol	0	2.3
Linoleyl Alcohol	23.8	76.2
Docosohexaenol	72.9	99.8
Cholesterol	6.6	31.3

Binding of [^{3}H]RO 5-4864 in the presence of lipids at the indicated concentrations was measured as described in Materials and Methods. Values represent percent inhibition of specific binding and are means of two experiments which varied by an average of 4%.

* HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Table 2. Inhibition by [^{3}H]RO 5-4864 binding to kidney membranes by glycerides and phospholipids

Compound	% Inhibition	
	10^{-5} M	10^{-4} M
Monoelaidin	37.8	53.6
Mono-oleoyl-rac-glycerol	40.8	85.1
Mono-stearyl-rac-glycerol	0	0
Distearin	2.9	0
1,3-Dioleyn	0.4	6.0
1,3-Dielaidin	1.1	12.3
1,2-Dioleoyl-rac-glycerol	5.6	19.0
Triolein	2.3	2.9
Trielaidin	1.7	31.0
Tristearin	0	0
Triarachidonin	72.5	94.0
Phosphatidic acid, dioleyl	18.3	61.2
Phosphatidylcholine, dioleoyl	2.4	3.8
Phosphatidylcholine, β-oleoyl, γ-palmitoyl	0	0
Phosphatidylethanolamine, dipalmitoyl	1.0	
Phosphatidylethanolamine, β-oleoyl,γ-palmitoyl	78.3	93.1
Phosphatidylserine, dipalmitoyl	0	
Phosphatidylinositol (soybean)	36.0	
Cardiolipin (bovine heart)	57.0	

Binding of [^{3}H]RO 5-4864 was measured in the presence of lipids at the indicated concentrations. Values represent percent inhibition of specific binding and are means of 2 to 3 experiments which varied by an average of 5%.

component fatty acids, with the exception of triarachidonin. The inhibitory potency of phospholipids was dependent upon both their head group and fatty acyl groups. Phosphatidylserine, -ethanolamine, and -choline species containing only saturated fatty acids (stearyl or palmitoyl) did not inhibit [^{3}H]RO 5-4864 binding. Phosphatidylethanolamine containing an unsaturated fatty acid at the 2-position (β-oleoyl, γ-palmitoyl) produced 70% inhibition of [^{3}H]RO 5-4864 binding at 10^{-5} M while the analogous phosphatidylcholine species (β-oleoyl, γ-palmitoyl) was not inhibitory (Table 2). Cardiolipin (from bovine heart) and phosphatidylinositol (from soybean) were somewhat weaker inhibitors than phosphatidylethanolamine, inhibiting [^{3}H]RO 5-4864 binding by 57 and 36%, respectively, at 10^{-5} M.

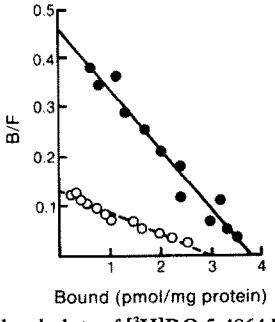

Fig. 1. Scatchard plots of [^{3}H]RO 5-4864 binding to kidney membranes in the presence and absence of 5×10^{-6} M phosphatidylethanolamine (β-oleoyl, γ-palmitoyl). Data are from a representative experiment. Key (●) control (mean ± SEM, N = 3): $B_{max} = 3.26 \pm 0.36$ pmol/mg protein, $K_D = 1.34 \pm 0.31$ nM; and (○) with phosphatidylethanolamine (N = 3): $B_{max} = 2.61 \pm 0.31$ pmol/mg protein, $K_D = 3.78 \pm 0.71$ nM.

Scatchard analysis of [^{3}H]RO 5-4864 binding in the presence of phosphatidylethanolamine or arachidonic acid indicated that these lipids produced a mixed-type inhibition of binding. Both apparent receptor density and affinity were decreased. In the presence of 5×10^{-6} M phosphatidylethanolamine (Fig. 1), the B_{max} was reduced significantly from 3.3 ± 0.4 to 2.6 ± 0.3 pmol/mg protein ($P < 0.05$, N = 3), while the K_D was increased significantly from 1.3 ± 0.3 to 3.8 ± 0.7 nM ($P < 0.05$, N = 3). Arachidonic acid had a similar effect of reducing both apparent receptor density and affinity (data not shown).

Examples of the several lipid classes were also tested for their abilities to inhibit the binding of [^{3}H]PK 11195 binding to PBRs in kidney membranes. Phospholipids were equipotent at inhibiting binding of [^{3}H]RO 5-4864 and [^{3}H]PK 11195 (Table 3). However, fatty acids and alcohols were considerably less potent at inhibition [^{3}H]PK 11195 than [^{3}H]RO 5-4864 binding (Table 3). PK 11195, RO 5-4864 and phosphatidylethanolamine produced nearly identical competition curves with either [^{3}H]RO 5-4864 or [^{3}H]PK 11195 as radioligand, whereas arachidonic acid was almost 50-fold weaker as an inhibitor of [^{3}H]PK 11195 than of [^{3}H]RO 5-4864 (Fig. 2).

