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Characterization of a peripheral-type benzodiazepine-binding site in the mitochondria of Chinese hamster ovary cells

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The isoquinoline carboxamide derivative [^{3}H]PK11195, a ligand for the peripheral-type benzodiazepine (BZD) receptor, binds to Chinese hamster ovary (CHO) cell mitochondria in a specific and saturable manner. Scatchard analysis showed the presence of a single-binding site with an apparent dissociation constant (K_d) of 12.0 ± 1.0 nM and a maximal binding capacity of 23.0 ± 2.0 pmol/mg protein. The pharmacological characterization of this CHO BZD-binding site, based on the displacement of [^{3}H]PK11195 by several drugs of known binding specificity, indicated that it is of the peripheral-type. The photoaffinity probe [^{3}H]PK14105, a nitrophenyl derivative of [^{3}H]PK11195, specifically labeled a 17 kDa CHO mitochondrial protein. This 17 kDa protein was purified from digitonin-solubilized mitochondria by gel-filtration chromatography and two reverse-phase HPLC steps. The purified material migrated as a single band on silver stained or autoradiographed SDS-polyacrylamide gels, and had an amino acid composition corresponding to a 17 kDa protein rich in Leu, Val, Ala, Gly, and Pro. Analysis of the amino-terminal sequence of the purified 17 kDa protein revealed a blocked amino-terminus.

Benzodiazepine; Receptor; Purification; HPLC; Photolabeling

1. INTRODUCTION

Besides their classical actions in the central nervous system, some BZDs interact with a second type of receptor which is mainly present in peripheral organs [1–4]. These peripheral-type BZD-binding sites can be selectively labeled either with a BZD derivative Ro5-4864 [5] or with an isoquinoline carboxamide derivative PK11195 [6].

Although insight into the physiological function of peripheral-type BZD receptors has recently emerged from several experimental approaches [7–9], the role of these receptors remains unclear. One approach to elucidating the molecular basis of the functional activity of the peripheral-type BZD receptors is to identify and purify them in order to clone and express these proteins.

It is the aim of this paper to describe a peripheral-type BZD-binding site present in the mitochondria of a cell easily cultured on a large scale, i.e. the CHO cell, and the purification and partial characterization of the putative binding protein with the aid of a nitrophenyl derivative of PK11195, the photoaffinity ligand [^{3}H]PK14105 previously described [10], that also binds with high affinity to the BZD-binding site present in these cells.

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2. MATERIALS AND METHODS

2.1. *Preparation of the CHO cell mitochondrial fraction*

The CHO cells [11] were cultured as described [12]. The cell layer was washed with PBS (65 mM Na_2HPO_4, 1.46 mM KH_2PO_4, 140 mM KCl, 140 mM NaCl) and incubated with 0.12% trypsin for 5 min at 37°C; the cells were removed from the roller bottles and trypsin was neutralized with 5% fetal calf serum. The cell suspension was centrifuged for 5 min at 500 × *g* and the pellet was washed 3 times with PBS at 37°C and finally suspended in 2 mM Hepes, 70 mM sucrose, 210 mM D-mannitol, 1 mM EDTA, 0.3 mM PMSF, pH 7.4, ice-cold buffer. Typically, 1.2×10^9 cells (0.3 g of protein) were homogenized in a glass homogenizer and then the mitochon-

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drial fraction was prepared as described by Anholt et al. [13]. Briefly, the homogenate was centrifuged 15 min at 650 × *g* at 4°C, the supernatant was centrifuged 15 min at 6500 × *g* at 4°C and the resulting pellet was, 3 times, resuspended in 2 mM Hepes, 70 mM sucrose, 210 mM D-mannitol, 1 mM EDTA, 0.3 mM PMSF, pH 7.4, buffer and centrifuged for 10 min at 10000 × *g* to obtain the mitochondrial preparation (25 mg of protein) that was stored at −20°C until used.

