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SPECIES DIFFERENCES AND HETEROGENEITY OF SOLUBILIZED PERIPHERAL-TYPE BENZODIAZEPINE BINDING SITES

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Abstract—The pharmacological characteristics of digitonin-solubilized peripheral-type benzodiazepine binding sites (PBS) from kidney membranes of various species were investigated to determine whether the species differences and heterogeneity observed in membrane-bound binding sites would be maintained after solubilization. [^{3}H]PK 11195 (0.05 to 10 nM) bound with high affinity to rat, guinea pig, calf, and cat kidney solubilized preparations yielding maximal numbers of binding sites (B_{max}) of 3,593 ± 381, 25,645 ± 1,795, 1,327 ± 141, and 2,446 ± 148 fmol/mg protein, respectively, and equilibrium dissociation constant (K_D) values of 1.74 ± 0.18, 2.15 ± 0.15, 0.85 ± 0.09, and 1.02 ± 0.06 nM, respectively. On the other hand, the respective B_{max} and K_D values for [^{3}H]Ro 5-4864 (1.25 to 40 nM) were 2,688 ± 275, 14,182 ± 1,134, 144 ± 23 and 205 ± 17 fmol/mg protein (about 75, 55, 11, and 8%, respectively, of that of [^{3}H]PK 11195) and 13.8 ± 1.5, 14.6 ± 1.1, 10.6 ± 1.7, and 19.9 ± 1.2 nM. Unlabeled Ro 5-4864 was two orders of magnitude more potent in displacing [^{3}H]PK 11195 binding from rat kidney solubilized preparations than from calf kidney solubilized preparations, whereas the potency of unlabeled PK 11195 in displacing [^{3}H]PK 11195 binding from both rat and calf kidney solubilized preparations was almost identical. Analysis of these displacement data revealed that PK 11195 bound to a single population of binding sites ($n_H \cong 1.0$), whereas Ro 5-4864 bound to two populations of binding sites ($n_H < 1.0$) in both rat and calf kidney solubilized preparations. These results indicate that PBS species differences and heterogeneity observed in membrane-bound binding sites are retained in the soluble state and are probably attributable to variations in the molecular structure of PBS rather than to differences in membrane environment.

Central-type benzodiazepine (BZ)† receptors (CBR) have been identified in the CNS [1, 2]. Another type of high-affinity BZ binding sites, termed peripheral-type BZ binding sites (PBS), has been demonstrated in peripheral tissues such as rat kidney, lung, liver, and heart [3–5], human term placenta [6], and also in the brain [7–9]. The two types of receptors for BZ differ in their subcellular localization, their distribution within the brain, and their ligand specificity. Ro 5-4864 (4′-chlorodiazepam) and PK 11195 [1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide] bind with high affinity to PBS, but not to CBR, whereas the reverse is true with regard to the BZ clonazepam. Additionally, CBR are coupled to γ-aminobutyric acid receptors and also to specific binding sites for anions and divalent cations [10–12], whereas PBS are not.

The physiological role of PBS has not yet been finally determined, although some physiologic and pharmacologic effects for PBS ligands have already been characterized, such as electrical and mechanical changes in isolated papillary muscle [13, 14], inhibition of cellular proliferation [15], induction of cellular differentiation [15, 16], anxiogenic and proconvulsant activity [17], and alteration in the release of several hormones [18–20]. Furthermore, recent studies indicate that PBS are subject to hormonal modulation [21–24]. Porphyrins have been identified recently as a possible PBS endogenous ligand [25].

Recent studies have demonstrated marked species differences and heterogeneity in membrane-bound PBS with respect to their density and distribution within the brain, as well as to their affinities for the prototype PBS high-affinity BZ ligand Ro 5-4864 [26–28]. In the present study we compared the binding characteristics of PK 11195 and Ro 5-4864 to digitonin-solubilized PBS from kidney membranes of various species, in order to determine whether PBS species differences and heterogeneity observed in the membrane-bound state would be retained after solubilization.

