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Photoaffinity Labeling of Peripheral Benzodiazepine Receptors in R-3327 Dunning Prostatic Tumors

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ABSTRACT. Photoaffinity labeling of peripheral benzodiazepine receptors (PBZr) in mitochondrial and microsomal fractions from AT-1, H, and G Dunning R-3327 tumor sublines was performed using the photoaffinity ligand ^{3}H-PK 14105. Subsequent sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) revealed specific labeling of a 17 kDa protein in the microsomal fractions from these tumors. In the mitochondrial fractions, however, two bands with the apparent molecular mass of 17 and 18 kDa in AT-1 and H tumors were identified, whereas only the 17 kDa band was present in G tumors. Using another ligand, ^{3}H-flunitrazepam, to photoaffinity label PBZr, proteins with molecular masses of 17, 30, 42, and 48 kDa were identified. These data suggest the possibility of different subclasses of PBZrs in Dunning prostatic tumors. BIOCHEM PHARMACOL 51;8:1009–1013, 1996.

KEY WORDS. mitochondria; PK 14105; benzodiazepine; photoaffinity labeling; neoplasm; Dunning tumors

There is considerable evidence that PBZr† are distinct, both pharmacologically and biochemically, from central-type benzodiazepine receptors. PBZr have been found in several tissues and it has been reported that PBZr density is higher in tumors, such as glioma, ovarian and colon carcinoma, than in the corresponding normal tissue [1–3]. The subcellular localization of PBZr has been found to be mainly in the outer mitochondrial membrane. The ligands most often used to detect and characterize PBZr are PK 11195, an isoquinoline derivative, and the benzodiazepine Ro5-4864. A photoaffinity ligand for PBZr (PK 14105), a derivative of PK 11195, has been developed and successfully used as a specific probe for PBZr in several tissues, such as rat heart [4], rat kidney [5], and human glioblastomas [6]. The 18 kDa mitochondrial protein labeled with ^{3}H-PK 14105 has been purified and cloned [7, 8], and this protein seems to be responsible for the isoquinoline-binding properties of the PBZr complex. Another photoaffinity ligand, the benzodiazepine ^{3}H-flunitrazepam, has also been used to label PBZr. Using this probe, 30 kDa and 32 kDa proteins that may partly be involved in the PBZr complex were labeled [9].

We have previously found extremely high concentrations of PBZr, using PK 11195 as a ligand, in Dunning rat prostatic tumors compared to the normal ventral or dorsolateral prostate [10, 11]. In contrast to normal prostate, where no binding to the microsomal fraction (p-fractions) could be detected, PBZr in prostatic tumors were also present in substantial amounts in p-fractions as well as in very high density in the mitochondrial fractions (m-fractions). A marked difference in the inhibition by diazepam and flunitrazepam of PK 11195 binding to PBZr in m- and p-fractions was also found [10, 11]. In the present study, we have used ^{3}H-PK 14105 and ^{3}H-flunitrazepam to examine whether or not the observed differences in ligand-binding properties of PBZr in the m- and p-fractions from the tumors may be attributed to different biochemical characteristics of PBZr.

MATERIAL AND METHODS

Chemicals

Tritiated PK 14105 [*N-methyl* ^{3}H] with a specific activity of 77 Ci/mmol was purchased from Dositek (Orsay, France). Flunitrazepam [*N-methyl*-^{3}H] with a specific activity of 85.8 Ci/mmol was supplied by NEN. Nonlabeled PK 11195 was a kind gift from Rhône-Poulenc Rorer R-D (Vitry Sur Seine, France) and nonlabeled flunitrazepam was a gift from Hoffman-La Roche (Basel, Switzerland). Ro5-4864 was obtained from Fluka (Buchs, Switzerland). The chemicals used for electrophoresis were from Pharmacia LKB (Uppsala, Sweden). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

Tumors and Subcellular Fractionation

The Dunning R-3327 prostatic tumor sublines, AT-1, H, and G tumor, with their varying androgen dependence,

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† *Abbreviations:* PBZr, peripheral benzodiazepine receptors; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; NEPHGE, non-equilibrium pH gradient electrophoresis; VDAC, voltage-dependent anion channel.