2.2. *Binding of [^{3}H]PK11195 and [^{3}H]PK14105 to the CHO mitochondrial preparation*

The mitochondrial preparation was washed and suspended in 25 mM Tris-HCl buffer, pH 7.4, at a final concentration of 100 μg of protein per ml. For the saturation experiments, the mitochondria were incubated with appropriate amounts of [^{3}H]PK11195 (66.0 Ci·mmol^{-1}, CEA, Gif-sur-Yvette, France) or [^{3}H]PK14105 (87.0 Ci·mmol^{-1}, CEA) for 1 h in the absence or presence of 500 times excess of unlabeled PK11195 (Sanofi). For the determination of the displacement potencies of PK11195, Ro5-4864 (Sanofi), diazepam (Sigma), flunitrazepam (Sigma), and clonazepam (Sigma), the mitochondrial preparation was incubated in the presence of [^{3}H]PK11195 (5 nM) with various concentrations of unlabeled drugs. In both cases, saturation and displacement, the experiments were performed on 0.26 ml aliquots of the prepared mitochondrial suspension in 96-well flat-bottomed microtiter plates at 4°C in a final volume of 0.3 ml, and the incubations were terminated by filtration under vacuum through GF/C glass fiber filters (Whatman) which had been previously soaked in 25 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100 and 0.1% bovine serum albumin. The filters were washed three times with ice-cold buffer, dried, and placed in 6 ml of Ready Safe liquid scintillation cocktail (Beckman), then the radioactivity was determined by liquid scintillation spectrometry. All values are expressed as specific binding obtained by the difference between the binding in the absence and presence of unlabeled PK11195. The triplicate determinations usually showed a variation of less than 10%.

2.3. *Photolabeling of the BZD-binding sites with [^{3}H]PK14105*

Photoaffinity labeling of the CHO mitochondrial BZD-binding sites was performed using [^{3}H]PK14105, essentially as described by Doble et al. [10]. Briefly, the mitochondrial preparation was washed 3 times with 5 mM Tris-HCl buffer, pH 7.4, containing 1 mM $MgCl_2$, 0.3 mM PMSF, 1 mM EDTA, 0.25 M sucrose, and resuspended in the same buffer at 0.4 mg of protein per ml. Aliquots of 0.25 ml were placed in 1.7 cm^2 cell culture dishes, [^{3}H]PK14105 was added to a final concentration of 100 nM, and samples were then irradiated at 4°C using an ultraviolet lamp (Model TF-35 L, Bioblock Scientific). In some experiments [^{3}H]PK14105 was preincubated with the mitochondria in the dark before ultraviolet irradiation. As controls, samples were incubated either with [^{3}H]PK14105 but not irradiated, or with [^{3}H]PK14105 in the presence of a 500 times excess of PK11195 during the irradiation. After the photolabeling, which was performed for times ranging from 10 to 120 min, the mitochondrial preparation was extensively washed with 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgCl_2$, 0.3 mM PMSF, 1 mM EDTA and subjected to SDS-PAGE followed by autoradiography, or used as a tracer for the purification as described below.

2.4. *Purification of a putative mitochondrial CHO BDZ peripheral binding site*

A fraction of freshly prepared CHO cell mitochondria (100 μg of protein) was photolabeled with [^{3}H]PK14105 by ultraviolet irradiation for 15 min at 4°C and, after extensive washing, added to unlabeled mitochondria (20 mg of protein). The mitochondrial preparation was resuspended in 2 mM Hepes, 70 mM sucrose, 210 mM D-mannitol, 1 mM EDTA, 0.3 mM PMSF, pH 7.4, buffer at a protein concentration of 2 mg·ml^{-1}, then extracted with digitonin (5 mg per mg protein) for 60 min at 4°C. The samples were then centrifuged at 15000 × *g*, the pellet was washed with the same buffer as described above, and then the supernatants were pooled. The clear pooled supernatant was concentrated to 2 ml in a Centricon 30 (Amicon) by centrifugation at 5000 × *g* at 4°C and loaded on a S300 Sephacryl (Pharmacia) gel filtration column (1.5 × 90 cm) equilibrated at 0.5 ml·min^{-1} in 5 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 0.3 mM PMSF, 0.1% Triton X-100. Eluting material was monitored at 278 nm and 2 ml fractions were collected. An aliquot of each fraction was used for radioactivity counting in a Beckman scintillation counter. The fractions containing radioactivity were pooled and applied to a radial compression separation system (Z Module, Waters Associates) equipped with a phenyl-μBondapack column (10 μm, 2.0 × 10.0 cm, Radial-PAK, Waters Associates). The column was washed with 0.1% trifluoroacetic acid (TFA) and then a gradient from 5 to 95% of acetonitrile, 0.1% TFA was applied in 60 min. The flow rate was 1.0 ml·min^{-1}. 1.0 ml fractions were collected and an aliquot of each was used for counting the radioactivity. A fraction eluting at 53% acetonitrile was diluted to 30% acetonitrile with 0.1% TFA and chromatographed on a BU 300 reverse-phase column (2.1 × 100 mm, Brownlee) using a gradient from 40 to 70% acetonitrile, 0.1% TFA, in 20 min, at 0.3 ml·min^{-1}. A main peak detected at 220 nm and containing more than 60% of the radioactivity injected was used for further analysis.