MATERIALS AND METHODS

Materials. [^{3}H]PK 11195 (85 Ci/mmol) and [^{3}H]Ro 5-4864 (78.9 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Unlabeled PK 11195 was a gift from Dr G. Le Fur (Pharmuka Laboratories, Gennevilliers, France). Unlabeled Ro 5-4864 was supplied by Drs H. Gutmann and E. Kyburz (Hoffmann-La Roche, Basel, Switzerland). Digitonin was purchased from the Sigma Chemical

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† Abbreviations: BZ, benzodiazepine; CBR, central-type BZ receptors; PBS, peripheral-type BZ binding sites; B_{max}, maximal number of binding sites; and K_D, equilibrium dissociation constant.

Co. (St Louis, MO, U.S.A.). Lumax was purchased from Lumac (Schaesberg, The Netherlands). All other compounds were purchased from commercial sources.

Membrane solubilization. Adult male Sprague–Dawley rats (N = 5; body wt 200–250 g), adult male outbred guinea pigs (N = 3; body wt 300–400 g), and 6-month-old male Holstein-Friesian calves (N = 3; body wt 150–200 kg) were killed by decapitation. Male common European house cats, 1 to 2-years-old (N = 3; body wt 3–4 kg), were killed by an overdose of pentobarbital. Kidneys were removed immediately and frozen at −70° until used for solubilization and binding studies. Samples from each kidney cortex were thawed and homogenized in 50 vol. of 50 mM Tris–HCl buffer, pH 7.4, at 4° with a Brinkmann Polytron (setting 10) for 15 sec. The homogenates were centrifuged at 49,000 *g* for 30 min, and the pellets were solubilized as previously described [29].

The pellets of washed membranes (15–20 mg tissue weight/ml) were homogenized in 50 mM Tris–HCl buffer, pH 7.4, containing 0.25% digitonin and were incubated for 30 min at 4°. After incubation, the homogenates were centrifuged at 100,000 *g* for 60 min. The resultant supernatant fraction was diluted 1:1 with the same buffer and used as the soluble preparation.

Binding assay for [^{3}H]*PK* 11195 *and* [^{3}H]*Ro* 5-4864. Binding assays for [^{3}H]PK 11195 and [^{3}H]Ro 5-4864 were conducted as previously described [27, 29]. [^{3}H]PK 11195 binding was conducted in 50 mM Tris–HCl buffer, pH 7.4, at 4°. Binding assay contained 400 μl soluble preparation (150–200 μg protein) and 25 μl [^{3}H]PK 11195 (0.05 to 10 nM) in the absence (total binding) or presence (non-specific binding) of 10 μM unlabeled PK 11195 or Ro 5-4864. After incubation for 60 min at 4°, samples were filtered under vacuum over Whatman GF/B filters pretreated with 0.3% polyethyleneimine [30] and then washed three times with 4 ml of ice-cold Tris buffer. Filters were placed in vials containing 5 ml of a 1:3 mixture of Lumax:xylene. Samples were counted for radioactivity after 12 hr. The binding assay for [^{3}H]Ro 5-4864 (1.25 to 40 nM) was similar to that described above for [^{3}H]PK 11195, except that non-specific binding was conducted using only 10 μM unlabeled PK 11195. Displacement curves were obtained using 1 nM [^{3}H]PK 11195 as a radioactive ligand in the presence of twenty to thirty concentrations (10^{-12}–10^{-4} M) of unlabeled PK 11195 or Ro 5-4864. In kinetic studies, 2 nM [^{3}H]PK 11195 was incubated with solubilized PBS from rat and calf kidney for different periods of time (association); after 60 min of incubation, 10 μM unlabeled PK 11195 or 10 or 100 μM unlabeled Ro 5-4864 was added (dissociation), and filtration was performed after different periods of time.

Data analysis of displacement experiments. Displacement experiments were conducted using [^{3}H]PK 11195 as radioligand and unlabeled PK 11195 or Ro 5-4864 as inhibitor. The displacement curves were analyzed by a non-linear least-squares curve-fitting procedure, using a model for either one or two binding sites. Theoretical displacement curves were fitted to the experimental data points using the non-linear least-squares-regression computer program BMDPAR (November 1978 revision), developed at the University of California Health Science Computing Faculty (Los Angeles, CA).

The goodness of fit was evaluated by comparison of the predictive error, given by the weighted sum of squared residuals, with the experimental error. The value of the Hill coefficient (n_H) was obtained by linear regression analysis of log I vs log $[P/(100 - P)]$, in which the variable I was the inhibitor concentration and P the bound value (in percent), and the slope of the line was n_H.