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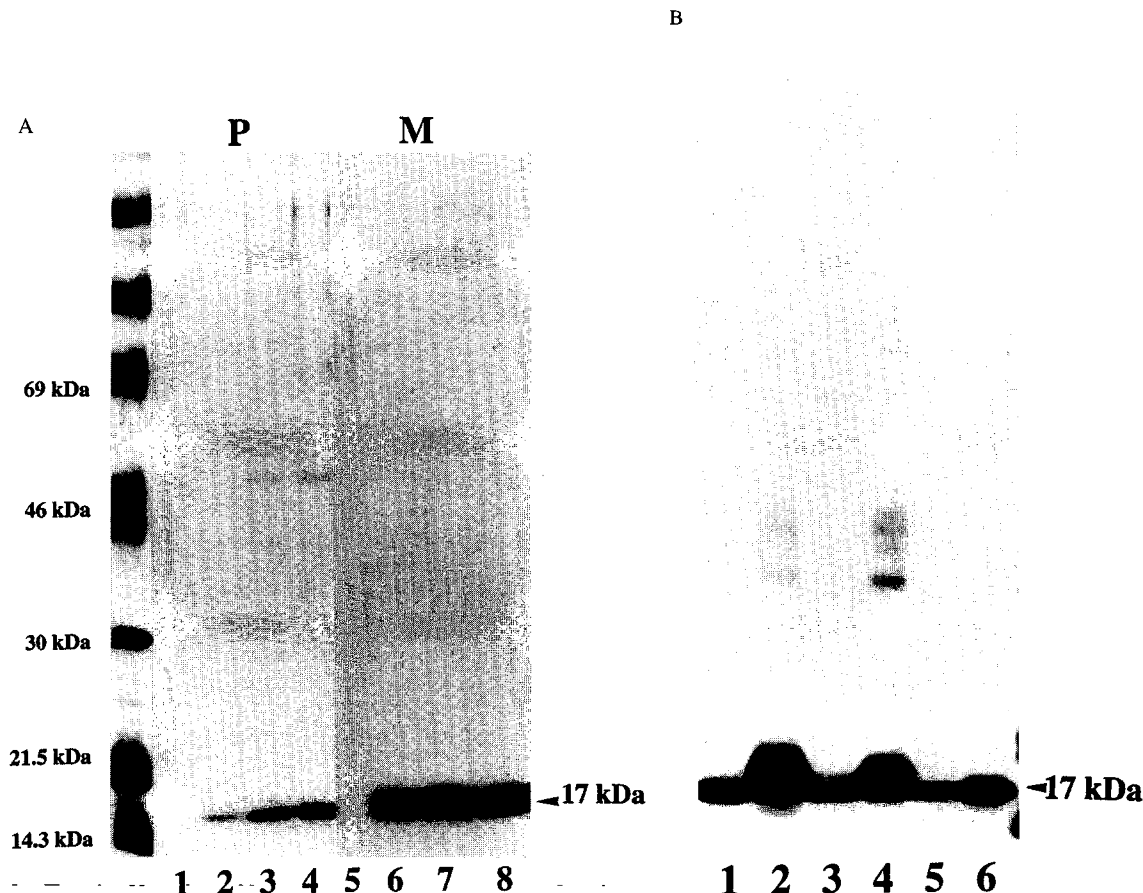

FIG. 1. Panel A, autoradiograms, of SDS-PAGE analysis of ^{3}H-PK 14105 photoaffinity-labeled PBZr in microsomal (P, Lanes 1–4) and mitochondrial (M, Lanes 5–8) fractions from AT-1 tumors. Lanes 1 and 5 show labeling in the presence of 10 µM PK 11195. Lanes 2 and 6 represent labeling after 30 min, lanes 3 and 7 after 60 min, and lanes 4 and 8 after 90 min irradiation. Arrows indicate position of the 17 kDa band. Panel B, visualization of photoaffinity-labeled proteins from AT-1, H, and G tumors in SDS gels after 60 min irradiation. Lanes 1, 3, and 5 show representative microsomal fractions and lanes 2, 4, and 6 data from mitochondrial fractions from AT-1, H, and G tumors, respectively.

histology, and growth rate, were kind gifts from Dr. John Isaacs (Johns Hopkins Hospital, Baltimore, MD). As previously reported by us, all three tumors contain high concentrations of PBZr in both the m- and p-fractions. Pieces of tumors, 1–2 mm^3, were implanted subcutaneously in Copenhagen × Fisher F_1 hybrids and allowed to grow for 27, 60–80, and 140–160 days for the AT-1, G, and H tumors, respectively. Mitochondrial and microsomal fractions from the tumors were prepared as described previously [10, 11]. Cytochrome oxidase activity in all fractions was measured according to Cooperstein and Lazarow [12].