2.5. *SDS-PAGE, amino acid and NH_2-terminal sequence analysis*

Aliquots of the purified ^{3}H-labeled binding site or the freshly labeled CHO mitochondria were subjected to SDS-PAGE [14]. After electrophoresis the gel was either soaked in the fluorographic reagent Amplify (Amersham) and autoradiographed using Hyperfilm-MP (Amersham), or silver-stained with a kit available from Bio Rad.

For amino acid analysis, the purified samples were dried in borosilicate tubes, hydrolysed with gaseous HCl at 160°C for 1 h, derivatized to PTC-amino acids on a 420A Derivatizer (Applied Biosystems Inc.), and then analysed on a 120A separation system (Applied Biosystems Inc.) using the standard programs.

For NH_2-terminal sequencing, the purified ^{3}H-photolabeled putative BZD-binding site was directly loaded onto a polybrene-coated filter and automatically sequenced with a gas-phase protein sequencer (model 470A, Applied Biosystems Inc.) equipped with a 120A on-line PTH-amino acid analyser (Applied Biosystems Inc.).

For amino acid analysis and sequencing of the proteins isolated by SDS-PAGE, the SDS gel was equilibrated for 3 min in 10 mM Caps buffer, pH 11.0, 10% ethanol, and electroblotted onto a PVDF membrane (Millipore) as described [15]. The membrane was either colored with Ponceau S [16] or dried and

subjected to autoradiography using Hyperfilm-^{3}H. In both cases the part of the PVDF membrane containing the 17 kDa band was cut into several small pieces and either subjected to amino acid analysis after vapor-phase hydrolysis as described above, or deposited on a polybrene-coated filter and automatically sequenced.

3. RESULTS

3.1. *Binding of [^{3}H]PK11195 to the CHO mitochondrial preparation*

Binding of [^{3}H]PK11195 to the crude mitochondrial preparation was detected at a very low concentration of ligand and was linear with increasing amounts of mitochondria in the range studied (10–500 μg of protein per tube). At less than saturating concentrations, more than 95% of the total binding was specific. When the binding of [^{3}H]PK11195 was determined at increasing concentrations, a plateau was reached. The linear regression analysis of the Scatchard plot showed a single component site with a K_d value of 12.0 ± 1.0 nM and a maximal binding capacity of 23.0 ± 3.0 pmol/mg protein (fig.1). The pharmacological properties of the peripheral-type BZD-binding site present in the CHO cells were evaluated in displacement experiments of [^{3}H]PK11195 with cold PK11195, Ro5-4864, diazepam, flunitrazepam, and clonazepam. The results, shown in table 1, indicate the following order of potency: PK11195 > Ro5-4864 > diazepam = flunitrazepam ≫ clonazepam.

