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Molecular basis for the different binding properties of benzodiazepines to human and bovine peripheral-type benzodiazepine receptors

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The 18 kDa peripheral benzodiazepine receptor (PBR) can be labelled by benzodiazepines, such as Ro5–4864, and isoquinoline carboxamides such as PK11195. These two compounds are reversible competitive inhibitors of each other. However, while the binding affinity of Ro5–4864 varies enormously across species, PK11195 always displays high affinity, suggesting that their binding domains are overlapping but not identical. We report here that recombinant human and bovine PBR produced in yeast, a microorganism devoid of endogenous PBR, can be labelled with [^{3}H]PK11195, but only the human receptor can be labelled with [^{3}H]Ro5–4864. Furthermore, we identified, through the binding analysis of human-bovine chimaeric receptors, a region near the C-terminal end of the PBR, with only five non-conserved amino acids between human and bovine sequences, as responsible for the difference in high affinity binding of Ro5–4864 to the two receptors.

Chimaeric; Mitochondrial receptor; Recombinant yeast; PK11195; Ro5–4864

1. INTRODUCTION

The psychoactive benzodiazepine (Bz) drugs exert sedative, anxiolytic and anticonvulsant effects through the central benzodiazepine receptor associated with the GABA-regulated chloride channels located in the central nervous system [1]. Bz also interacts with a second receptor originally described in rat peripheral tissues and designated as the peripheral benzodiazepine receptor (PBR) [2]. This receptor is expressed in almost all tissues and abundantly in steroid-producing cells [3,4]. PBR is a small protein (18 kDa) located mainly in the outer membrane of the mitochondria [5], but it is also present on the membrane of erythrocytes and adrenal cells [6,7]. A physiological role for the mitochondrial PBR has been demonstrated in adrenocortical cells [8,9]. A more general regulatory role has been suggested for the PBR present in the glial cells in the brain [10], but no clear function has been ascribed either to the mitochondrial receptor present in non steroid-producing cells, or to the cell membrane receptor. The cDNAs for the rat [11], bovine [12], and human [13] proteins have been recently cloned and the deduced amino acid sequences show that the 18 kDa PBR is a hydrophobic protein with five potential transmembrane regions. PBR can be labelled not only with Bz, such as Ro5–4864, but also with isoquinoline carboxamides (IQC), such as PK11195. In fact, IQC, which are structurally different from Bz, have much greater selectivity for PBR than for the central benzodiazepine receptor [14]. Although Bz and IQC are able to displace each other from PBR [11–14,16], there is evidence that suggests that the binding domains of the two compounds are not identical. For example, Bz and IQC binding to rodent receptors can be differentially perturbed by several enzymatic or chemical modifications [17–19]. Furthermore, it has been shown that high affinity binding for the IQC PK11195 is conserved across species, while marked differences have been described for the binding of the Bz Ro5–4864 [20]. It is difficult to rule out that other proteins in the membrane environment may be responsible for this Ro5–4864 binding behaviour since it has been shown that several proteins are closely associated to the 18 kDa PBR [21,22]. The present study examines the molecular basis of the differences in the Bz Ro5–4864 binding to the human and bovine 18 kDa PBR. We constructed chimaeric receptors with segments of the human PBR, which binds PK11195 and Ro5–4864 [13], and segments of the bovine receptor which binds only PK11195 [12]. Then, the human, bovine, and chimaeric receptors were produced in yeast, thus providing a homogeneous cellular environment devoid of endogenous PBR [23], and analysed for binding of PK11195 and Ro5–4864. Our results show that the differential binding of Ro5–4864 to the human and bovine receptors is due to amino acid differences between the two PBRs; that Bz and IQC binds to overlapping but not identical sites on the PBR; and that a small sequence near the

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Abbreviations: PBR, peripheral benzodiazepine receptor; Bz, benzodiazepine; IQC, isoquinoline carboxamide.

C-terminal end of the molecule is involved in the binding of Ro5–4864 but not of PK11195.

2. MATERIALS AND METHODS

2.1. *Bacteria and yeast strains*

Escherichia coli strains were TG1 (*hsdS, proAB, lacZX174*, {F' pro+, DM15}) and S17–1 (*recA, thi, pro, hsdR [RP4 : 2-Tc :: Mu ; Kn :: Tn7]* [24]. C13ABYS86 (*MATa, ura3, leu2, his3, pra1, prb1, prc1, cps1*) is a multiprotease-deficient strain of *Saccharomyces cerevisiae* obtained from D. Wolf [25]. Bacterial strains were grown in LB medium containing 100 μg/ ml ampicilline, or in 2YT [26]. Yeast strains were grown in complex YP (1% yeast extract, 2% bacto-peptone) or minimal YNB (0.67% yeast nitrogen base) media supplemented with either 2% glucose or 2% glycerol and 2% galactose.

