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COFRACTIONATION OF THE 17-kD PK 14105 BINDING SITE PROTEIN WITH SOLUBILIZED PERIPHERAL-TYPE BENZODIAZEPINE BINDING SITES

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Abstract—To examine the relationship between PKBS, a 17-kD protein covalently photolabeled by [^{3}H]PK 14105, and its association with peripheral-type benzodiazepine binding sites, rat adrenal mitochondrial fractions were photolabeled with [^{3}H]PK 14105, solubilized in digitonin, and subjected to anion-exchange chromatography over Q-Sepharose. The chromatographic behavior of PKBS was evident as principally two major fractions, signified as Q-I and Q-II. Specific binding sites for [^{3}H]Ro5-4864 and [^{3}H]PK 11195 were also assayed and found to cofractionate with each other and in a manner which coincided with the photolabeled PKBS profile. The Q-I and Q-II fractions were further distinguished based on their different molecular sizes observed by gel filtration, yet both fractions were characterized as containing peripheral-type benzodiazepine recognition sites according to the following criteria. Scatchard analysis of both subpopulations revealed a single class of binding sites for [^{3}H]Ro5-4864 with an apparent K_D of 14 nM for Q-I and 22 nM for Q-II; these affinities were slightly lower than those found in mitochondrial membrane preparations used as the starting material for solubilization. The rank order of potency to inhibit [^{3}H]Ro5-4864 binding in both subpopulations was PK 11195 > Ro5-4864 > diazepam > clonazepam, in connection with the pharmacological specificity of membrane-associated peripheral-type benzodiazepine binding sites. These studies provide direct biochemical evidence that the recognition sites for benzodiazepines and isoquinoline carboxamides cofractionate in unison with the 17-kD PKBS protein, demonstrating an intimate relationship between this protein and the binding domains for peripheral-type benzodiazepine ligands.

Peripheral-type benzodiazepine receptors (PBR) have been distinguished from central-type benzodiazepine receptors due to their differing specificities to bind various benzodiazepine derivatives with high affinity [1–3]. PBR appear to be present in virtually all mammalian tissues [4–7] and are localized primarily on mitochondrial membranes [8, 9]. Recently, this receptor has been found to be coupled to intramitochondrial cholesterol transport during steroid synthesis [10, 11], a phenomenon which may be linked to the reported effects on mitochondrial respiration by PBR ligands [12].

In addition to benzodiazepines, PBR are coupled to recognition sites for isoquinoline carboxamides [13, 14], protoporphyrins [15, 16], thiazide diuretics [17], and a host of other compounds [18]. The binding of benzodiazepines and isoquinoline carboxamides to these sites exhibits differential sensitivity to temperature [14], detergents [19], unsaturated fatty acids [20], phospholipase A_2 treatment [21], and the histidine-modifying reagent diethylpyrocarbonate [20, 22], suggesting that the binding domains for these two classes of compounds are different. The isoquinoline carboxamide photoaffinity probe, PK 14105, specifically photolabels a protein of 17–18 kD from mitochondrial preparations containing PBR [9, 23, 24]. In contrast, two different benzodiazepines, flunitrazepam [15] and AHN 086 [25], were reported to covalently modify proteins of 30–35 kD. These findings imply that different proteins constitute the binding domains for benzodiazepines and isoquinoline carboxamides; however, additional work is required to substantiate this proposal. This becomes a particularly important consideration because these studies did not include supporting data to determine whether these identified proteins subfractionate with PBR ligand binding activity.

While several groups have attempted the solubilization and purification of the PBR complex, they did not correlate the presence of any specific proteins which correspond with the solubilized binding activity [26–31]. In previous studies we had purified and cloned the protein of 17–18 kD which is specifically photolabeled by PK 14105 and entitled this protein PKBS (PK binding site) [32, 33]. Transfection studies with the corresponding cDNA for this protein demonstrated the expression of binding sites for PBR ligands [33], but did not rule out the possibility that this protein complements cryptic binding sites already present in the cells. In this report, we present further biochemical evidence that, following solubilization, PKBS cofractionates in a manner which coincides precisely with the subfractionation of PBR, supporting the proposal that PKBS constitutes binding domains for PBR ligands.

METHODS

Isolation and photolabeling of rat adrenal mitochondrial fractions. Adrenals from adult Sprague–Dawley rats were homogenized at 0° in 10 vol. of

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25 mM Tris–HCl (pH 7.4), 0.32 M sucrose by ten strokes in a Potter–Elvehjem tissue grinder. The homogenates were first centrifuged at 770 *g* for 15 min, and the resulting supernatant fractions were recovered and centrifuged again at 5,200 *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. These mitochondrial fractions were stored at −70° until they were used. Protein quantitation of the membrane fractions was performed according to the method of Bradford [34] using ovalbumin with a reduced mannose content (Sigma Chemical Co., St. Louis, MO) as a standard. Photolabeling of mitochondrial fractions with [^{3}H]PK 14105 was performed as described elsewhere [11].