Photolabelling of Membranes

Mitochondrial and microsomal preparations, 50 µg protein, were preincubated on ice in 1 mL ice-cold sucrose (0.25 M) -HEPES (10 mM) buffer (pH 9.0) containing 100 nM ^{3}H-PK 14105 in the absence or presence of 10 µM PK 11195 as described by Doble *et al.* [4]. After preincubation in the dark for 15 min, membrane suspensions were exposed to UV-light (Spectrolinker Xl 1000, Spectronics Corp., Westbury, NY) at 15 cm for 30, 60, and 90 min. Based on preliminary results (Fig. 1A) 60-min irradiation was found to be optimal. Membranes were then isolated by centrifugation. The m-fractions were centrifuged at 10,000 *g* for 15 min in an SW-60Ti rotor and the p-fractions at 105,000 *g* for 35 min. The pellets were washed once in the above buffer. Prior to electrophoresis the pellets were solubilized in 30 µL sample buffer (4% SDS; 10 mM dithiothreitol; 0.01% bromophenol blue; 100 mM Tris-HCl; pH 6.8) and 30 µL water, then heated at 95°C for 5 min. For photoaffinity experiments with flunitrazepam, the conditions were the same as for PK 14105, except that Ro5-4864 and nonlabeled flunitrazepam were used for determination of nonspecific binding.

Protein Electrophoresis

SDS-PAGE was run in a Protean II xi cell (length, 20 cm; Bio-Rad) using a standard buffer system and 14% homogeneous gels. Rainbow ^{14}C-molecular weight marker from Amersham (U.K.) was used for determination of molecular

mass. The gels were fixed, treated with Amplify (Amersham), dried and opposed to Kodak XAR5 film at –70°C for 1–3 weeks.

Two-dimensional nonequilibrium pH electrophoresis was run according to the method of O'Farrell *et al.* [13]. First dimension nonequilibrium pH gradient electrophoresis (NEPHGE) gels included 1.5% Ampholine 7–9 and 0.8% Ampholine 9–11 and were run for 4.5 hr at 400 V. The tube gels were incubated for 5 min at room temperature in equilibrium buffer (0.06 M Tris-HCl; pH 6.8; 2% SDS; 0.1 M dithiothreitol; 10% glycerol) before application to the second-dimension gel electrophoresis. Second-dimension gel electrophoresis was run according to the SDS-PAGE protocol described above.

RESULTS

PBZr from isolated mitochondrial and microsomal membrane fractions from Dunning AT-1, G, and H tumors were photoaffinity labeled with ^{3}H-PK 14105. Autoradiograms of the SDS gels are shown in Fig. 1. A single 17 kDa band was detected when photoaffinity-labeled proteins from p-fractions were separated by gel electrophoresis. However, in contrast to the single 17 kDa band in the m-fraction from G tumors, the same fractions from AT-1 and H tumors displayed two bands at 17 and 18 kDa after photoaffinity labeling with PK 14105. There was a small difference in molecular mass, approximately 1 kDa, between the two bands. No difference in molecular mass was found between the bands in p-fractions from the various tumors. Similarly no difference was detected in the position of the respective bands in m-fractions, two for each AT-1 and H tumor and one for the G tumor.

Data in Table 1 show cytochrome oxidase activity in m- and p-fractions from AT-1, H, and G tumors. Depending on the tumor, the activity of cytochrome oxidase in the m-fraction was 5–10 times higher than in the corresponding p-fraction.

The photoaffinity-labeled bands in m- and p-fractions from the AT-1 tumor were further characterized by two-dimensional NEPHGE (Fig. 2). In m-fractions from the AT-1 tumor, 2 major spots and 1 minor spot could be visualized in the autoradiogram, indicating a difference in isoelectric point. In the gels containing photoaffinity-labeled membranes from the p-fractions, a single spot was identified, which indicates the interaction of PK 14105 with only one protein species.