Results are expressed as means of three separate experiments with less than 15% variability.

RESULTS

Binding of [^{3}H]*PK* 11195 *and* [^{3}H]*Ro* 5-4864 *to solubilized PBS from kidney membranes of various species.* Saturation curves and Scatchard analyses of [^{3}H]PK 11195 and [^{3}H]Ro 5-4864 binding to digitonin-solubilized PBS from rat and calf kidney membranes are presented in Fig. 1. [^{3}H]PK 11195 (0.05 to 10 nM) bound with high affinity to solubilized PBS from both rat and calf kidney membranes. Specific binding of [^{3}H]PK 11195 to solubilized PBS from rat and calf kidney membranes was saturated at 4–6 nM, with equilibrium dissociation constants (K_D) of 1.74 ± 0.18 and 0.85 ± 0.09 nM, respectively, and maximal numbers of binding sites (B_{max}) of 3,593 ± 381 and 1,327 ± 141 fmol/mg protein, respectively (Fig. 1). On the other hand, [^{3}H]Ro 5-4864 (1.25 to 40 nM) bound with high affinity (K_D = 13.8 ± 1.5 nM) to rat kidney solubilized preparations, yielding B_{max} value of 2,688 ± 275 fmol/mg protein, whereas in calf kidney solubilized preparations the B_{max} value for [^{3}H]Ro 5-4864 was only 144 ± 23 fmol/mg protein, and the K_D value was 10.6 ± 1.7 nM (Fig. 1).

We also compared the binding capacity of [^{3}H]Ro 5-4864 to digitonin-solubilized PBS from kidney membranes of various species to that of [^{3}H]PK 11195 (Table 1). [^{3}H]Ro 5-4864 binding capacity in solubilized preparations of rat, guinea pig, calf, and cat kidney membranes was about 75, 55, 11, and 8%, respectively, of that of [^{3}H]PK 11195.

The following results should be noted. First, non-specific binding of [^{3}H]PK 11195 was less than 10% of the total binding in rat, guinea pig, calf, and cat kidney solubilized preparations, whereas non-specific binding of [^{3}H]Ro 5-4864 was less than 10% of the total binding in rat and guinea pig kidney solubilized preparations and about 78% of the total binding in calf and cat kidney solubilized preparations. No difference in non-specific binding was obtained when the unlabeled ligand was Ro 5-4864 or PK 11195 (10 μM). Second, digitonin solubilized the same amount of PBS (about 30% recovery of the initial binding activity) from rat, guinea pig, calf, and cat kidney membranes (data not shown). Third, GF/B filters pretreated with 0.3% polyethyleneimine restored the same binding activity of [^{3}H]PK 11195 as did filtration upon precipitation by the polyethelene glycol method (data not shown).

Displacement studies. The potencies of unlabeled PK 11195 and Ro 5-4864 in displacing [^{3}H]PK 11195