Solubilization of mitochondrial membrane fractions. Mitochondrial membrane preparations were resuspended at a concentration of 10 mg of protein/mL of 25 mM Tris–HCl (pH 8.0), 1% digitonin (w/v) and incubated at 0° for 30 min. The samples were then centrifuged at 100,000 *g* for 30 min, and the supernatant fractions were recovered for subsequent experiments.

Membrane and soluble binding assays. Incubation of mitochondrial membranes or digitonin-solubilized samples with a radioligand was performed in 25 mM Tris–HCl (pH 7.4) at 0°. Specific details concerning the conditions used for each experiment are described in the text. Membrane samples were diluted in 5 mL of buffer and subjected to vacuum filtration on Whatman GF/C filters (2.4 cm); the filters were immediately washed three times with 5 mL of ice-cold buffer.

For the determination of soluble binding activity, the method developed by Bruns *et al.* [35] was used. Whatman GF/B filters (2.4 cm) were pretreated for 1 hr with 0.5% polyethylenimine. The filter was placed on the support and dried under reduced pressure, after which the vacuum line was closed off and 200 μL of the sample was applied onto the filter. The vacuum was then reintroduced, and the filter was washed with 25 mL of cold buffer and subjected to liquid scintillation counting.

Molecular exclusion chromatography. In calibrating all gel filtration columns, blue dextran and [^{3}H]γ-aminobutyric acid were used to determine the void volumes and total column volumes respectively. For Sepharose CL-6B and Sephadex G-200 the following proteins were used as molecular size standards: thyroglobulin (669 kD), apoferritin (440 kD), catalase (232 kD), alcohol dehydrogenase (150 kD), bovine serum albumin (68 kD), and carbonic anhydrase (29 kD). For calibration of Sephadex G-75 the following standards were used: bovine serum albumin (68 kD), ovalbumin (43 kD), carbonic anhydrase (29 kD), and cytochrome *c* (12.4 kD).

Materials. [*N-methyl*-^{3}H]PK 11195 and [*N-methyl*-^{3}H]Ro5-4864 were purchased from New England Nuclear. [*N-methyl*-^{3}H]PK 14105 was a gift from Dr. H. Van Hove (Commissariat a l'Energie Atomique, Gif-sur-Yvette Cedex, France). PK 11195 and PK 14105 were donated by Dr. C. Gueremy (Pharmuka Laboratories, Groupe Rhone Poulenc Sante, Gennevilliers, France). Diazepam, clonazepam, and Ro5-4864 were gifts from Dr. P. Sorter of Hoffmann-LaRoche (Nutley, NJ). Gel filtration molecular size standards were obtained from either the Sigma Chemical Co. or Pharmacia (Piscataway, NJ).

RESULTS

Fractionation of solubilized PBR. To solubilize and fractionate rat adrenal mitochondrial PBR, an approach was taken which we used previously in the purification of PKBS [32], a protein associated with PBR and presumed to constitute the binding domain for isoquinoline carboxamides. This procedure involved photolabeling adrenal mitochondrial fractions with [^{3}H]PK 14105, combining these photolabeled mitochondrial preparations with a 100-fold excess of unlabeled mitochondrial membranes, solubilizing the samples in 1% digitonin, and performing anion-exchange chromatography over Q-Sepharose. After washing the column with buffer, the solubilized mitochondrial protein which had bound to the Q-Sepharose matrix was eluted with a linear gradient of 0–0.6 M NaCl followed by a step-wise elution at 1.0 M NaCl.

The UV absorbance and [^{3}H]PK 14105 profiles that were obtained from one experiment are shown in Fig. 1A. An initial peak of radioactivity, corresponding to material that did not bind to Q-Sepharose, was due to the presence of nonspecifically photolabeled adducts and free [^{3}H]PK 14105 which had remained associated with the mitochondrial membranes despite repeated washings. Upon application of a salt gradient, two additional major peaks of radioactivity were observed to elute at approximate NaCl concentrations of 50 and 250 mM. These two [^{3}H]PK 14105-labeled fractions are termed Q-I and Q-II respectively. If photolabeling was performed in the presence of 10 μM PK 14105 or 10 μM PK 11195, the radioactivity associated with Q-I and Q-II was reduced by >90%, while the [^{3}H]PK 14105 associated with the first peak (found in the flow-through fractions) was not lowered. Occasionally additional labeled species, eluting at higher salt concentrations, were observed (as can be seen in Fig. 1); however, their levels in comparison to those of Q-I and Q-II were much less and they were also detected less consistently.