The abilities of arachidonate and phosphatidylethanolamine to inhibit binding to digitonin-solubilized PBRs were tested. Affinities for [^{3}H]PK 11195 ($K_D = 1.7$ nM, $B_{max} = 3.4$ pmol/mg protein) and [^{3}H]RO 5-4864 ($K_D = 7.6$ nM, $B_{max} = 1.6$ pmol/mg protein) binding to solubilized PBRs were similar to their affinities for intact membranes. Phosphatidylethanolamine inhibited binding of both [^{3}H]RO 5-4864 and [^{3}H]PK 11195 to solubilized PBRs with IC_{50} values of $1–3 \times 10^{-6}$ M (Fig. 3). Arachidonic acid

Table 3. Inhibition of [^{3}H]PK 11195 binding to kidney membranes by lipids

Compound	Concn (M)	% Inhibition [^{3}H]PK 11195	% Inhibition [^{3}H]RO 5-4864
Myristic acid	10^{-4}	12.4	31.2
Stearic acid	10^{-4}	7.3	21.9
Oleic acid	10^{-4}	0	90.2
Linoleic acid	10^{-4}	17.9	94.9
Arachidonic acid	10^{-4}	13.5	97.9
Docosohexaenoic acid	10^{-4}	25.6	98.5
Linoleyl alcohol	10^{-4}	24.1	76.2
Docosohexaenol	10^{-4}	80.2	99.8
Phosphatidylcholine, dioleoyl	10^{-5}	5.3	2.4
Phosphatidylethanolamine β-oleoyl, γ-palmitoyl	10^{-5}	82.1	78.3
Phosphatidyl inositol	10^{-5}	36.5	36.0
Cardiolipin	10^{-5}	56.5	57.0

Values represent percent inhibition of specific [^{3}H]PK 11195 or [^{3}H]RO 5-4864 binding measured as described in Materials and Methods. Values are the means of two experiments which varied by an average of 5%. Values for inhibition of [^{3}H]RO 5-4864 are from Tables 1 and 2.

inhibited the binding of [^{3}H]RO 5-4864 to solubilized PBRs with an IC_{50} of 4×10^{-6} M. However, specific [^{3}H]PK 11195 binding to solubilized PBRs was increased in the presence of the same concentrations of arachidonic acid that inhibited [^{3}H]RO 5-4864 binding (Fig. 4). Thus, the differential effects of these lipids upon PBRs in membranes also occur with solubliized PBRs, with arachidonic acid again having a distinctly different effect upon the binding of the two ligands.

The effects of oxidizing and reducing agents were studied to determine the possible contribution of membrane lipid oxidation to the inhibition of PBR binding. Vitamin E (0.1%) reduced [^{3}H]PK 11195 binding by 17.3% but did not alter [^{3}H]RO 5-4864 binding. Butylated hydroxytoluene (0.01%) reduced [^{3}H]PK 11195 binding by 32.9% and [^{3}H]RO 5-4864 binding by 8.9%. The binding of neither ligand was affected by 0.03% hydrogen peroxide, 0.1% ascorbic acid, or 0.1% β-mercaptoethanol. Ferrous sulfate (10^{-3} M) and ferric nitrate (10^{-3} M), agents which promote lipid peroxidation, did not affect [^{3}H]RO 5-4864 binding but decreased [^{3}H]PK 11195 binding by 12.3 and 37.9% respectively. Thus, lipid oxidizing and reducing agents had virtually no effect upon the binding of [^{3}H]RO 5-4864 and slightly inhibited [^{3}H]PK 11195 binding. Therefore, the effects of lipids upon PBR binding were not due to alterations in lipid peroxidation. Cardiolipin and phosphatidylethanolamine at 100 μg/ml did not inhibit signifi-

