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Binding characteristics of [^{3}H]DAA1106, a novel and selective ligand for peripheral benzodiazepine receptors

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Abstract

Here, we investigated the binding characteristics of [^{3}H]*N*-(2,5-dimethoxybenzyl)-*N*-(5-fluoro-2-phenoxyphenyl)acetamide ([^{3}H]DAA1106), a potent and selective ligand for peripheral benzodiazepine receptors, in mitochondrial fractions of the rat brain. [^{3}H]DAA1106 bound to the mitochondrial fraction of the rat brain in a saturable manner. The dissociation constant (K_d) and maximal number of binding sites (B_{max}) obtained from Scatchard plot analysis of the saturation curve of [^{3}H]DAA1106 binding were 0.12 ± 0.03 nM and 161.03 ± 5.80 fmol/mg protein, respectively. [^{3}H]DAA1106 binding to mitochondrial preparations of the rat cerebral cortex was inhibited by several peripheral benzodiapine receptor ligands, and DAA1106 was the most potent inhibitor in inhibiting [^{3}H]DAA1106 binding among the peripheral benzodiazepine receptor ligands we tested. The binding of [^{3}H]DAA1106 was not affected by several neurotransmitter-related compounds, including adrenoceptor, γ-aminobutyric acid (GABA), dopamine, 5-hydroxytryptamine (5-HT), acetylcholine, histamine, glutamate and central benzodiazepine receptor ligands even at a concentration of 10 μM. In the cerebral cortex of rhesus monkeys, DAA1106 and 1-(2-chlorophenyl)-*N*-methyl-(1-methylpropyl)-3-isoquinoline carboxamide (PK11195) potently inhibited [^{3}H]DAA1106 binding, while 7-chloro-5-(4-chlorophenyl)-1-methyl-1,3-dihydrobenzo[*e*][1,4]diazepin-2-one (Ro5-4864) did not. The highest [^{3}H]DAA1106 binding was observed in the olfactory bulb, followed by the cerebellum. In autoradiographic studies, practically the same results were obtained, in that the highest binding of [^{3}H]DAA1106 was in the olfactory bulb. Potent labeling was also noted in ventricular structures such as the choroid plexus. Thus, [^{3}H]DAA1106 is a potent and selective ligand for peripheral benzodiazepine receptors and should prove useful for elucidating the physiological relevance of events mediated through peripheral benzodiazepine receptors.

Keywords: DAA1106; PK11195; Benzodiazepine receptor, peripheral; Receptor binding

1. Introduction

Benzodiazepines are widely prescribed anxiolytics and anticonvulsants, for which binding sites were initially identified in nervous tissues (Braestrup and Squires, 1977; Mohler and Okada, 1977). These sites are mainly localized on the extracellular domain of the γ-aminobutyric acid $(GABA)_A$ receptor, and modulate the GABA- regulated opening of the Cl^- channel (Tallman et al., 1978, 1980). In addition to these central benzodiazepine receptors linked to the $GABA_A$ receptor, a second type of benzodiazepine receptor has been identified (Schoemaker et al., 1981). This binding site has distinct pharmacological properties from the central benzodiazepine receptor in that it has a low affinity for most benzodiazepines. Certain benzodiazepines, such as 7-chloro-5-(4-chlorophenyl)-1-methyl-1,3-dihydrobenzo[*e*][1,4]diazepin-2-one (Ro5-4864), and isoquinoline derivatives, such as 1-(2-chlorophenyl)-*N*-methyl-(1-methylpropyl)-3-isoquinoline carboxamide (PK-11195), interact weakly with the central benzodiazepine receptor and have nanomolar affinity for this binding site (Schoemaker et al., 1981; Benavides et al., 1983). The binding sites were shown to be distributed abundantly in peripheral tissues and to be located on the mitochondrial outer membrane in secretory tissues (Papadopoulos et al., 1991), and thus, were defined as peripheral-type benzodiazepine receptors.

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Protein purification and molecular cloning revealed that peripheral benzodiazepine receptor consists of three different protein subunits, 18-, 30- and 32-kDa proteins (McEnery et al., 1992; Parola et al., 1993). The 18-kDa protein is labeled by isoquinoline carboxamides such as PK11195, while the 30- and 32-kDa subunits are presumed to be essential for affinity for benzodiazepines (Parola et al., 1993; Garnier et al., 1994). Selective antibodies and reagents identified the 32- and 30-kDa proteins as the voltage-dependent anion channels (VDAC) and the adenine nucleotide carrier, respectively (McEnery et al., 1992).