The fractions recovered from the Q-Sepharose column were also assayed for specific binding of [^{3}H]Ro5-4864 and [^{3}H]PK 11195 by a method to measure soluble binding activity for ligands [34]. In other studies it was determined that ≥90% of the [^{3}H]PK 14105-adducts from Q-I and Q-II were retained on the filters under these conditions. Panels B and C of Fig. 1 demonstrate the profiles of specific Ro5-4864 and PK 11195 binding, respectively, that were observed. The profiles obtained with both ligands were essentially identical and different from the profile of total protein eluted from the column. Furthermore, they closely resembled the profile of [^{3}H]PK 14105-adducts with the exception that the peak found in the flow-through fractions was virtually absent. These results demonstrate that the two major fractions of solubilized PBR observed contained binding sites for benzodiazepines and isoquinoline carboxamides. Therefore, the identification of the

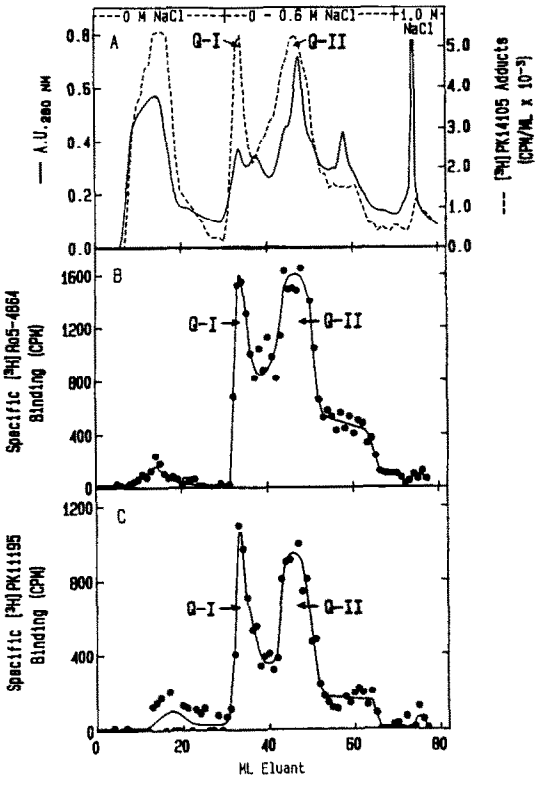

Fig. 1. Q-Sepharose chromatography of solubilized PBR. Eight milliliters of a digitonin-solubilized extract of 50 mg of mitochondrial membrane protein, of which 0.5 mg was photolabeled with [^{3}H]PK 14105, was applied at 9 mL/hr to a Q-Sepharose column (0.6 × 30 cm) equilibrated in 25 mM Tris–HCl (pH 8.0), 0.1% digitonin (w/v). After application of the sample, the column was washed with 8 mL of buffer and a linear gradient to 0.6 M NaCl in 50 mL of column buffer was run followed by a step-wise elution with 1.0 M NaCl. The upper portion of panel A indicates the duration of each elution step in this scheme. Fractions (1 mL) were collected and 25-μL aliquots of each were directly subjected to liquid scintillation counting (A). Additional 50-μL aliquots were added to 450 μL of 25 mM Tris–HCl (pH 7.4) containing 5 nM [^{3}H]Ro5-4864 or 5 nM [^{3}H]PK 11195 (final concentrations), incubated for 90 min at 0°, and processed for soluble binding activity as described in Methods. Specific binding data are shown as the mean of duplicate determinations (B and C) using a 10 μM concentration of the respective nonradioactive ligand to determine nonspecific binding in parallel assays. Curves drawn for B and C represent approximations of the binding activity profile.

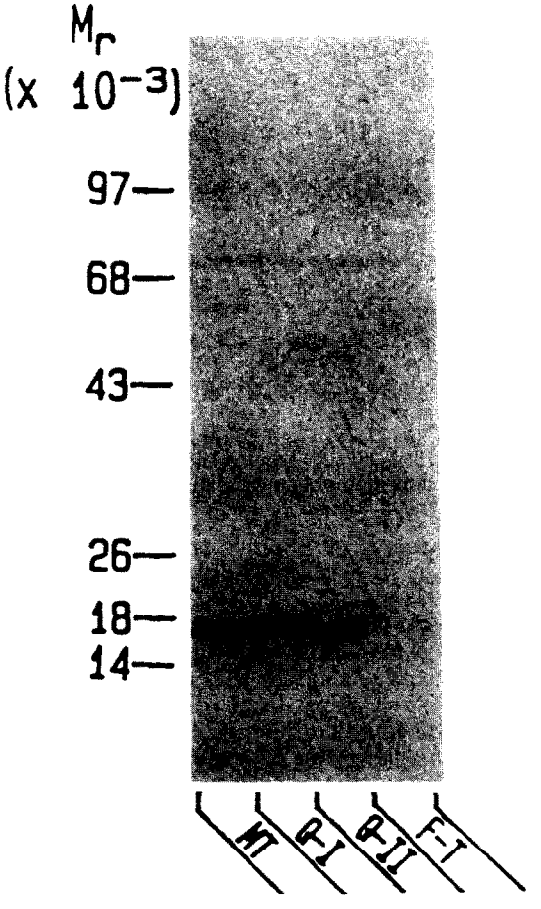

Fig. 2. Identification of PKBS as the photolabeled adduct in Q-I and Q-II. The flow-through fractions (F-T), Q-I and Q-II were obtained by subfractionation of [^{3}H]PK 14105 photolabeled mitochondrial digitonin-extracts on Q-Sepharose. Samples of all three fractions were concentrated on Centricon-30 units and lyophilized. Approximately 5000 cpm of each fraction and an additional sample from photolabeled mitochondrial membranes (MT) were electrophoresed on a 5–15% polyacrylamide gel, transferred to nitrocellulose, and autoradiographed as described [32]. Positions of molecular size markers (Bethesda Research Laboratories, Gaithersburg, MD) are indicated.