3.2. *Photolabeling of the mitochondrial BZD-binding sites with [^{3}H]PK14105*

The [^{3}H]PK14105 photoaffinity ligand binds to the mitochondrial peripheral-type BZD-binding sites in a saturable manner. The linear regression analysis of the Scatchard plot showed a single component site with a K_d value of 28.0 ± 3.0 nM and a maximal binding capacity of 21.0 ± 3.0 pmol/mg protein (fig.1). Photoaffinity labeling of the peripheral-type BZD-binding sites was performed, with or without preincubation in the dark of the mitochondrial preparation, with saturating concentrations of [^{3}H]PK14105 before ultraviolet irradiation. After irradiation, performed for different periods of time ranging from 10 to 120 min, the mitochondrial preparation was extensively washed and subjected to SDS-PAGE analysis followed by autoradiography. Typically,

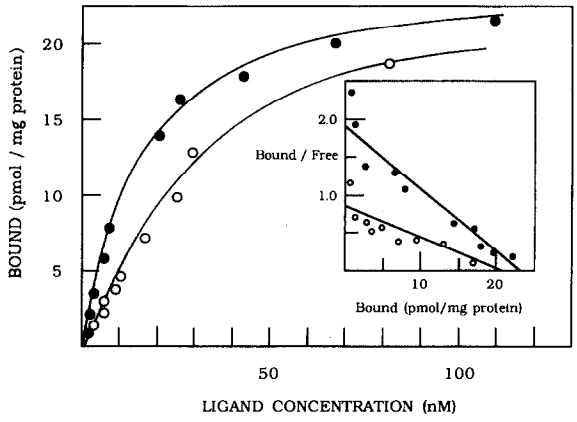

Fig.1. Saturation and Scatchard plots of [^{3}H]PK11195 (●) and [^{3}H]PK14105 (○) binding to the CHO cell mitochondrial preparation.

50–70% of the specific [^{3}H]PK14105 binding in the dark was irreversible after either 10 or 120 min of irradiation. The results shown in fig.2 indicated that a protein with an electrophoretical mobility corresponding to 17 kDa was labeled whether or not a preincubation in the dark was performed (lines 1 and 2). With increasing irradiation times, the 17 kDa band did not change, but several new minor bands were labeled (lines 3 and 4). The labeling of the main 17 kDa protein, as well as the minor ones, could be completely blocked by coincubation of the mitochondria with PK11195 during the irradiation (line 5). In the absence of irradiation, almost no radioactive bands were observed (line 6).

3.3. *Purification and chemical analysis of a putative BZD-binding site*

Freshly prepared CHO cell mitochondria (100 μg of protein) were photolabeled with

Table 1

Potency of several drugs in displacing [^{3}H]PK11195 binding from the CHO mitochondrial-binding sites

Drug	IC_{50}[a]	Relative potency
PK11195	3.2×10^{-8} M	100
Ro5-4864	7.9×10^{-7} M	4
Diazepam	1.6×10^{-5} M	0.2
Flunitrazepam	2.0×10^{-5} M	0.2
Clonazepam	$>1.0 \times 10^{-4}$ M	<0.01