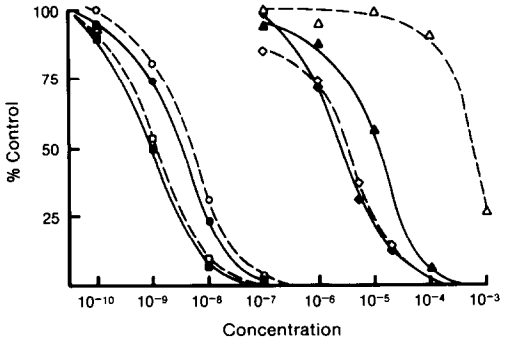

Fig. 2. Inhibition of [^{3}H]RO 5-4864 and [^{3}H]PK 11195 binding to PBRs in kidney membranes by drugs and lipids. Values represent the percent of control specific binding obtained in the presence of the following compounds: (■, □) = PK 11195; (●, ○) = RO 5-4864; (▲, △) = arachidonic acid; (◆, ◇) = phosphatidylethanolamine (β-oleoyl, γ-palmitoyl). Closed symbols represent binding of [^{3}H]RO 5-4864. Open symbols represent binding of [^{3}H]PK 11195. Control values were as follows: [^{3}H]PK 11195, 56.5 ± 4.4 fmol/mg protein; [^{3}H]RO 5-4864, 89.1 ± 5.1 fmol/mg protein (means ± SEM, N = 4 from a single experiment).

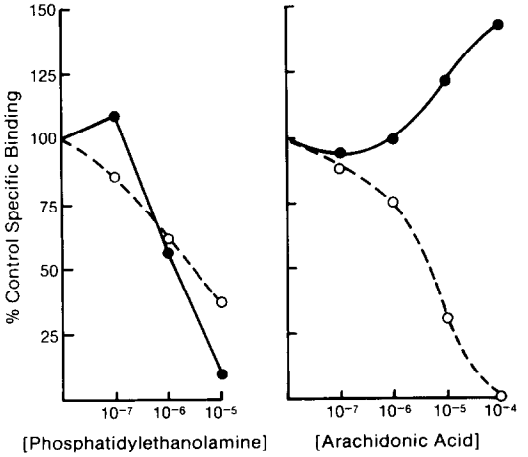

Fig. 3. Effect of lipids on digitonin-solubilized PBRs. The binding of [^{3}H]RO 5-4864 (○) and [^{3}H]PK 11195 (●) to PBRs solubilized with digitonin from rat mitochondrial membranes was determined in the presence of increasing concentrations of β-oleoyl, γ-palmitoyl phosphatidylethanolamine (left panel) or arachidonic acid (right panel). Control values were as follows: [^{3}H]PK 11195, 320 ± 21 fmol/mg protein; [^{3}H]RO 5-4864, 74 ± 5 fmol/mg protein (mean ± SEM, N = 4 from a single experiment).

cantly the binding of [^{3}H]flunitrazepam (2 nM) to membranes from rat forebrain. At the same concentration, these lipids inhibited greater than 80% of specific [^{3}H]flunitrazepam binding (as well as both [^{3}H]RO 5-4864 and [^{3}H]PK 11195 binding) to rat kidney membranes. This indicates that lipids do not inhibit benzodiazepine receptor binding by reducing the available free ligand concentration.

DISCUSSION

While characterizing hydrophobic inhibitors of PBRs present in urine and serum, we found that certain lipids inhibit ligand binding to PBRs. Fatty acids and alcohols inhibited [^{3}H]RO 5-4864 binding to PBRs at concentrations of 10^{-6} M to 10^{-4} M. Inhibitory potencies of fatty acids and alcohols were highly correlated with the degree of unsaturation, and *cis*-isomers were more potent than *trans*-isomers. Inhibitory potencies of fatty acids and alcohols were thus somewhat correlated with structures that tend to increase membrane fluidity. The binding of the proposed antagonist ligand [^{3}H]PK 11195 was much less sensitive to inhibition by fatty acids and alcohols than was the binding of [^{3}H]RO 5-4864.

Certain phospholipids also inhibited PBRs. Phosphatidylethanolamine (β-oleoyl, γ-palmitoyl), phosphatidylinositol and cardiolipin inhibited ligand binding to PBRs at concentrations of 10^{-6} to 10^{-4} M. Inhibition by phospholipids was again dependent upon the presence of unsaturated bonds in the fatty acyl moiety. The phospholipid head group was also important, since phosphatidylcholine was not inhibitory, whereas phosphatidylethanolamine with the identical fatty acyl groups was a potent inhibitor. In contrast to the fatty acids and alcohols, phospholipids were equally effective at inhibiting [^{3}H]RO 5-4864 and [^{3}H]PK 11195 binding. Arachidonic acid and phosphatidylethanolamine reduced both the affinity and apparent binding density of [^{3}H]RO 5-4864 binding.

Lipids had similar inhibitory effects upon solubilized PBRs. Phosphatidylethanolamine inhibited the binding of [^{3}H]RO 5-4864 and [^{3}H]PK 11195 to digitonin-solubilized PBRs with an IC_{50} of 1–3×10^{-6} M. As with PBRs in intact membranes, arachidonic acid was a much more potent inhibitor of [^{3}H]RO 5-4864 binding than of [^{3}H]PK 11195 binding to solubilized PBRs. Linoleic and docosohexaenoic acid were also much more potent as inhibitors of [^{3}H]RO 5-4864 than of [^{3}H]PK 11195 binding to solubilized receptors (data not shown).