In contrast to the clarification of the molecular properties of peripheral benzodiazepine receptors, the precise physiological significance of events mediated through peripheral benzodiazepine receptors has not been fully elucidated. Peripheral benzodiazepine receptors are abundant in glandular and secretory tissues such as the adrenal and salivary glands and kidney (Schoemaker et al., 1983; Anholt et al., 1985). Peripheral benzodiazepine receptors enhance the synthesis of pregnenolone, a parent compound of all steroid hormones, by facilitating the access of cholesterol to the side-chain cleavage enzyme cytochrome *P*-450scc (Krueger and Papadopoulos, 1990, 1992). Neurosteroids modulate neurotransmitter-gated ion channel activity at the $GABA_A$ receptor (Lambert et al., 1995) and the *N*-methyl-D-aspartate receptor (Wu et al., 1991), an event which results in indirect modulation of GABAergic and glutamatergic transmission. Endogenous neurosteroids and the synthetic neuroactive steroid, Co 3-0593, are anticonvulsants and are anxiolytic in experimental animals (Wieland et al., 1995, 1997; Carter et al., 1997), suggesting that peripheral benzodiazepine receptors might be involved in certain pathophysiological events, such as anxiety, by stimulating the production of neuroactive steroids in glial cells in the brain.

The indoleacetamide derivative, 2-hexyl-indole-3-acetamide-*N*-benzenetricarboxylic acid (FGIN-(1-27)), was found to be a selective agonist for peripheral benzodiazepine receptors, with nanomolar affinity, and to enhance the production of neurosteroids in glioma cells and in the rat brain (Romeo et al., 1992). FGIN-(1-27) has also been reported to have anxiolytic-like effects in an elevated plus maze, without unwanted side effects such as sedation, ataxia and muscle relaxation (Romeo et al., 1993; Auta et al., 1993). Moreover, the anxiolytic-like effect of FGIN-(1-27) was attenuated by the peripheral benzodiazepine receptor antagonist, PK11195, but not by a central benzodiazepine receptor antagonist (Romeo et al., 1993). Thus, FGIN-(1-27) elicits pharmacological effects by selectively stimulating peripheral benzodiazepine receptors. Ligands which potently and selectively act on peripheral benzodiazepine receptors could be effective anxiolytics without the side effects which can occur with classical benzodiazepines.

Recently, we identified a novel, potent and selective ligand for peripheral benzodiazepine receptors, *N*-(2,5-dimethoxybenzyl)-*N*-(5-fluoro-2-phenoxyphenyl)acetamide (DAA1106) (Okuyama et al., 1999), which has subnanomolar affinity for peripheral benzodiazepine receptors. We now report characterization of the binding of radiolabeled DAA1106 in the rat brain.

2. Materials and methods

2.1. Materials

PK11195, Ro5-4864, (−)-musimol, (+)-bicuculine, (±)-8-hydroxy-2-(di-*n*-propylamino)tetraline hydrobromide (8-OH-DPAT) and pyrilamine maleate were purchased from Sigma (St. Louis, MO, USA). *R*(+)-baclofen hydrochloride, (+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride (SCH23390), haloperidol, prazosin hydrochloride, propranolol, ritanserin, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo-[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate (MK-801) and 3α,21-dihydroxy-5α-pregnan-20-one (THDOC) were purchased from Research Biochemicals (Natick, MA, USA). Yohimbine hydrochloride and L-glutamic acid monosodium salt were purchased from Nakarai Tesque (Kyoto, Japan). Diazepam and calbachol were purchased from Wako Pure Chemical (Osaka, Japan). Famotidine was prepared from commercially available drug Gaster®, obtained from Yamanouchi Pharmaceutical (Tokyo, Japan). DAA1106, *N*,*N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)-phenyl]-ethylamine monohydrochloride (NE-100) and 2-hexyl-indole-3-acetamide-*N*-benzenetricarboxylic acid (FGIN-(1-27)) were synthesized in our research laboratory. [^{3}H]DAA1106 (Specific radioactivity: 84 Ci/mmol) was synthesized at Amersham International (Buckinghamshire, England). All other chemicals were of the highest purity available.