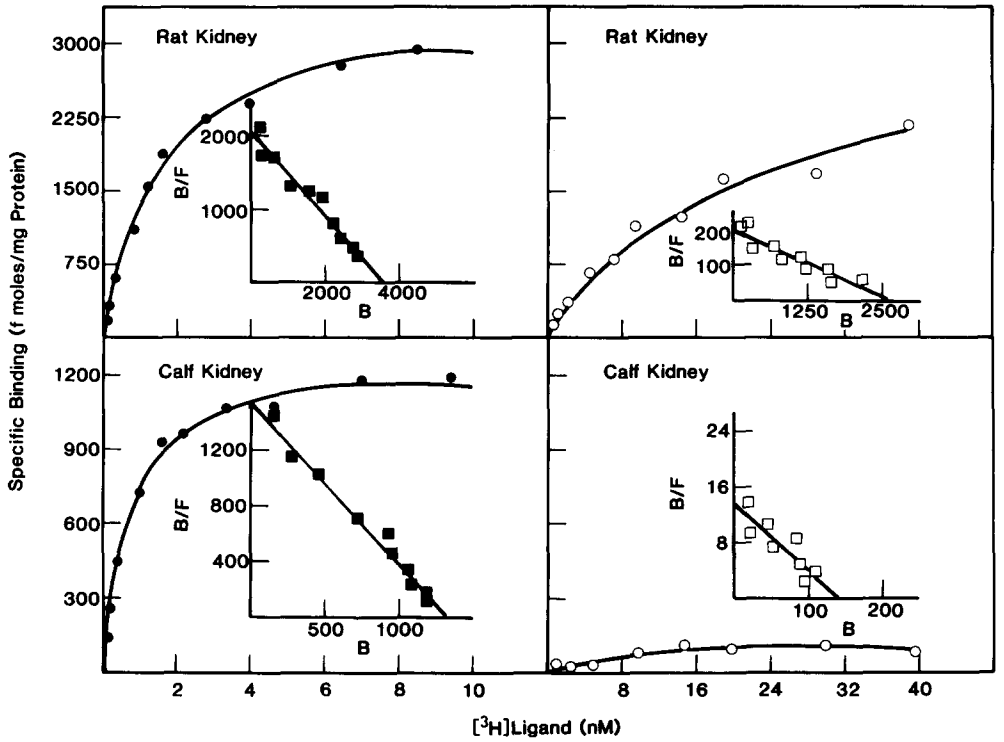

Fig. 1. Saturation curves of [³H]PK 11195 and [³H]Ro 5-4864 binding to digitonin-solubilized PBS from rat and calf kidney membranes. [³H]PK 11195 (●) (0.05 to 10 nM) and [³H]Ro 5-4864 (○) (1.25 to 40 nM) binding at 4° for 60 min was determined for solubilized preparations of rat and calf kidney membranes. Details of the binding procedure are described under Materials and Methods. Results are the means of three separate experiments with less than 15% variability. Insets: Scatchard plots of saturation curves of [³H]PK 11195 (■) and [³H]Ro 5-4864 (□) specific binding to solubilized preparations from rat and calf kidney membranes. Abbreviations: B, bound; and B/F, bound/free.

Table 1. [³H]PK 11195 and [³H]Ro 5-4864 binding capacity in digitonin-solubilized preparations from kidney membranes of various species

Species	B_{max} (fmol/mg protein)	
	[³H]PK 11195	[³H]Ro 5-4864
Calf	1,327 ± 141	144 ± 23
Rat	3,593 ± 381	2,688 ± 275
Guinea pig	25,645 ± 1,795	14,182 ± 1,134
Cat	2,446 ± 148	205 ± 17

Specific binding of [³H]PK 11195 (0.05 to 10 nM) and [³H]Ro 5-4864 (1.25 to 40 nM) was detected in digitonin-solubilized PBS from kidney membranes of various species. Incubation was performed for 60 min at 4° as described under Materials and Methods. Non-specific binding of [³H]PK 11195 was < 10% of the total binding in all mammalian kidney solubilized preparations tested, whereas non-specific binding of [³H]Ro 5-4864 was < 10% of the total binding in rat and guinea pig kidney solubilized preparations and ~78% in calf and cat kidney solubilized preparations. Data are the mean ± SD of three separate experiments with less than 15% variability.

binding from rat and calf kidney solubilized preparations are presented in Fig. 2. While unlabeled PK 11195 exhibited similar potency in displacing [³H]PK 11195 from both rat and calf kidney solubilized preparations, unlabeled Ro 5-4864 was two orders of magnitude more potent in displacing [³H]PK 11195 from rat kidney solubilized preparations than from calf kidney solubilized preparations.

Analysis of these displacement data revealed that PK 11195 bound to a single population of binding sites ($n_H \cong 1.0$), whereas Ro 5-4864 bound to two populations of binding sites ($n_H < 1.0$) in both rat and calf kidney solubilized preparations (Table 2).