[a] 50% binding inhibiting concentration

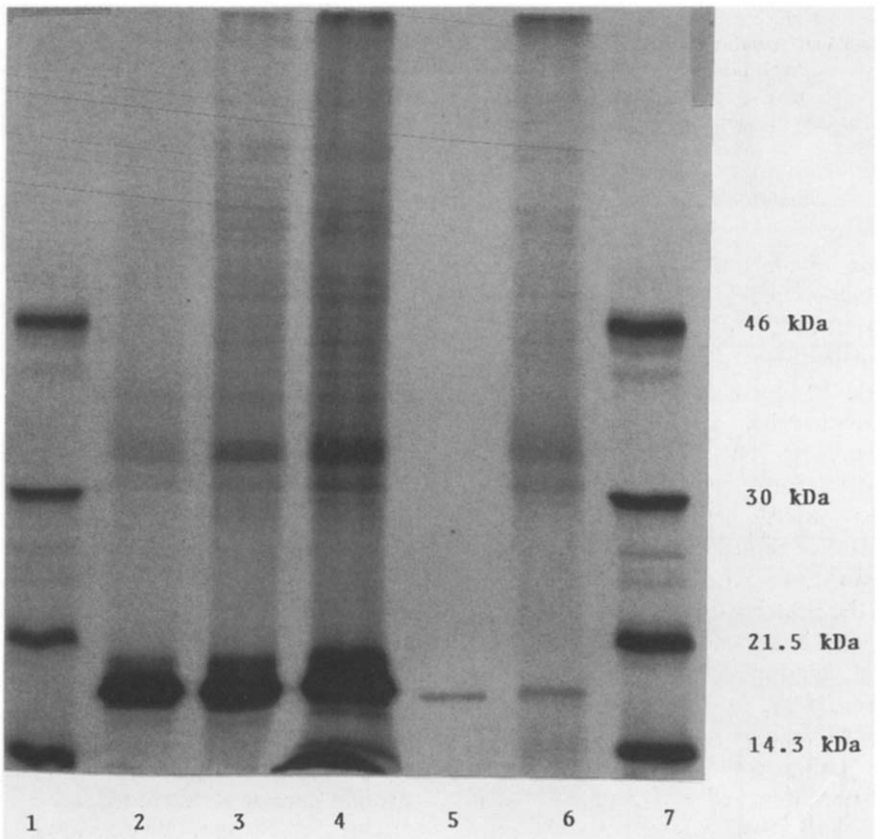

Fig.2. Autoradiograms of SDS-PAGE analysis of [^{3}H]PK14105 photolabeled CHO mitochondrial preparation. Lanes: 1 and 7, molecular mass standards; 2, membranes preincubated for 1 h in the dark at 4°C with [^{3}H]PK14105 and then UV irradiated for 10 min; 3, membranes incubated with [^{3}H]PK14105 and UV irradiated for 90 min; 4, membranes incubated with [^{3}H]PK14105 and UV irradiated for 120 min; 5, membranes incubated with [^{3}H]PK14105 in the presence of 10^{-6} M PK11195 and UV irradiated for 90 min; 6, membranes incubated with [^{3}H]PK14105 for 90 min but not UV irradiated.

[^{3}H]PK14105 and added to unlabeled mitochondria (20 mg of protein). The preparation was then extracted with digitonin, concentrated, and loaded on a gel-filtration column. A main peak, containing more than 90% of the loaded radioactivity, was found between the elution positions of bovine serum albumin and human growth hormone; the rest of the radioactivity eluted in the dead volume of the column (fig.3A). The radioactive fractions of the main peak were pooled and chromatographed on a phenyl reverse-phase HPLC column. Radioactivity determination of the collected fractions showed three main peaks (fig.3B). SDS-PAGE followed by autoradiography indicated that the 17 kDa protein was present in the peak eluting at 40 min. Silver-stained SDS-PAGE analysis of the material eluting in this peak showed a major band of 17 kDa and several minor bands with lower and higher molecular masses. These minor contaminants were eliminated by a second HPLC step on a butyl reverse-phase column. The material eluting with the single radioactive peak at 17 min (fig.3C), showed on silver-stained SDS-PAGE a single 17 kDa band that co-migrated with the radiolabeled protein (fig.4). The amino acid composition of the HPLC purified material and the 17 kDa protein electroblotted from SDS-PAGE are shown in table 2. NH_2-terminal sequence analysis of either the purified material, or the 17 kDa band electroblotted from SDS-PAGE onto a PVDF membrane, usually failed to give a sequence. In two out of six purifications, however,