[^{3}H]PK 14105-labeled protein is apparently indicative of the chromatographic behavior of PBR.

After having performed over twenty solubilization studies, we typically noticed Q-I and Q-II as the major forms, while at times additional species were also observed. In fact, the experiment shown in Fig. 1 is an example of one preparation where additional species eluting later than Q-II were detected. It is noteworthy that despite these additional peaks the profile of [^{3}H]PK 14105-labeled adducts follows the same contours found with the profiles of [^{3}H]Ro5-4864 and [^{3}H]PK 11195 binding activity. Furthermore, the relative quantities of Q-I and Q-II also exhibited substantial variation in different experiments where Q-I was usually the most abundant. Despite this variation, the relative quantities of Q-I and Q-II detected by [^{3}H]Ro5-4864 and [^{3}H]PK 11195 binding consistently correlated with those found with the photolabeled adducts.

The recovery of [^{3}H]PK 14105-labeled adducts from the Q-Sepharose column was typically 90–95%. A lower recovery of specific Ro5-4864 binding activity from Q-Sepharose chromatography, being approximately 25% of that found in the original membrane fractions, was presumably due to interference of digitonin in Ro5-4864 binding [19].

Identification of PKBS in Q-I and Q-II. Identification of the radioactively-labeled PK 14105-adduct found in Q-I and Q-II, when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography, was verified to be the 17 kD

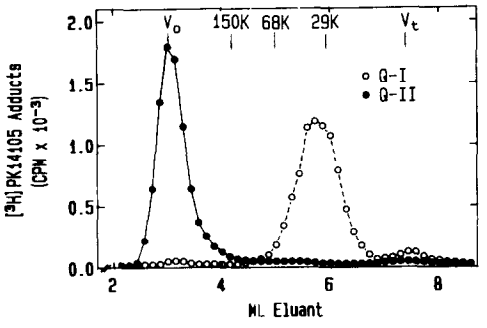

Fig. 3. G-200 chromatography of Q-I and Q-II. Aliquots of 0.5 mL from fractions of isolated Q-I (○) and Q-II (●) were concentrated to 50 μL on Centricon-30 units and chromatographed on a G-200 superfine column (0.6 × 30 cm) in 25 mM Tris–HCl (pH 7.4), 0.5 M NaCl at a flow rate of 450 μL/hr. Fractions collected were subjected to liquid scintillation counting. Elution volumes of gel filtration molecular size standards are indicated.

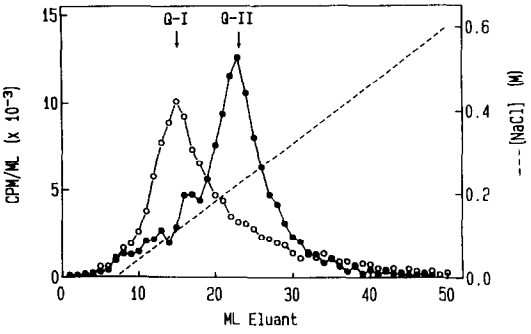

Fig. 4. Conversion of Q-II to Q-I. A preparation of Q-II from photolabeled mitochondrial membranes was obtained by Q-Sepharose chromatography using buffers containing a protease inhibitor mixture including 0.1 mM phenylmethylsulfonyl fluoride, 10^{-7} M pepstatin A, 1 μg/mL leupeptin, and 1 μg/mL aprotinin. This Q-II fraction was divided into two 2-mL portions. One portion was concentrated on a Centricon-30 membrane to a volume of 200 μL, diluted to 2 mL with 25 mM Tris–HCl (pH 8.0) containing protease inhibitors, reconcentrated to 200 μL, and again brought to 2 mL with Tris buffer. Both 2-mL samples were then incubated at 4° for 24 hr. At this time the second portion was desalted, as was performed for the first portion, and both samples were loaded to duplicate Q-Sepharose columns (0.6 × 10 cm) and eluted with 0–0.6 m NaCl gradients in Tris buffer. The profiles for the samples desalted before (○) and after (●) the incubation period are shown during the gradient elution. Arrows indicate the respective locations at which Q-I and Q-II eluted from the Q-Sepharose column.

PKBS protein (Fig. 2). These results indicate that the PKBS protein is common to both major subpopulations of solubilized PBR generated by this procedure. The initial peak of [^{3}H]PK 14105-adducts, which did not bind the Q-Sepharose, did not contain a detectable labeled protein adduct for the reason mentioned above; it was comprised of free [^{3}H]PK 14105 and other nonspecifically photolabeled adducts. For the reason that PKBS accounts for >90% of the [^{3}H]PK 14105-protein adducts from mitochondrial membranes, as shown in Fig. 2, the detection of [^{3}H]PK 14105-adducts during application of the salt gradient was equivalent to recovery of photolabeled PKBS.