2.2. *Plasmid constructions, yeast transformation and* E. coli–S. cerevisiae *DNA transfer*

All the yeast expression vectors used throughout this study are derived from pEMR614 [23]. The non-translated sequences located at the 3'-end of the PBR cDNA were deleted from pEMR614 to yield pEMR763. Plasmid pEMR780 differed from pEMR763 by the addition of the RP4-derived 760 bp fragment that contained *oriT*. This small fragment, which flanks the 3'-end of the *PGK* terminator in pEMR780 (Fig. 1), is required for RP4-encoded transacting proteins to direct the in vivo transfer of the shuttle plasmid from a *E. coli* donor to a *Saccharomyces cerevisiae* recipient, namely S17–1 and C13ABYS86, respectively. A synthetic bovine PBR gene was constructed by standard methods [27], the published sequence [12] being slightly modified to introduce restriction sites found in the human sequence to facilitate the obtention of chimaeras (Fig. 2B). A truncated human receptor S-II was obtained by introducing a stop codon after arginine-156 by site directed mutagenesis (Amersham).

For the transkingdom-conjugation mid-exponentially growing donor cells were harvested and resuspended in 10 mM $MgSO_4$ to a final density of 2×10^9/ml. Recipient c13ABYS86 cells were grown overnight in YPG, pelleted and resuspended in 10 mM $MgSO_4$ to 10^8 cells/ml. Donors and recipient cells were mixed in a ratio of 1:1 on a HP25 millipore filter, incubated on YPG-agar plates for 8 to 16 h, resuspended in 10 mM $MgSO_4$ and finally spread onto selective YNBG medium at 30°C. Exconjugants were identified by their ability to form colonies on a pyrimidine-free or a leucine-free growth medium.

2.3. *Recombinant receptor expression, subcellular fractionation, and binding of [^{3}H]PK11195 and [^{3}H]Ro5–4864*

Yeast transformants were grown in YNBG supplemented with 0.5% casaminoacids for 16 h. Cells were washed and resuspended in YP medium containing galactose and glycerol. Cells were then grown at 30°C overnight. Mitochondria were isolated as described [28], and used for the binding experiments.

Saturation experiments with [^{3}H]PK11195 (66.0 Ci/mmol, CEA, Gif-sur-Yvette, France) or with [^{3}H]Ro5–4864 (63.0 Ci/mmol, CEA, Gif-sur-Yvette, France) were performed on the mitochondrial fraction of the cell-free extracts as previously described [22,23]. Specific binding was calculated as the difference between the binding in the absence and in the presence of unlabeled PK11195 (Sanofi) or Ro5–4864 (Sanofi); non-specific binding represented less than 10% of total binding. Scatchard analysis was performed as previously described [23].

3. RESULTS

Human and bovine PBRs are 169 amino acid proteins with 5 putative transmembrane domains (Fig. 2A). Recombinant human PBR expressed in yeast binds the IQC [^{3}H]PK11195, and the Bz [^{3}H]Ro5–4864, while the recombinant bovine PBR expressed in the same system

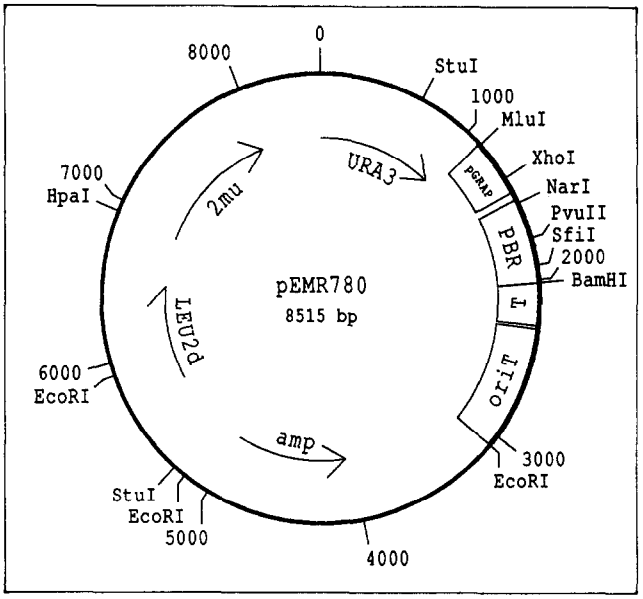

Fig. 1. Structure of pEMR780. The pEMR780 plasmid (8515 bp) is derived from the already described pEMR614 [24]. This plasmid contains the ampicillin-resistance gene of pBR322 (amp), the defective LEU-α gene cloned inside the 2μ fragment that contains the origin of replication and the cis-acting STB locus, the URA3 marker and the PBR-expression cassette.