2.2. Methods

2.2.1. Preparation of crude mitochondrial fraction

Male Wistar rats (Japan SLC, Hamamatsu, Japan; 200–250 g) and male rhesus monkeys (Hamuri Japan, Ibaraki, Japan; 3.2–5.2 kg) were used in this study. All experiments were reviewed by The Taisho Pharmaceutical, Animal Care Committee, and met the Japanese Experimental Animal Research Association Standards, as defined in the Guidelines for Animal Experiments (1987). The crude mitochondrial fraction from rat or monkey brain was prepared as described earlier (Romeo et al., 1993). Rats were killed by decapitation, and the cerebral cortex, olfactory bulb, cerebellum, hypothalamus, striatum, hippocampus and thalamus were rapidly dissected according to Glowinski and Iversen (1966). Rhesus monkeys were killed by intravenous injection of an overdose of pentobarbital, and the brain was rapidly removed. Unless otherwise men-

tioned, the cerebral cortex was routinely used for the crude mitochondrial preparation. The brain was homogenized with 10 vol. of ice-cold 0.32 M sucrose–10 mM HEPES buffer (pH 7.4), using a Teflon glass homogenizer, and centrifuged at $900 \times g$ for 10 min at 4°C, after which the supernatant was centrifuged at $9000 \times g$ for 10 min at 4°C. The pellet was suspended in 50 mM HEPES buffer (pH 7.4) at a protein concentration of 1 mg/ml and centrifuged at $12{,}000 \times g$ for 10 min at 4°C. The pellet obtained was then suspended in 50 mM HEPES buffer (pH 7.4) and served as a crude mitochondrial fraction.

2.2.2. Receptor binding assay

For a typical binding experiment, the reaction was initiated by incubating 1 ml of the crude mitochondrial preparation with [^{3}H]DAA1106. The reaction mixture was incubated for 90 min at 4°C, a time period that allowed for equilibration. The reaction was terminated by rapid filtration through Whatman GF/B glass fiber filters presoaked with 0.3% polyethyleneimine, after which the filters were washed three times with 3 ml of ice-cold 50 mM HEPES buffer (pH 7.4), using a multi cell harvester M-24R (Brandel Biomedical Research and Development Laboratories, Gaithersburg, MD, USA). Aquazol-2 scintillator (Du Pont/New England Nuclear) (10 ml) was added, and filter-bound radioactivity was counted in a liquid scintillation spectrometer (LS6000TA, Beckman Instruments, Fullerton, CA, USA). Nonspecific binding was determined in the presence of 10 μM DAA1106. Specific binding was determined by subtracting nonspecific from total binding and was approximately 90% of total binding.

In the time course experiment, the reaction was carried out at 4, 25 or 37°C, using 0.5 nM of [^{3}H]DAA1106. For determination of the equilibrium dissociation constant (K_d), the crude mitochondrial preparation was incubated with 0.07–0.94 nM of [^{3}H]DAA1106 at 4°C for 90 min. Saturation binding data were analyzed by Scatchard plot analysis, and the K_d and the maximal number of binding sites (B_{max}) were calculated.

In the competition binding assay, the reaction was carried out at 4°C for 90 min, using 0.5 nM of [^{3}H]DAA1106. The concentration of the test compound that caused 50% inhibition of specific binding of [^{3}H]DAA1106 (IC_{50} value) was determined from each concentration–response curve. IC_{50} values were determined by the Marquardt–Levenberg nonlinear least-squares curve-fitting procedure of the MicroCal ORIGIN program (MicroCal, Northampton, MA, USA) running on a Microsoft Windows NT. K_i values for each test compound were calculated according to the equation of Cheng and Prusoff (1973), using the K_d value obtained from Scatchard analysis.