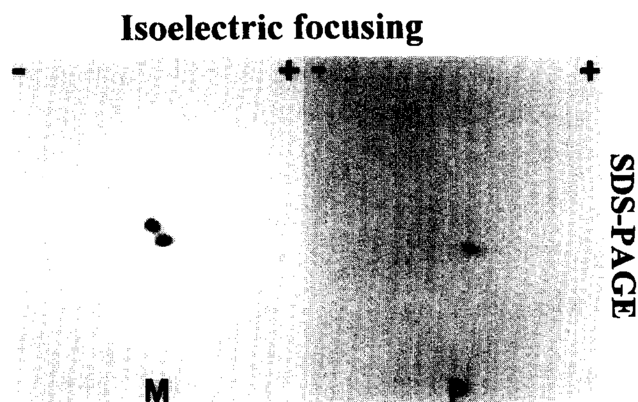

FIG. 2. Autoradiograms after two-dimensional NEPHGE analysis of ^{3}H-PK 14105 photoaffinity-labeled PBZr in mitochondrial (M) and microsomal (P) fractions from AT-1 tumors. Samples were irradiated for 60 min.

When flunitrazepam was used for photoaffinity labeling of proteins, two major bands with apparent molecular masses of 42 and 48 kDa could be distinguished in p-fractions (Fig. 3). In the m-fractions, a 30 kDa protein was identified as the most pronounced band. In contrast to the clearly visible 17 kDa band in the m-fraction from G tumor, no 17 kDa band was detected in m-fractions from AT-1 and H tumors. The specificity was generally low, but the labeling of some of the bands could be effectively blocked by an excess of flunitrazepam (Fig. 3).

DISCUSSION

The present data on various prostatic tumors are the first to show that two different proteins could be labeled by PK 14105 in a mitochondrial fraction. The photoaffinity-labeling data of m-fractions from the tumors using PK 14105 indicated two apparent bands in m-fractions from AT-1 and H tumors, but only one band in G tumor. Determination of cytochrome oxidase activity showed some contamination by mitochondria in the p-fraction, which was variable (10–20%) depending on the tumor. However, as shown in the photographs of the gels, only one band was present in the photolabeled proteins from p-fractions, sug-

TABLE 1. Cytochrome oxidase activity in isolated m- and p-fractions from AT-1, H, and G tumors

	Cytochrome oxidase activity (dA* min × mg protein)	
Tumor	**m-fraction**	**p-fraction**
AT-1	0.505 ± 0,004	0,105 ± 0,074**
H	2,18 ± 0,248	0,224 ± 0,055**
G	1,09 ± 0,167	0,084 ± 0,021**

* Change in absorbance. Values are means ± SEM of 3 separate determinations. Significance of difference from the corresponding m-fraction is denoted by ** $P < 0.001$.

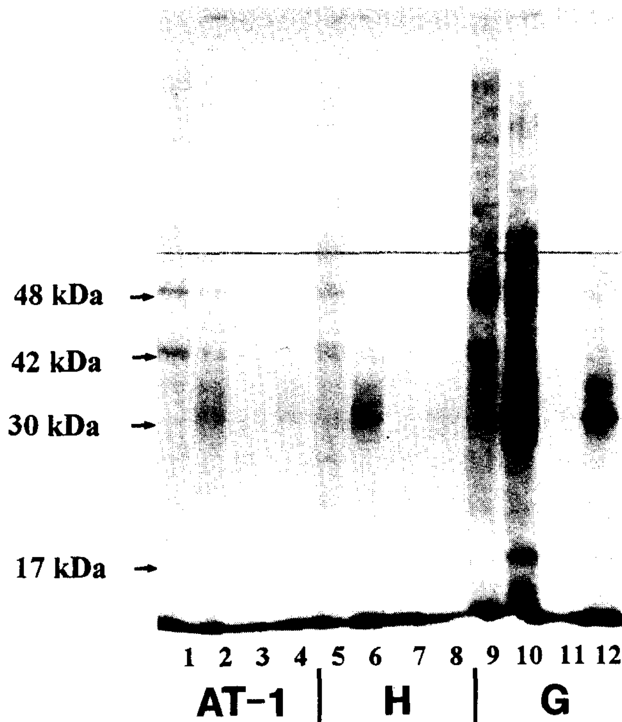

FIG. 3. SDS-PAGE analysis of ^{3}H-flunitrazepam photoaffinity-labeled proteins in microsomal (lanes 1, 5, and 9) and mitochondrial (lanes 2, 6, and 10) fractions from AT-1, H, and G tumors, respectively. Lanes 3, 7, and 11 and lanes 4, 8, and 12 represent microsomal and mitochondrial fractions from AT-1, H, and G tumors, respectively, labeled in the presence of 10 μM flunitrazepam. Samples were irradiated for 60 min.

gesting that contamination by mitochondria was of no significance. Two-dimensional NEPHGE analysis of labeled proteins from m-fractions from AT-1 tumors showed a difference in isoelectric point between the two major spots. This difference might be due to a different degree of phosphorylation or amino-acid content/composition.