Molecular exclusion chromatography of Q-I and Q-II. Molecular properties which distinguished Q-I from Q-II were also detected by gel filtration chromatography. As shown in Fig. 3, when these subfractions were chromatographed over Sephadex G-200 (in the absence of detergent), the [^{3}H]PK 14105-labeled adduct of Q-II eluted at near the void volume of this column, while the adduct of Q-I behaved as a much smaller entity. Additional gel filtration columns were used to obtain a more accurate estimate of the sizes of both species. On Sephadex G-75 the apparent molecular size estimation of Q-I was 30 ± 6 kD and the size of Q-II, as determined on Sepharose CL-6B, was in the range of 200 ± 20 kD (data not shown). In all gel filtration experiments calibration of the columns was also performed in the absence of detergent to obviate aberrant chromatographic behavior of proteins associating with detergent micelles.

Conversion of Q-II to Q-I. As discussed earlier we have observed great variation in the relative quantities of Q-I compared to Q-II in different experiments. This led us to hypothesize that one subpopulation may be converted to that of the other by some unknown mechanism. In fact, in some preliminary studies we had observed a decrease in the relative quantity of Q-II and a concomitant increase in Q-I with time following solubilization, suggesting that the PBR of Q-II can be converted to the Q-I fraction. To provide more convincing evidence that proteolysis does not account for this conversion, we first considered that Q-II appeared quite stable inasmuch as a large complex was still observed by gel filtration 48 hr following Q-Sepharose chromatography. This implied that the NaCl, which is used to elute the protein from the ion-exchange resin, may stabilize this species.

The following experiment was then devised using this principle including a mixture of protease inhibitors in all buffers. [^{3}H]PK 14105-photolabeled mitochondrial membranes were solubilized and subjected to Q-Sepharose chromatography, and the fractions corresponding to Q-II were pooled. One-half of the pooled material was desalted using a Centricon-30 unit. Both halves of the sample were then kept at 4° for 24 hr. After this time the other half of the sample was also desalted and both fractions were applied to identical Q-Sepharose columns and chromatographed in parallel. The profiles obtained from both protocols are shown in Fig. 4.

The portion of sample that was kept in the presence of NaCl during the 24-hr incubation continued to elute from the Q-Sepharose column at a position characteristic of Q-II, although a fraction of this sample had apparently converted to Q-I. The other half of the preparation, which was desalted prior to the incubation, eluted from the column at lower ionic strength as would be expected for Q-I. Q-II is only apparent in this profile as a shoulder of the major peak. Ultrafiltration studies on membranes with a 100,000 molecular weight cut-off provided additional support that the respective sizes of these derivatives

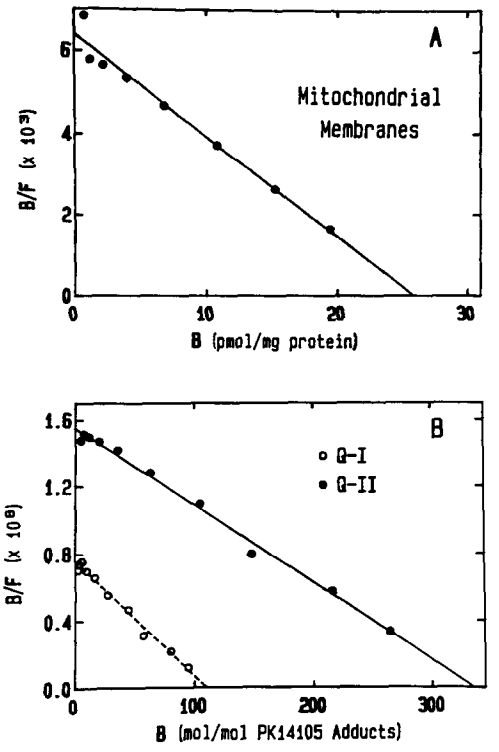

Fig. 5. Scatchard analysis of specific [^{3}H]Ro5-4864 binding. All incubations for radioligand binding assays were at 0° for 120 min, a time determined beforehand as sufficient to attain equilibrium. Nonspecific binding was measured by including 10 μM Ro5-4864 in parallel samples. Binding studies on a mitochondrial fraction were performed using 5 μg of membrane protein in 500 μL of buffer and are presented as the means of triplicate determinations (A). For Q-I and Q-II, for which the mean of ten replicates is shown (B), the abscissa is expressed relative to moles of [^{3}H]PK 14105-adducts in order to normalize the B_{max} to the quantity of photolabeled PKBS in each fraction. This parameter was chosen for comparison because binding relative to the amount of total protein in each fraction is of no significance due to differing degrees of purification of PBR in Q-I and Q-II by Q-Sepharose chromatography.

Table 1. Competition of specific [^{3}H]Ro5-4864 binding

Compound	IC_{50} (nM)		
	Mitochondria	Q-I	Q-II
PK 11195	2.9	14	8.9
Ro5-4864	2.2	41	32
Diazepam	68	890	1,200
Clonazepam	>10,000	>10,000	>10,000

Specific binding using 3 nM [^{3}H]Ro5-4864 was measured as described in the legend of Fig. 5. Binding in the presence of each competitor at six different concentrations was used to determine the IC_{50} values listed.

appear consonant with the molecular sizes observed for Q-I and Q-II by gel filtration (data not shown). These results provide evidence that NaCl can prevent at least partially the conversion of Q-II to Q-I, indicating that this conversion is not apparently due to proteolytic activity.