2.2.3. Autoradiographic study

Male Wistar rats were killed by decapitation, and the brain was rapidly removed and frozen using finely powdered dry ice, delicately applied. Sections 10-μm thick were obtained by cutting the brain on a microtome cryostat, then thaw-mounted onto chrome alum/gelatin-coated slides and stored at −80°C until use. The slide-mounted brain sections were incubated with [^{3}H]DAA1106 (1 nM) in 50 mM HEPES buffer (pH 7.4) for 60 min at room temperature. After incubation with the radioligand, the slide-mounted sections were washed with ice-cold 50 mM HEPES buffer for two consecutive periods of 2 min, and dipped into ice-cold water. Nonspecific binding was determined in the presence of 10 μM DAA1106. The slides were dried with a stream of cold air, desiccated and exposed to tritium-sensitive film for 7–21 days at 4°C. The films were developed using Rendol and Super Fujifix (Fuji Photo film, Tokyo, Japan). Autoradiograms were analyzed quantitatively using an image analysis system (MCID Imaging Research, Ontario, Canada).

3. Results

3.1. Binding of [^{3}H]DAA1106 as a function of protein concentration and time course

The specific binding of [^{3}H]DAA1106 to mitochondrial preparations from the rat cerebral cortex increased linearly,

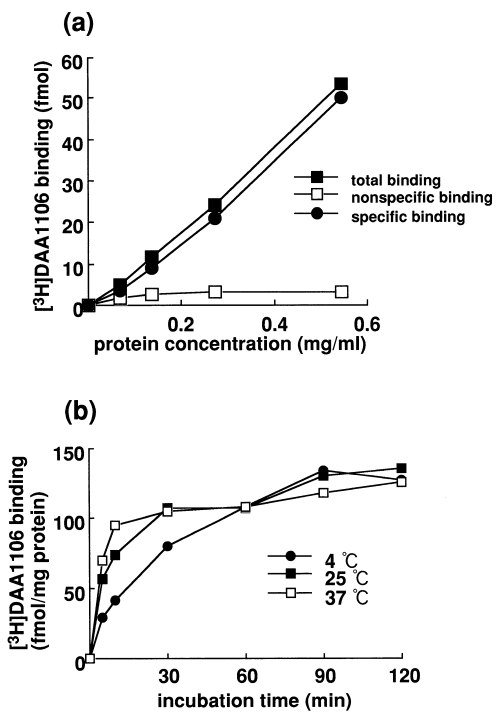

Fig. 1. Protein linearity and time course of [^{3}H]DAA1106 binding to mitochondrial fractions of rat brain. Mitochondrial fractions were incubated with 0.5 nM [^{3}H]DAA1106 at 4°C with increasing concentrations of protein (a) or were incubated at 4, 25 or 37°C (b). The data are means of duplicate determinations.

depending on the protein concentration, at least up to approximately 0.6 mg/ml (Fig. 1a). The specific binding of [^{3}H]DAA1106 exceeded 85% of the total binding (Fig. 1a). The binding of [^{3}H]DAA1106 was increased in a time-dependent manner and reached a plateau phase at 30 min at 25° and 37°C (Fig. 1b). At 4°C, [^{3}H]DAA1106 binding reached a plateau after 90 min of incubation (Fig. 1b).

3.2. Saturation binding of [^{3}H]DAA1106 to mitochondrial preparation of the rat cerebral cortex

The characteristics of the binding of [^{3}H]DAA1106 to mitochondrial fractions were then investigated. Saturation studies of [^{3}H]DAA1106 binding showed that [^{3}H]DAA-1106 binding increased in a saturable manner with increasing concentrations of [^{3}H]DAA1106 (Fig. 2a). Scatchard plot analysis of the saturation curve of [^{3}H]DAA1106 revealed the existence of a single population of binding sites (Fig. 2b). The K_d and B_{max} values computed from Scatchard plot analysis were 0.12 ± 0.03 nM and 161.03 ± 5.80 fmol/mg protein, respectively.