Kinetic experiments. The time course of [³H]PK 11195 binding to rat and calf kidney solubilized preparations is shown in Fig. 3. [³H]PK 11195 (2 nM) binding reached equilibrium after 15 and 30 min of incubation at 4° with calf and rat kidney solubilized preparations, respectively. The time course of [³H]PK 11195 dissociation from rat and calf kidney solubilized preparations after addition of 10 μM unlabeled PK 11195 or 10 or 100 μM unlabeled Ro 5-4864 is also shown in Fig. 3. When 10 μM unlabeled PK 11195 or Ro 5-4864 was added to rat kidney solubilized preparations, half the amount of specific [³H]PK 11195/PBS complexes was dissociated after 78 min. When 10 μM unlabeled PK 11195 was added to calf kidney solubilized preparations, half the amount of specific [³H]PK 11195/PBS complex was dissociated after 90 min. In contrast, even when up to 100 μM unlabeled Ro 5-4864 was added to calf kidney solubilized preparations and incubation lasted for 9 hr, not all of the specific [³H]PK 11195/PBS complex was dissociated. Therefore, its half-life time could not be calculated. Higher concentrations of unlabeled Ro 5-4864 could not be

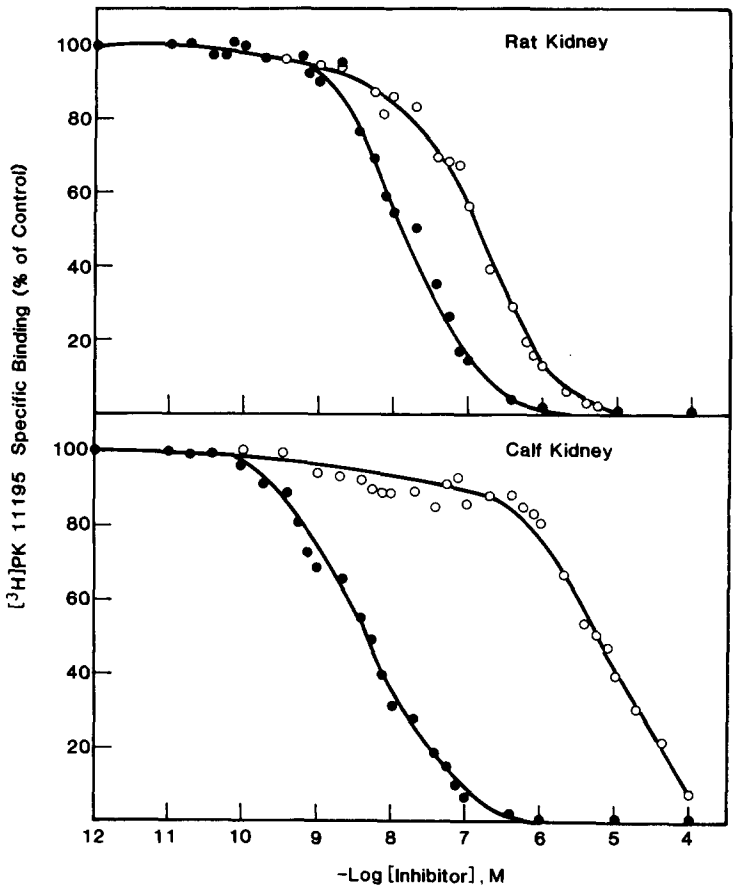

Fig. 2. Displacement of [^{3}H]PK 11195 binding from digitonin-solubilized PBS from rat and calf kidney membranes by unlabeled PK 11195 or Ro 5-4864. Binding assays were conducted with 1 nM [^{3}H]PK 11195 in the presence of the indicated concentrations of unlabeled PK 11195 (●) or Ro 5-4864 (○). Controls (100% binding) for rat and calf kidney solubilized membranes gave values of 1,387 ± 124 and 720 ± 69 fmol/mg protein, respectively. Details of the binding assays are described under Materials and Methods. Results are the means of three separate experiments, each of which consisted of triplicate measurements with less than 15% varaibility.

Table 2. Binding characteristics of PK 11195 and Ro 5-4864 to digitonin-solubilized PBS from rat and calf kidney membranes

Species	K_i (nM)	α	K_H (nM)	K_L (nM)	n_H
Rat					
PK 11195	9.5 ± 0.5	1.0 ± 0.05	9.5 ± 0.5		~1.0
Ro 5-4864	67 ± 8	0.2 ± 0.02	1.0 ± 0.1	107 ± 11	0.49 ± 0.05
Calf					
PK 11195	2.3 ± 0.2	1.0 ± 0.06	2.3 ± 0.2		~1.0
Ro 5-4864	2750 ± 183	0.15 ± 0.01	74 ± 5	3537 ± 236	0.46 ± 0.03

For each displacement curve, we used [^{3}H]PK 11195 (final concentration, 1 nM) as a radioactive ligand and twenty to thirty concentrations of unlabeled PK 11195 or Ro 5-4864 (final concentration, 10^{-12}–10^{-4} M) as inhibitor. The binding parameters (mean ± SD values from three separate experiments) were calculated independently by the non-linear regression procedure for one- and two-site models. K_i, inhibition constant; α, proportion of high-affinity sites for PK 11195 and Ro 5-4864; K_H and K_L, equilibrium dissociation constants of high- and low-affinity binding sites, respectively; and n_H, slopes of Hill plots.