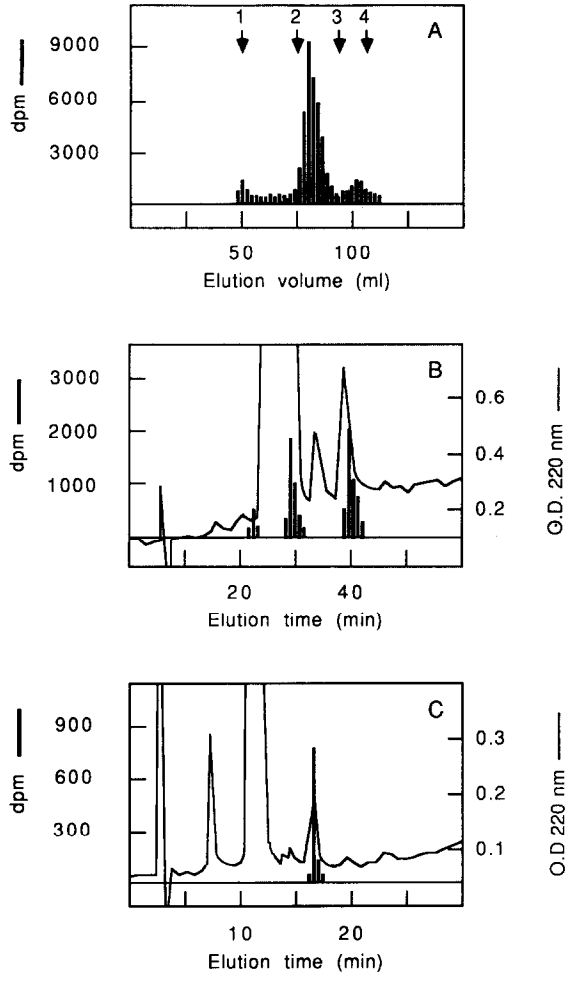

Fig.3. Purification of the 17 kDa putative peripheral BDZ-binding site from the photolabeled CHO mitochondrial preparation. Typical elution profile from: (A) gel-filtration chromatography (molecular mass markers are 1, blue dextran 2000 (2×10^6); 2, bovine serum albumin (6.7×10^4); 3, human growth hormone (2.2×10^4); and 4, tryptophan (2.0×10^2); (B) reverse-phase HPLC on phenyl-μBondapack column; (C) reverse-phase HPLC on C4 Brownlee column.

a single sequence was detected, but the initial yields in both cases were very low, 3–6% (calculated from the amino acid analysis of the material deposited on the filter). This sequence was Ala-Pro-Ser-X-Val-Pro-Ala-Val-Gly-Leu. The repetitive yields, calculated from the linear regression analysis of the amount of PTH-amino acids recovered after each degradation cycle, were 88–90%. These sequencing experiments suggest

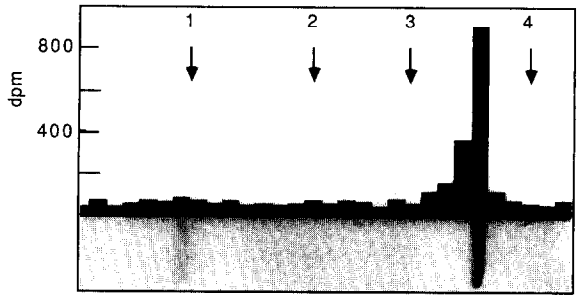

Fig.4. SDS-PAGE of the purified photolabeled 17 kDa putative peripheral BDZ-binding site. The same sample was loaded in parallel lanes of a gel and then either silver-stained (A) or sliced and used for tritium localization (B). The arrows correspond to the following molecular mass markers: 1, ovalbumin (46.0×10^3); 2, carbonic anhydrase (30.0×10^3); 3, trypsin inhibitor (21.5×10^3); and 4, lysozyme (14.3×10^3).

that the purified protein has the N-terminal blocked, and that the sequence obtained may correspond to an internal sequence of the putative BZD-binding site resulting from N-terminal degradation of the protein during purification. The presence of a contaminating protein with a sequenceable N-terminal that co-purified with this protein cannot be excluded.

Table 2

Amino acid composition of the purified putative benzodiazepine-binding site

Amino acid	HPLC purified protein[a]	Blotted protein[a]
Asx	7.5 ± 0.7	9.0 ± 1.0
Glx	9.0 ± 1.4	11.5 ± 3.5
Ser	12.7 ± 0.6	6.5 ± 0.6
Gly	17.2 ± 1.3	15.5 ± 0.7
His	5.3 ± 0.2	2.0 ± 1.0
Arg	13.5 ± 1.0	9.0 ± 1.0
Thr	9.2 ± 1.6	9.5 ± 2.1
Ala	16.3 ± 0.6	21.0 ± 0.7
Pro	12.0 ± 1.0	15.5 ± 0.7
Tyr	8.3 ± 0.6	9.5 ± 0.7
Val	6.0 ± 0.5	10.0 ± 1.4
Met	1.5 ± 0.5	3.0 ± 1.4
Ile	2.0 ± 0.3	2.5 ± 0.7
Leu	16.7 ± 4.0	23.5 ± 3.5
Phe	5.7 ± 0.6	6.0 ± 1.5
Lys	3.7 ± 0.6	2.0 ± 1.4