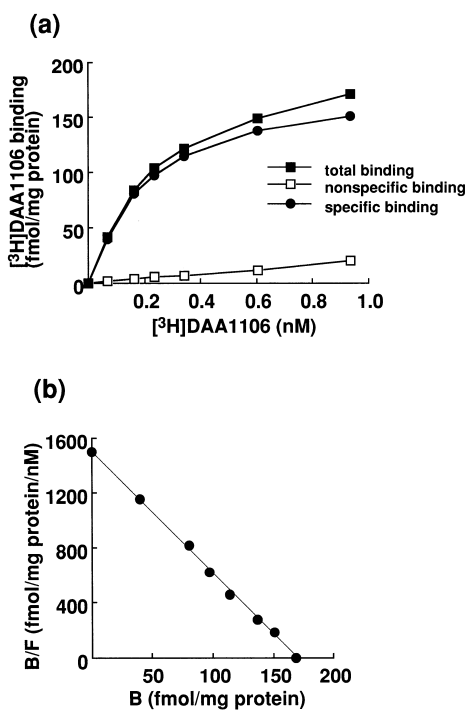

Fig. 2. Saturation isotherm and Scatchard plot of [^{3}H]DAA1106 binding to mitochondrial fractions of the rat cerebral cortex. Mitochondrial fractions were incubated with increasing concentrations of [^{3}H]DAA1106 at 4°C for 90 min. (a) Representative saturation curves for equilibrium binding of [^{3}H]DAA1106 are shown. Essentially, the same results were obtained in three independent experiments, done in duplicate. Total binding, nonspecific binding and specific binding are indicated as ■, □ and ●, respectively. (b) Scatchard plot of the same data as in (a). Mean values of K_d and B_{max} are 0.12 ± 0.03 nM and 161.03 ± 5.80 fmol/mg protein, respecively.

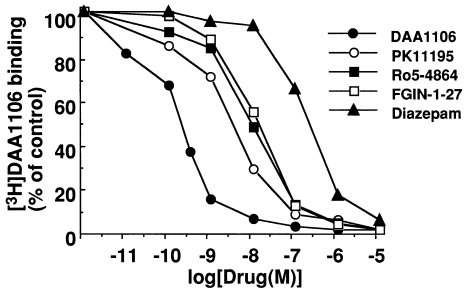

Fig. 3. Effect of several representative peripheral benzodiazepine receptor ligands on [^{3}H]DAA1106 binding to mitochondrial fractions of the rat cerebral cortex. Mitochondrial fractions were incubated with increasing concentrations of peripheral benzodiazepine receptor ligands in the presence of 0.5 nM [^{3}H]DAA1106 at 4°C for 90 min. The results given are the mean values of three separate experiments, each done in duplicate. Key: DAA1106 (●), PK1195 (○), Ro5-4864 (■), FGIN-(1-27) (□) and Diazepam (▲).

3.3. Displacement of [^{3}H]DAA1106 binding by various peripheral benzodiazepine receptor ligands and neurotransmitter-related compounds

Several structurally dissimilar peripheral benzodiazepine receptor-related ligands were examined for their potential to displace [^{3}H]DAA1106 binding to mitochondrial preparations of the rat cerebral cortex. [^{3}H]DAA1106 binding was dose dependently inhibited by unlabeled DAA1106 and several peripheral benzodiazepine receptor ligands (Fig. 3, Table 1). DAA1106 was the most potent inhibitor of [^{3}H]DAA1106 binding, the K_i value being 0.043 ± 0.005 nM (Fig. 3, Table 1). The rank order of potency of peripheral benzodiazepine receptor ligands in inhibiting [^{3}H]DAA1106 binding was consistent with findings obtained for the bindings of [^{3}H]PK11195 and [^{3}H]Ro5-4864, the other peripheral benzodiazepine receptor-specific ligands. The effects of numerous neurotransmitter-related compounds, including GABAergic, adrenergic, dopaminergic, serotonergic, cholinergic, histaminergic and glutamatergic agents, on [^{3}H]DAA1106 binding were tested to investigate the specificity of [^{3}H]DAA1106 binding to peripheral benzodiazepine receptors. Of all the compounds studied for their ability to displace [^{3}H]-

Table 1
Effect of PBR ligands on [^{3}H]DAA1106 binding to mitochondrial fraction of rat and monkey cerebral cortex

Compounds	K_i (nM)	
	Rat	Monkey
DAA1106	0.043 ± 0.005	0.188 ± 0.027
PK11195	0.766 ± 0.116	0.738 ± 0.131
Ro5-4864	1.815 ± 0.112	> 1000
FGIN-1-27	2.285 ± 0.345	N.D.
Diazepam	37.650 ± 10.03	N.D.

N.D.: not determined.
Data is the mean ± S.E. from three separate experiments, each done in duplicate.