Data from some previous studies strongly suggest that mitochondrial PBZr is a heterogeneous protein because the molecular mass ranged from 17 to 19 kDa, depending on the tissue examined [8, 14, 15]. Our data, showing bands in m-fractions at positions corresponding to 17 and 18 kDa proteins, are consistent with these observations.

We previously found extremely high levels of PBZr in m-fractions and fairly high levels in p-fractions from AT-1, H, and G Dunning prostatic tumors as compared to normal prostate. The G tumor had the highest density, followed by the H and AT-1 tumors [10, 11]. Data on the inhibition by diazepam and flunitrazepam in the above studies showed a highly significant difference in K_i values for these competitors in the m- and p-fraction from these tumors. These observations suggested the possibility of multiformity of PBZr in m- and p-fractions.

Riond *et al.* [16] have identified mitochondrial proteins in the range of 30 to 35 kDa in CHO cells that could be labeled with ^{3}H-PK 14105. Proteins of 30 to 35 kDa have also been photolabeled by ^{3}H-flunitrazepam in kidney mitochondria [9]. More recently, McEnery *et al.* [17] presented evidence indicating that the 18 kDa protein was associated with two proteins of 30 and 32 kDa. These proteins were identified as the adenine nucleotide carrier and the voltage-dependent anion channel (VDAC), respectively. On the basis of these observations, it was suggested that isoquinoline carboxamide and benzodiazepine bind to the 18 kDa PBZr and 32 kDa VDAC protein, respectively. Photoaffinity labeling of the m-fractions from the tumors with ^{3}H-flunitrazepam showed that although only one band with apparent molecular mass of 30 kDa was labeled in AT-1 and H tumors, a faint band corresponding to 17 kDa was also observed in G tumors. The nature or/and significance of this latter band is not clear because a 17 kDa band should normally not be labeled by ^{3}H-flunitrazepam. The 30 kDa protein found in our mitochondrial fractions might be identical to the adenine nucleotide carrier described by McEnery *et al.* [17]. However, we were not able to detect the 32 kDa VDAC protein. Recently, an interaction between benzodiazepine binding and adenine nucleotide carrier in vas deferens mitochondria was reported [18, 19]. We were, however, unable to see such an interaction in our competition studies using various nucleotides in m- or p-fractions from AT-1 tumors (unpublished data).

In our photolabelled p-fractions, bands with apparent molecular masses of 42 and 48 kDa were the most pronounced. Thomas *et al.* [20] have described a 48 kDa protein in membranes prepared from glioma cells by pelliting a fraction at 20,000 *g*. It is possible that the protein detected by us in the p-fraction is similar to the 48 kDa species described by these authors. Although the subcellular localization of this protein cannot be ascertained from the present data, it is not unlikely that it resides in the cell membrane because our p-fraction is enriched with plasma membrane. The possibility that this protein represents a subunit of the central benzodiazepine receptor (CBZr)) was investigated by using a specific ligand for CBZr (Ro 151788). No specific binding was detected in the p-fraction or even m-fraction, indicating the absence of central benzodiazepine proteins in our fractions (data not shown).

In summary, our data reinforce the suggestion that PBZr are composed of different proteins and/or are part of different protein complexes in different cell types. Our determination of proteins with different molecular mass labeled with ^{3}H-PK 14105 and ^{3}H-flunitrazepam in m- and p-fractions may, possibly, explain the variable binding spectrum of isoquinolines and benzodiazepines in preparations from different tissues. The finding of the difference in the pattern of photolabeled proteins in m- and p-fractions, particularly with ^{3}H-flunitrazepam, emphasizes that caution should be exercised when comparing data from studies where the identity of the subcellular fraction is poorly defined. Although highly elevated levels of PBZr are found in some tumors, such as prostatic tumors [10, 11], further studies are needed to define the role of PBZr in cell growth and tumorigenesis.

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