Characterization of PBR associated with Q-I and Q-II. To provide further support that the Q-I and Q-II fractions of PKBS protein correspond to solubilized PBR, Scatchard analysis of specific [^{3}H]Ro5-4864 binding to these fractions was performed (Fig. 5). A single class of binding sites was observed with apparent dissociation constants of 14 and 22 nM for Q-I and Q-II respectively. These values are 4 to 5-fold higher than the value of 2–4 nM determined for the membrane preparations. A decrease in affinity was typically observed for detergent-solubilized receptors. The B_{max} for the solubilized samples, expressed relative to the moles of [^{3}H]PK 14105-adducts present in each fraction, was approximately three times higher for Q-II. The reason for this is unknown but may indicate a higher proportion of the photolabeled PKBS protein relative to nonlabeled PKBS subfractionating with Q-I. Because of high nonspecific binding of [^{3}H]PK 11195 to the filters, reliable Scatchard analyses with this ligand were difficult to perform on the solubilized fractions, although specific binding at nanomolar concentrations was detected easily.

In addition to Ro5-4864 and PK 11195, PBR also exhibit nanomolar affinity for diazepam, but not for clonazepam, a ligand which is much more selective for central-type benzodiazepine receptors. These compounds were tested for their ability to compete with specific [^{3}H]Ro5-4864 binding in rat adrenal mitochondrial membrane fractions and in preparations of Q-I and Q-II (Table 1). Q-I and Q-II exhibited the same specificity observed for membrane fractions, showing that both solubilized fractions retained the pharmacological specificity characteristic of PBR. It should also be noted that in agreement with the results of Awad and Gavish [19], digitonin solubilization significantly lowered the apparent affinity for benzodiazepine derivatives in comparison to the affinity for PK 11195.

DISCUSSION

The studies described in this report provide direct biochemical evidence that fractionation of PKBS, the protein identified by photolabeling with [^{3}H]PK 14105, parallels the fractionation of PBR ligand binding activity. When rat adrenal mitochondrial membrane fractions were solubilized in digitonin and subjected to chromatography over Q-Sepharose, very similar patterns were obtained when comparing the [^{3}H]PK 14105-PKBS profile with that of the [^{3}H]Ro5-4864 and [^{3}H]PK 11195 binding profiles. These results suggest that the binding sites for benzodiazepines and for isoquinoline carboxamides continue to be in association with each other following solubilization and that PKBS cofractionates in unison with these recognition sites. Hence, this work demonstrates that the recognition sites for benzodiazepines and for isoquinoline carboxamides subfractionate as identical molecular entities and that there is a very intimate association of the PKBS protein with these binding sites.

The fractionation of PKBS and PBR ligand binding activity were found to predominate in two fractions using anion-exchange chromatography. These fractions, signified as Q-I and Q-II herein, were verified as corresponding to PBR by several methods. Both fractions exhibited specific high-affinity binding for PBR ligands of the benzodiazepine and isoquinoline carboxamide classes. The rank order of potency PK 11195 > Ro5-4864 > diazepam > clonazepam determined for these fractions is consistent with the binding specificity of PBR. Scatchard analysis of Q-I and Q-II detected a single class of high-affinity binding sites for Ro5-4864, as was observed with mitochondrial membranes. Solubilization therefore retains, for the most part, the distinctive binding properties of PBR as observed in both fractionated subpopulations.

The characteristics which distinguish Q-I from Q-II were apparent, using chromatographic techniques. Q-I was eluted from Q-Sepharose at salt concentrations lower than those required for the elution of Q-II. As determined by molecular exclusion chromatography in the absence of digitonin, the apparent molecular weight of Q-I was approximately 30,000 as compared to 200,000 for Q-II.

The reasons for observing a heterogeneous population of solubilized PBR are not clear at this point. It is possible that Q-I may represent a dissociated entity derived from Q-II. In agreement with this possibility was the demonstration that Q-II can be converted to Q-I under mild conditions. It is unlikely that this conversion was due to proteolytic action for two reasons. First, the conversion occurred in the presence of a mixture of protease inhibitors and second the conversion was reduced significantly by NaCl.

An alternative possibility to account for the two different fractions is that PBR may simply be incorporated into two classes of protein-detergent micelles. Support for this possibility is given by the observation that Q-I and Q-II initially bound to Q-Sepharose, whereas PKBS is predicted to be a basic protein based on its amino acid composition [33], indicating an association of PKBS with separate micelles having different anionic character. This possibility may account for our experience where even after taking precautions to ensure using precisely defined conditions during solubilization, we contined to observe a wide variation in the relative amounts of Q-II to Q-I, although the protein elution profiles remained relatively constant between separate experiments. This variation was still obtained in different solubilized preparations when using a single lot of digitonin and the same mitochondrial fractions.

Whereas each experiment varied in the relative quantities of Q-I and Q-II observed, there was always a close correlation between the profiles of photolabeled PKBS with those of specific PK 11195 and Ro5-4864 binding. These findings reinforce the suggestion that the PKBS protein is physically associated with PBR and most probably contains the binding domains for PBR ligands [33].