[a] The values are the mean of triplicate determinations and the compositions are calculated for a 17–18 kDa protein. Cys and Trp are not determined

4. DISCUSSION

The isoquinoline carboxamide derivative [^{3}H]PK11195, a selective ligand for the peripheral-type BZD-binding site, binds to CHO cell mitochondria in a specific and saturable manner with an apparent K_d of 12.0 ± 1.0 nM and a maximal binding capacity of 23.0 ± 2.0 pmol/mg protein. This receptor density is similar to the one described for the rat testis and lung mitochondria (25–35 pmol/mg protein) [3,4,19,20], two of the tissues, together with the adrenal gland (90–120 pmol/mg protein), for which the highest density of binding sites has been reported [3,4,17,20]. The pharmacological characterization of this CHO BZD-binding site, based on the displacement of [^{3}H]PK11195 by several drugs of known binding specificity, indicated that it is of the peripheral-type [1–4,17].

We extended the characterization of the CHO mitochondria-binding site with the use of another isoquinoline carboxamide compound, [^{3}H]PK-14105, a molecule whose pharmacological profile is nearly identical to that of PK11195 [10–19]. PK14105 is a nitrophenyl derivative of PK11195 that can be covalently incorporated into the BZD-binding site when exposed to ultraviolet light [10]. [^{3}H]PK14105 binds with high affinity to the CHO mitochondrial preparation and, in our experimental conditions, 50–70% of the specific binding in the dark was irreversible after irradiation. When the mitochondria were incubated with the photoaffinity probe and irradiated for 10 min, a 17 kDa protein was predominantly labeled providing further evidence for the identification of the CHO-binding site with those previously described [10,18–20]. However, with longer irradiation times several other proteins were labeled, the most important of which were in the 31–35 kDa range. Whether or not these proteins are associated with the 30 kDa protein or with the voltage-dependent anion channel, labeled with [^{3}H]flunitrazepam in rat kidney mitochondria, described by Snyder et al. [21] is currently being studied in our laboratory. In conclusion, these labeling experiments showed that the 17 kDa protein is the main binding site of [^{3}H]PK14105, but also that other proteins can be part of, or be associated with the BZD receptor present in the CHO cells.

Since the BZD-binding sites are abundant in the CHO mitochondria, we used these cells as the starting material for the purification of the main binding protein. Using the photolabeled material as tracer, a 17 kDa protein was purified from digitonin-solubilized CHO mitochondria by gel-filtration chromatography and two reverse-phase HPLC steps. The purified material showed a single band on silver-stained SDS gels that corresponded to the only band detected by radioautography of the same gel. Analysis of the purified protein in several different chromatographic systems failed to separate the 17 kDa protein from the radioactivity (data not shown), strongly suggesting identity of the silver-stained and radioactive material. The amino acid analysis of the HPLC purified protein and of the 17 kDa protein electroblotted after SDS-PAGE are similar and within the expected variability when working in the low picomolar range. Minor differences in His, Pro and Val contents may reflect the presence of residual contaminants in the HPLC purified material that, in some preparations, were detected in SDS-PAGE. Attempts to sequence the N-terminal of the 17 kDa protein usually failed. However, in some batches a sequence was obtained that did not show any homology when compared to the sequences present in the GenBank data bank. The lack of an N-terminal sequence and, when observed, the very low initial yield in the sequencing experiments suggest that the N-terminal of the purified protein is blocked, and that the sequence obtained may correspond either to an internal sequence of the BZD-binding site resulting from N-terminal degradation of the protein during the purification, or to a non-blocked contaminant protein that co-purified with this protein. Work is in progress in our laboratory to obtain, purify, and sequence peptides from this protein, as well as to determine whether or not the labeled protein is the functional BZD receptor.

In conclusion, we described a peripheral-type BZD-binding site in CHO cells, and the purification and partial characterization of a putative 17 kDa binding protein from the mitochondria of these cells. Since CHO cells have a high density of binding sites and are easily cultured on a large scale, they provide an interesting source for this receptor which may facilitate its complete characterization and lead to the cloning of the gene.

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