binds only the IQC (Table I). To identify the regions of the human PBR responsible for the high affinity binding of benzodiazepines we carried out a systematic exchange of segments of the human with the corresponding bovine PBR segments (Fig. 2B). The resulting chimaeric receptors were expressed in yeast, and a mitochondrial enriched fraction was tested for binding with [^{3}H]PK11195 and [^{3}H]Ro5–4864. The first two mutants constructed, C-I and C-II (Fig. 2B) combined the N- and C-terminal halves of the human sequence with the C- and N-terminal halves of the bovine receptor sequences. Scatchard analysis of the saturation curves showed that both C-I and C-II chimaeric receptors were

Table I

Affinity of the chimeric human-bovine and deleted human PBR for the isoquinoline carboxamide PK11195 and the benzodiazepine Ro5-4864

	Receptor	K_d (nM)	
		PK11195	Ro5-4864
Wh	h1-169	8 ± 2	14 ± 7
Wb	b1-169	18 ± 8	> 200
C-I	b1-88-h89-169	13 ± 5	22 ± 9
C-II	h1-88-b89-169	22 ± 10	> 200
C-III	h1-88-b89-143-h144-169	10 ± 4	14 ± 4
C-IV	h1-143-b144-169	5 ± 3	> 200
C-V	b1-143-h144-169	14 ± 4	13 ± 3
S-II	h1-156	9 ± 7	12 ± 5

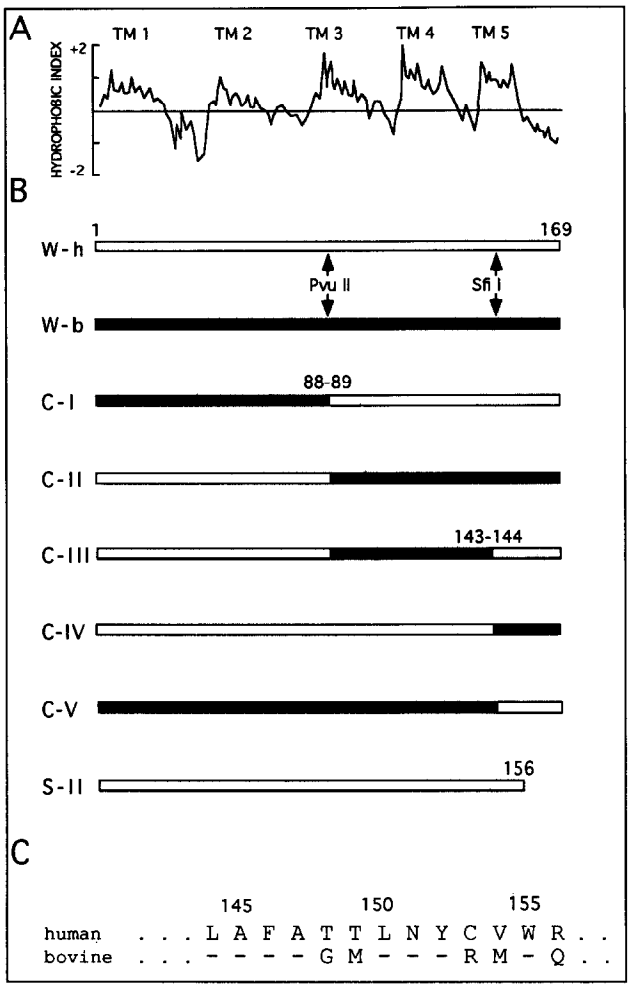

Fig. 2. (A) Putative transmembrane regions (TM) deduced from the hydrophatic profile of the human and bovine PBR sequences. (B) Recombinant chimaeric receptors (C-I to C-V) constructed with segments from the human (Wh) and bovine (Wb) receptors; and short (S-II) form of the human receptor. The restriction sites *Pvu*II and *Sfi*I used for the gene constructions are indicated for the Wh and Wb. (C) Amino acid differences between the human and bovine PBR in the region 144–156 of the sequences.

able to bind PK11195 with affinities similar to those shown by the wild type human (Wh) and bovine (Wb) receptors. However, only the C-I receptor (bovine-human) was able to bind Ro5–4864, the C-II receptor (human-bovine) having lost the capacity to recognise this ligand (Table I). These results indicated that some of the 21 residues that differ in the human and bovine C-terminal 80 amino acid sequence are necessary for the binding of Ro5–4864 but not of PK11195.