Table 2
Effect of numerous neurotransmitter-related compounds on [^{3}H]DAA1106 binding to mitochondrial fractions of the rat cerebral cortex

Compound	1 μM	10 μM
	% of control	
CBR		
Clonazepam	92.07 ± 0.72	64.77 ± 0.87
GABAergic		
Mucimol	87.43 ± 3.60	91.70 ± 2.04
Bicuculine	94.43 ± 0.62	92.63 ± 2.27
Baclofen	93.77 ± 1.21	93.63 ± 1.48
Neurosteroid		
THDOC	94.83 ± 1.41	91.40 ± 0.21
Dopaminergic		
SCH23390	94.27 ± 1.63	91.43 ± 1.68
Haloperidol	89.9 ± 3.87	87.53 ± 3.80
Adrenergic		
Prazosin	96.67 ± 1.72	96.47 ± 0.90
Yohimbin	96.33 ± 0.68	97.07 ± 0.84
Serotonergic		
Propranolol	96.53 ± 1.95	94.77 ± 2.00
8-OH-DPAT	97.87 ± 1.65	93.6 ± 2.52
Ritanserin	91.2 ± 3.35	97.93 ± 6.61
Sigma		
NE-100	100.97 ± 6.1	102.4 ± 0.65
Glutamatergic		
MK-801	102.07 ± 2.44	106.93 ± 2.48
L-Glutamate	101.03 ± 5.31	103.67 ± 1.13
Cholinergic		
Carbachol	100.9 ± 3.82	102.37 ± 1.98
Histaminergic		
Pyrilamine	85.4 ± 2.90	96.6 ± 1.40
Famotidine	94.53 ± 0.63	98.8 ± 2.38

DAA1106 binding, none had significant effect on binding at concentrations of 1 and 10 μM, although clonazepam, a central benzodiazepine receptor ligand, at 10 μM slightly inhibited [^{3}H]DAA1106 binding (Table 2).

3.4. Effect of peripheral benzodiazepine receptor ligands on [^{3}H]DAA1106 binding to mitochondrial fraction of the rhesus monkey cerebral cortex

It has been reported that isoquinoline derivatives such as PK11195 show no species variation, while benzodiazepines such as Ro5-4864 have a much higher affinity for rodent peripheral benzodiazepine receptors than for peripheral benzodiazepine receptors from other species (Parola et al., 1993). To address species differences, we investigated [^{3}H]DAA1106 binding to the mitochondrial fraction of the rhesus monkey cerebral cortex, and the effect of DAA1106 on [^{3}H]DAA1106 binding. [^{3}H]DAA1106 bound to mitochondrial fractions of the rhesus monkey cerebral cortex in a saturable manner (data not shown), and K_d and B_{max} values computed from Scatchard plot analysis were 0.426 ± 0.036 nM and 701.34 ± 70.479 fmol/mg protein, respectively. DAA1106 and PK11195 had high affinity for peripheral benzodiazepine receptors of the rhesus monkey, while Ro5-4864 did not inhibit [^{3}H]DAA1106 binding, even at 1 μM (Table 1).

3.5. Regional distribution of [^{3}H]DAA1106 binding in the rat brain

To further investigate the characteristics of [^{3}H]DAA1106 binding in the rat brain, the regional distribution of [^{3}H]DAA1106 binding was studied. The highest density of binding sites was observed in the olfactory bulb (Fig. 4). A high level of [^{3}H]DAA1106 binding was also detected in the cerebellum, and a moderate but significant level of binding sites was observed in the cerebral cortex, hypothalamus, striatum, hippocampus and thalamus (Fig. 4). The level of [^{3}H]DAA1106 binding in the thalamus was the lowest among the regions examined in this study. To investigate the regional distribution of [^{3}H]DAA1106 binding sites in detail, we used autoradiographic analysis. The specific binding of [^{3}H]DAA1106 binding to slide-mounted brain sections was observed when nonradiolabeled DAA1106 was used to determine nonspecific binding (Fig. 5a,b). Likewise, both 10 μM PK11195 and Ro5-4864 completely inhibited the specific binding of [^{3}H]DAA1106 (data not shown). As shown in the study with the mitochondrial preparation, [^{3}H]DAA1106 bound mainly to the olfactory bulb (Fig. 5a,b). Strong labeling also occurred in ventricular structures such as the choroid plexus (Fig. 5a), and moderate binding was also observed in the cerebellum