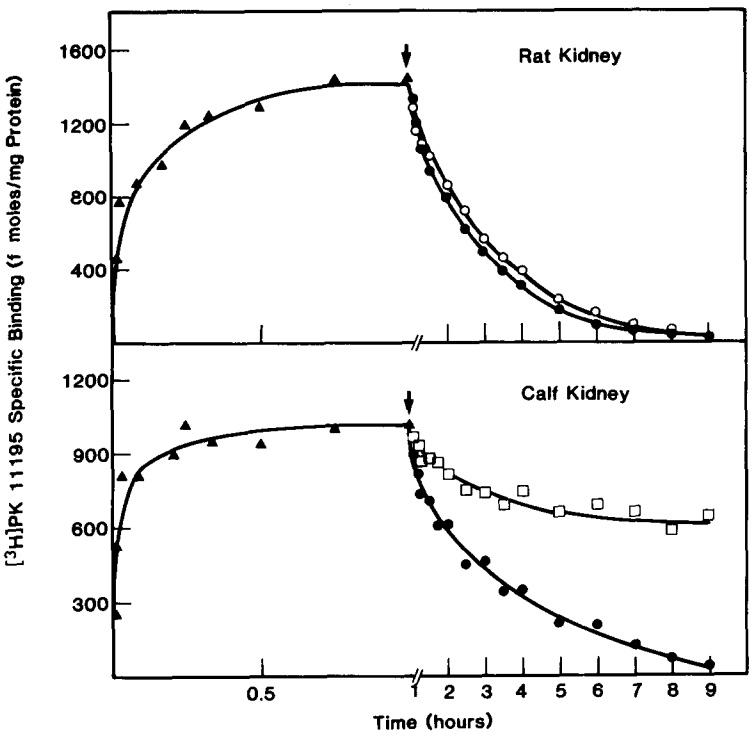

Fig. 3. [^{3}H]PK 11195 association to and dissociation from digitonin-solubilized PBS from rat and calf kidney membranes. Binding of 2 nM [^{3}H]PK 11195 (▲) to solubilized PBS from rat and calf kidney membranes was measured as a function of time at 4°. Dissociation was begun after 60 min of incubation (arrow) by addition of 10 μM unlabeled PK 11195 (●) or of 10 μM (○) or 100 μM (□) unlabeled Ro 5-4864. Specific binding was determined as described under Materials and Methods. Results are the means of three separate experiments with less than 15% variability.

used, because of the insolubility of the compound in water. These results are similar to those previously demonstrated in membrane-bound PBS of rat and calf kidney [27].

DISCUSSION

Earlier reports have suggested qualitative and quantitative species differences and heterogeneity in PBS through comparison of their density and distribution within the brain and peripheral tissues of various species, as well as of their binding affinity for the prototype PBS high-affinity BZ ligand Ro 5-4864. Autoradiographic studies have shown significant differences in the density and distribution of PBS within human and rat brain [26]. These results were further supported by our recent demonstration of differences in PBS binding capacity in the cerebral cortex, heart, and kidney of various species [27]. Using [^{3}H]PK 11195 as a radioactive ligand, the rank order of PBS binding capacity in the cerebral cortex of various species was calf > rabbit > rat, whereas the rank order of binding capacity in the heart and kidney was rat > calf > rabbit and rat > calf = rabbit, respectively [27].