Alterations in lipid peroxidation or membrane reducing environment are not likely causes for the effects of the lipids tested upon PBR binding, since agents which stimulate lipid peroxidation (ferrous sulfate, hydrogen peroxide) or alter the reducing equivalents present (vitamin E, ascorbic acid, butylated hydroxytoluene) had little or no effect on [^{3}H]RO 5-4864 binding and, unlike the fatty acids or phospholipids, had a greater effect upon binding of [^{3}H]PK 11195. Inhibition of binding by lipids was not caused by sequestering of ligand in lipid vesicles or reduction of free ligand concentration by lipids, since cardiolipin and phosphatidylethanolamine at 100 μg/ml did not inhibit [^{3}H]flunitrazepam binding to central-type BZD receptors in brain membranes but did inhibit [^{3}H]flunitrazepam binding to PBRs in kidney membranes by greater than 80%. Inhibition was not due to loss of receptors from membranes since the major effect of lipids was one of reduced affinity rather than reduced density, and solubilized receptors collected on polyethyleneimine-treated filters were also inhibited by fatty acids.

Havoundjian *et al.* [33] have demonstrated recently that treatment of membranes with phospholipase A_2 reduces the apparent affinity of [^{3}H]RO 5-4864 binding to PBRs while increasing the binding of [^{3}H]PK 11195 binding to these receptors. Our results indicate that this effect may be attributable to unsaturated fatty acids released by phospholipase A_2, since these fatty acids inhibit [^{3}H]RO 5-4864 to a much greater extent than [^{3}H]PK 11195 binding to membranes and increase [^{3}H]PK 11195 binding to solubilized receptors. One could speculate from the present results that release of unsaturated fatty acids by phospholipase A_2 from phosphatidylcholine, which is not itself a PBR inhibitor, would reduce PBR affinity for RO 5-4864. However, release of fatty acids from phosphatidylethanolamine, which is a strong inhibitor, would have no effect, or even increase apparent PBR affinity for RO 5-4864 (compare reductions in [^{3}H]RO 5-4864 binding of 38.5% by oleic acid, 2.4% by β-oleoyl-phosphatidylcholine, and 78.3% by β-oleoyl-phosphatidylethanolamine, each lipid present at 10^{-5} M). The affinity of the RO 5-4864 binding site of the PBR in mitochondria may therefore be affected by the relative concentration of different phospholipids.

Since only a few representatives of each lipid class were tested, natural sources are likely to contain several lipids which inhibit PBRs at concentrations even lower than 10^{-6} M. Urine from normal individuals contains an average of 4.8×10^{-6} M phospholipid and 1.2×10^{-4} M free fatty acid [34], concentrations at which the more potent of these compounds significantly inhibit PBRs. We have found that hydrophobic material in urine that adsorbs to Sep-pak C18 cartridges can be further separated by reverse phase HPLC into numerous peaks of inhibitory activity, indicating that the inhibitors are a heterogeneous group of compounds (unpublished results). Some of these inhibitory compounds may be fatty acids or phospholipids with effects similar to the lipids reported here.

The various inhibitory fatty acids, alcohols and phospholipids do not appear to share common structural features with either RO 5-4864 or PK 11195. Therefore, the inhibition of ligand binding may not be produced by a direct interaction with the binding site(s) for these drugs but rather through conformational changes in the receptor which lower binding site affinity. This may represent a nonspecific effect of lipids upon membrane fluidity and surface charge to which the PBR is more sensitive than the CBR. Alternatively, the two ligands as well as unsaturated lipids may associate in a mutually exclusive manner with overlapping parts of a hydrophobic binding pocket in the PBR. A more interesting possibility is that PBRs may functionally act upon or be modulated by unsaturated lipids. The early finding that RO 5-4864 stimulates phospholipid methylation

in glioma cells [15] suggests that PBRs may be functionally linked with lipid metabolism. Benzodiazepines are also reported to potentiate the activation of phospholipase A_2 by muscimol in glioma cells [35]. Recently, Shibata *et al.* [36] have shown that diazepam reduces potassium-induced aldosterone secretion from adrenal glomerulosa cells, although these authors postulate an effect of PBRs upon voltage-dependent calcium channels rather than upon mitochondrial lipid metabolism. The results reported here raise the possibility that PBRs may be involved in mitochondrial lipid metabolism or that PBR function may be regulated by lipids. Furthermore, polyunsaturated fatty acids and phospholipids may contribute to PBR inhibitory activity present in biological fluids and tissue extracts. Polyunsaturated fatty acids selectively inhibit RO 5-4864 binding, indicating another difference between this benzodiazepine and the PBR ligand PK 11195.

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