In contrast to photolabeling of the 17-kD PKBS protein by the isoquinoline carboxamide PK 14105, two different benzodiazepine affinity-labeling probes have identified a protein(s) of 30–35 kD. This suggests that different proteins contain the binding domains for these two classes of compounds; however, the possibility that these probes may also label (and thereby identify) other proteins either not associated or in close proximity with PBR cannot be discounted. The findings of this study, therefore, verify that PKBS is, in fact, associated with PBR. Similar investigations are also required with the benzodiazepine probes to verify if the 30–35 kD protein(s) is an integral component of the PBR complex.

The identification of a 30 kD PKBS-containing species from Q-I following gel filtration (permitting removal or release from detergent micelles) makes it compelling to determine whether this 30 kD species has specific binding sites for Ro5-4864 and/or PK 11195. Unfortunately, attempts to provide direct evidence for this are inconclusive because this 30 kD species, isolated after gel filtration, fails to be retained on polyethylenimine-treated filters as would be expected for a positively charged protein. Other methods to detect soluble binding activity have thus far proven unsuitable in our hands. Thus, our present data neither support nor refute whether the 30 kD species can bind PBR ligands; however, McEnery *et al.* [36] recently have achieved purification and reconstitution of PKBS in a native state and demonstrated that it does bind Ro5-4864 and PK 11195. Since the 30 kD species reported here has not been purified to homogeneity, it is still not known whether PKBS is the only protein component of this molecular unit or whether PKBS is represented as a monomer or dimer in this form.

REFERENCES

1. Braestrup C and Squires RF, Specific benzodiazepine receptors in rat brain characterized by high affinity [^{3}H]diazepam binding. *Proc Natl Acad Sci USA* **74**: 3805–3809, 1977.
2. Wang JKT, Taniguchi T and Spector S, Structural requirements for the binding of benzodiazepines to their peripheral-type sites. *Mol Pharmacol* **25**: 349–351, 1984.
3. Marangos PJ, Patel J, Boulenger JP and Clark-Rosenberg R, Characterization of peripheral-type benzodiazepine binding sites in brain using [^{3}H]Ro5-4864. *Mol Pharmacol* **22**: 26–32, 1982.
4. Shoemaker H, Boles RG, Horst WD and Yamamura HI, Specific high-affinity binding sites for [^{3}H]Ro5-4864 in rat brain and kidney. *J Pharmacol Exp Ther* **225**: 61–69, 1983.
5. De Souza EB, Anholt RRH, Murphy KMM, Snyder SH and Kuhar MS, Peripheral-type benzodiazepine receptors in endocrine organs: Autoradiographic localization in rat pituitary, adrenal and testis. *Endocrinology* **116**: 567–573, 1985.
6. Anholt RRH, De Souza EB, Oster-Granite ML and Snyder SH, Peripheral-type benzodiazepine receptors: Autoradiographic localization in whole-body sections of neonatal rats. *J Pharmacol Exp Ther* **223**: 517–526, 1985.
7. Anholt RRH, Mitochondrial benzodiazepine receptors as potential modulators of intermediary metabolism. *Trends Pharmacol Sci* **7**: 506–511, 1986.
8. Anholt RRH, Pedersen PL, De Souza EB and Snyder SH, The peripheral-type benzodiazepine receptor. *J Biol Chem* **261**: 576–583, 1986.