Subsequent chimaeric receptors were designed to investigate whether the first or second part of the C-terminal 80 amino acid sequence was involved in the binding of Ro5–4864. Scatchard analysis of the saturation curves of the C-III receptor, where the human sequence 89–143 was replaced by the corresponding bovine one, showed that it behaved as the Wh receptor. Both [^{3}H]PK11195 and [^{3}H]Ro5–4864 labelled the chimaeric receptor with K_d of 10 and 20 nM, respectively, indicating that the 10 amino acid differences between human and bovine receptors located in this region are irrelevant for the binding of Bz. Analysis of C-IV, where the last 26 amino acids of the human sequence were replaced by the corresponding bovine sequence, showed a K_d of 5 nM for PK11195, but Ro5–4864 was not able to bind to this receptor. Thus, C-IV behaved like the Wb receptor. The human and bovine 26 amino acid C-terminal sequences differ in 11 residues. Of those 11, 6 are not essential either for the binding of Bz or for IQC, since a recombinant receptor lacking the last 13 amino acids (S-II) could be labelled with [^{3}H]PK11195 as well as [^{3}H]Ro5–4864. As seen in Table I, IQC and Bz bound to S-II with a Kd of 9 nM and 12 nM, respectively. Thus, the candidate amino acids that make possible the binding of Ro5–4864 to the human receptor can be reduced to the five residues that differ in the 144–156 portion of the protein. These amino acids are: threonine-148, threonine-149, cysteine-153, valine-154, and arginine-156, that in the bovine sequence are replaced by glycine, methionine, arginine, methionine, and glutamine, respectively (Fig. 2C). To investigate if these residues could confer Ro5–4864 binding capacity we constructed C-V, a chimaeric bovine receptor in which those C-terminal amino acids were replaced by the corresponding ones in the human sequence. Analysis of the saturation experiments of the recombinant receptor showed that those changes were sufficient to confer the capacity to bind Ro5–4864 to the bovine receptor, while the capacity to bind PK11195, as expected, was not affected (Table I).

4. DISCUSSION

We have presented here a study aimed at the identification of the molecular basis of the differential binding behaviour of Ro5–4864 to the PBR across species. When expressed in yeast, a cell devoid of endogenous PBR, the human PBR can be labelled with PK11195 and Ro5–4864, but the bovine PBR can be labelled only with Ro5–4864, suggesting that the non-conserved amino acids between both sequences are involved in the binding of the Bz. To test this hypothesis, and identify those amino acids, we constructed human-bovine chimaeric receptors. The chimaeric receptors were expressed in yeast and analysed for binding with [^{3}H]PK11195 and [^{3}H]Ro5–4864. The results pointed to the 26 C-terminal residues as responsible for the binding of Ro5–4864 to the human receptor. We confirmed that this region was responsible for the differential binding of Ro5–4864 to the human and bovine receptors, by constructing a bovine receptor with the human C-terminal 26 amino acids. This chimaera indeed behaved as the Wh receptor. However, since the last 13 residues of the receptor are not essential for the binding of the Bz,

the region was narrowed down to 13 residues, of which only five are different between the human and bovine molecules. Those non-conserved amino acids are threonine-148, threonine-149, cysteine-153, valine-154, and arginine-156 in the human sequence, that are replaced by glycine, methionine, arginine, methionine, and glutamine, respectively, in the bovine sequence. Interestingly, the rat PBR, which binds IQC and Bz with high affinity, shares the threonine-148, valine-154, and arginine-156, with the human sequence. Thus, we speculate that one or more of these residues may be the key to the binding of Ro5–4864 to the human PBR and work is in progress to test this hypothesis. It should also be noted that all the chimaeric receptors bound PK11195 with high affinity, suggesting that the modifications do not alter either the targeting of the receptor to the mitochondrial membrane or the membrane topology of the receptor. Although other mitochondrial membrane proteins closely associated with the 18 kDa protein [21,22] may contribute to the binding properties of PBR, our results show that the Bz and IQC binding differences observed between the human and bovine PBR can be attributed to the non-conserved amino acids between the two sequences. Thus, since PK11195 and Ro5–4864 displace each other from the human receptor [24,28] and since our results clearly show that certain amino acids are implicated only in the binding of Ro5–4864, an appropriate model is that of two overlapping sites, as previously suggested [17,18]. However, an alternative model of two different conformational states, one that binds preferentially IQC and the other Bz [18], cannot be excluded. We have previously reported, using [^{3}H]PK14105, a photoaffinity probe derived from PK11195, that amino acids in the N-terminal region of the human PBR were involved in the binding of isoquinolines [13]. Those results, together with the ones described here, suggest that the N- and C-terminal regions of the molecule may be closely located and exposed to the cytoplasm once it is inserted in the mitochondrial membrane. We recently described a tridimensional model, based on molecular dynamic simulations, in which the putative 5 membrane regions delimited a pore [30]; in that model the first and fifth transmembrane regions are close. Interestingly, the amino acids identified in this work as essential for the binding of Ro5–4864 are in the interface between the membrane and the cytoplasm in the models based on the hydropathic profile of PBR [30,31]. A better understanding of the Bz and IQC binding sites on the PBR may contribute to the synthesis of new specific ligands for one or the other binding site that should help to a better understanding of the structure and physiological function of this receptor.

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