Species differences and heterogeneity in PBS have also been demonstrated in the binding affinity of [^{3}H]Ro 5-4864 to tissues of various species and as compared to the binding affinity of [^{3}H]PK 11195. Similarly, species differences and heterogeneity in PBS have also been demonstrated in the rank order of potencies of unlabeled Ro 5-4864 in inhibiting [^{3}H]PK 11195 specific binding to tissues of various species and as compared to the potency of unlabeled PK 11195. The binding affinity of Ro 5-4864 to cow brain membranes has been found to be 200 times lower than its affinity to rat brain membranes [31]. Recently we demonstrated that [^{3}H]PK 11195 binds with nanomolar affinity to both rat and calf cerebral cortex and peripheral tissues, whereas [^{3}H]Ro 5-4864 binds with nanomolar affinity to rat and guinea pig cerebral cortex and peripheral tissues, but with micromolar affinity to calf, rabbit, cat, and dog tissues [27]. Similar differences in the binding affinity of [^{3}H]PK 11195 and [^{3}H]Ro 5-4864 have also been obtained in rat and calf pineal gland [28]. Additionally, the potency of unlabeled Ro 5-4864 in inhibiting [^{3}H]PK 11195 specific binding to cat brain sections and membranes has been found to be 140 times lower than that of unlabeled PK 11195 [32], whereas in rat brain the potency of Ro 5-4864 is similar to that of PK 11195 [33]. Recently we found that the rank order of potency of unlabeled Ro 5-4864 in inhibiting [^{3}H]PK 11195 specific binding to cerebral cortex membranes of various species is rat > guinea pig > cat = dog > rabbit > calf [27]. Unlabeled Ro 5-4864 was three orders of magnitude more potent in inhibiting specific [^{3}H]PK 11195 binding to rat cerebral cortex membranes than to calf cerebral cortex membranes. On the other hand, the potency

of unlabeled PK 11195 in inhibiting [^{3}H]PK 11195 specific binding to cerebral cortex and kidney membranes of various species was almost identical [27]. Analysis of these displacement data revealed that PK 11195 binds with high affinity to a single population of binding sites in both rat and calf cerebral cortex and kidney ($n_H \cong 1.0$), whereas Ro 5-4864 binds to two populations of membrane binding sites ($n_H < 1.0$), with high affinity to one population and with lower affinity to the other. It was also found that Ro 5-4864 binds to a single population of binding sites in dog and cat cerebral cortex, but to two populations of binding sites in rat, guinea pig, rabbit, and calf cerebral cortex [27]. Furthermore, such species differences and heterogeneity in PBS have also been characterized in human cerebral cortex and peripheral tissues (unpublished data).

These dramatic species differences and heterogeneity in PBS may theoretically be related to either (1) variations in PBS molecular structure resulting from slight evolutionary or mutational variations, (2) variations in the microenvironmental composition of PBS phospholipids in the membrane, or (3) variations in receptor–modulator coupling. The present study was designed to elucidate which of these possibilities may be substantiated.

PBS have been solubilized in an "active" form from rat kidney and adrenal gland membranes by cholic acid, Triton X-100, and digitonin [29, 34–36].

The major findings in the present study were that (1) while PK 11195 bound with high affinity to solubilized PBS from rat, guinea pig, calf, and cat kidney membranes, Ro 5-4864 bound with high affinity to solubilized PBS from rat and guinea pig kidney membranes but not from calf and cat kidney membranes; (2) Ro 5-4864 binding capacity, as compared to PK 11195, in the soluble extract of kidney of various species was markedly different; and (3) Ro 5-4864 bound to two populations of binding sites in both rat and calf kidney solubilized preparations ($n_H < 1.0$).

These results indicate that species differences and heterogeneity in PBS observed in membrane-bound binding sites are retained in the solubilized binding sites. Such species differences and heterogeneity are therefore probably not attributable to variations in the PBS environment within the membrane or receptor–modulator coupling. Additionally, the finding that digitonin solubilized the same amount of PBS from rat, guinea pig, calf, and cat kidney membranes rules out the possibility that the species differences and heterogeneity in PBS observed in solubilized binding sites are due to differential solubilization. Such species differences and heterogeneity in PBS are probably attributable to structural characteristics. Benavides *et al.* [36] did not find differences in the molecular weights of digitonin-solubilized PBS from rat and calf adrenal gland by gel-filtration column using [^{3}H]PK 11195 as radioactive ligand. Recently it was reported that PBS are purified after photoaffinity labeling from rat adrenal gland [37]. Comparison of the amino acid composition and sequence of PBS purified from rat and calf tissues will give a better understanding of the origin of the species differences and heterogeneity in PBS.

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