9. Antkiewicz-Michaluk L, Guidotti A and Krueger KE, Molecular characterization and mitochondrial density of a recognition site for peripheral-type benzodiazepine ligands. *Mol Pharmacol* **34**: 272–278, 1988.
10. Mukhin AG, Papadopoulos V, Costa E and Krueger KE, Mitochondrial benzodiazepine receptors regulate steroid biosynthesis. *Proc Natl Acad Sci USA* **86**: 9813–9816, 1989.
11. Papadopoulos V, Mukhin AG, Costa E and Krueger KE, The peripheral-type benzodiazepine receptor is functionally linked to Leydig cell steroidogenesis. *J Biol Chem* **265**: 3772–3779, 1990.
12. Hirsch JD, Beyer CF, Malkowitz L, Beer B and Blume AL, Mitochondrial benzodiazepine receptors mediate inhibition of mitochondrial respiratory control. *Mol Pharmacol* **35**: 157–163, 1989.
13. Le Fur G, Perrier ML, Vaucher N, Imbault F, Flamier A, Uzan A, Renault C, Dubroeucq MC and Gueremy C, Peripheral benzodiazepine binding sites: Effects of PK 11195, 1-(2-chlorophenyl)-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide. I. *In vitro* studies. *Life Sci* **32**: 1839–1847, 1983.
14. Le Fur G, Vaucher N, Perrier ML, Flamier A, Benavides J, Renault C, Dubroeucq MC, Gueremy C and Uzan A, Differentiation between two different ligands for peripheral benzodiazepine binding sites, [^{3}H]Ro5-4864 and [^{3}H]PK 11195, by thermodynamic studies. *Life Sci* **33**: 449–457, 1983.
15. Snyder SH, Verma A and Trifiletti RR, The peripheral-type benzodiazepine receptor: A protein of mitochondrial outer membranes utilizing porphyrins as endogenous ligands. *FASEB J* **1**: 282–288, 1987.
16. Verma A and Snyder SH, Characterization of porphyrin interactions with peripheral type benzodiazepine receptors. *Mol Pharmacol* **34**: 800–805, 1988.
17. Lukeman DS and Fanestil DD, Interactions of diuretics with a renal benzodiazepine binding site in rat kidney. *J Pharmacol Exp Ther* **241**: 950–955, 1987.
18. Hirsch JD, Beyer CF, Malkowitz L, Loullis CC and Blume AL, Characterization of ligand binding to mitochondrial benzodiazepine receptors. *Mol Pharmacol* **35**: 164–172, 1989.
19. Awad M and Gavish M, Differential effect of detergents on [^{3}H]Ro5-4864 and [^{3}H]PK 11195 binding to peripheral-type benzodiazepine binding sites. *Life Sci* **43**: 167–175, 1988.
20. Beaumont K, Skowronski R, Vaughn DA and Fanestil DD, Interactions of lipids with peripheral-type benzodiazepine receptors. *Biochem Pharmacol* **37**: 1009–1014, 1988.
21. Havoundjian H, Cohen RM, Paul SM and Skolnick P, Differential sensitivity of "central" and "peripheral" type benzodiazepine receptors to phospholipase A_2. *J Neurochem* **46**: 804–811, 1986.
22. Benavides J, Begassat F, Phan T, Tur C, Uzan A, Renault C, Dubroeucq MC, Gueremy C and Le Fur G, Histidine modification with diethylpyrocarbonate induces a decrease in the binding of an antagonist, PK 11195, but not of an agonist, Ro5-4864, of the peripheral benzodiazepine receptors. *Life Sci* **35**: 1249–1256, 1984.
23. Doble A, Ferris O, Burgevin MC, Menager J, Uzan A, Debroeucq MC, Renault C, Gueremy C and Le Fur G, Photoaffinity labeling of peripheral-type benzodiazepine binding sites. *Mol Pharmacol* **31**: 42–49, 1987.
24. Skowronski R, Fanestil DD and Beaumont K, Photoaffinity labeling of peripheral-type benzodiazepine receptors in rat kidney mitochondria with [^{3}H]PK 14105. *Eur J Pharmacol* **148**: 187–193, 1988.
25. McCabe RT, Schoenheimer JA, Skolnick P, Newman AH, Rice KC, Reig J-A and Klein DC, [^{3}H]AHN 086 acylates peripheral benzodiazepine receptors in the rat pineal gland. *FEBS Lett* **244**: 263–267, 1989.
26. Martini C, Giannaccini G and Lucacchini A, Solubilization of rat kidney benzodiazepine binding sites. *Biochim Biophys Acta* **728**: 289–292, 1983.
27. Benavides J, Menager J, Burgevin MC, Ferris O, Uzan A, Gueremy C, Renault C and Le Fur G, Characterization of solubilized "peripheral-type" benzodiazepine binding sites from rat adrenals by using [^{3}H]PK 11195, an isoquinoline carboxamide derivative. *Biochem Pharmacol* **34**: 167–170, 1985.
28. Gavish M and Fuares F, Solubilization of peripheral benzodiazepine binding sites from rat kidney. *J Neurosci* **5**: 2889–2893, 1985.
29. Anholt RRH, Aebi U, Pedersen PL and Snyder SH, Solubilization and reassembly of the mitochondrial benzodiazepine receptor. *Biochemistry* **25**: 2120–2125, 1986.
30. Doble A, Burgevin MC, Menager J, Ferris O, Begassat F, Renault C, Dubroeucq MC, Gueremy C, Uzan A and Le Fer G, Partial purification and pharmacology of peripheral-type benzodiazepine receptors. *J Recept Res* **7**: 55–70, 1987.
31. Parola AL, Putnam CW, Russell DH and Laird HE II, Solubilization and characterization of the liver peripheral-type benzodiazepine receptor. *J Pharmacol Exp Ther* **250**: 1149–1155, 1989.
32. Antkiewicz-Michaluk L, Mukhin AG, Guidotti A and Krueger KE, Purification and characterization of a protein associated with peripheral-type benzodiazepine binding sites. *J Biol Chem* **263**: 17317–17321, 1988.
33. Sprengel R, Werner P, Seeburg PH, Mukhin AG, Santi MR, Grayson DR, Guidotti A and Krueger KE, Molecular cloning and expression of cDNA encoding a peripheral-type benzodiazepine receptor. *J Biol Chem* **264**: 20415–20421, 1989.
34. Bradford MM, 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.
35. Bruns RF, Lawson-Wendling K and Pugsley TA, A rapid filtration assay for soluble receptors using polyethylenimine-treated filters. *Anal Biochem* **132**: 74–81, 1983.
36. McEnery MW, Snowman AM and Snyder SH, Resolution and identification of the putative subunits of the peripheral benzodiazepine receptor of rat kidney mitochondria. *Soc Neurosci Abstr* **15**: 642, 1989.