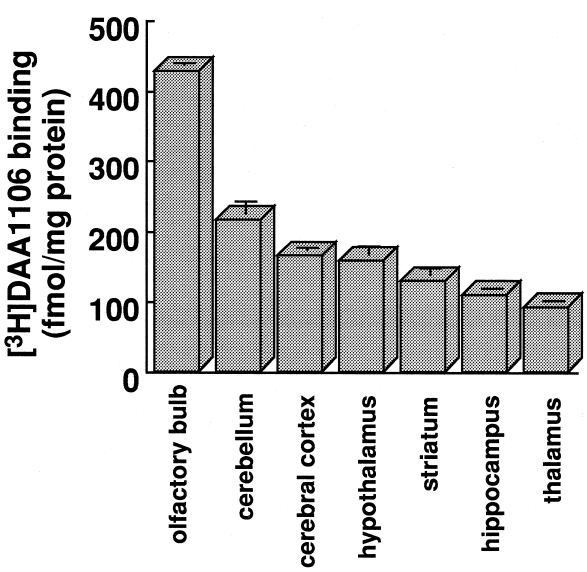

Fig. 4. Regional distribution of [^{3}H]DAA1106 binding in mitochondrial fractions of the rat brain. Mitochondrial fractions were incubated with 0.5 nM [^{3}H]DAA1106 at 4°C for 90 min, and results are given as fmol/mg protein. Values are means ± S.E. of three separate experiments, each done in duplicate.

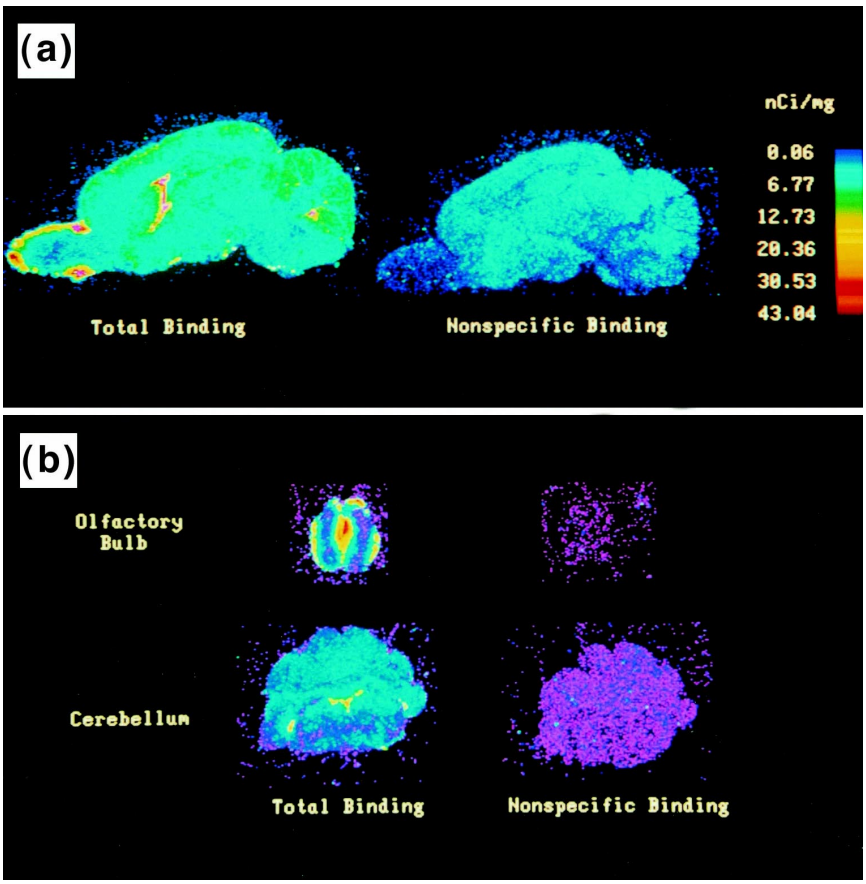

Fig. 5. Autoradiographic studies of [^{3}H]DAA1106 binding in the rat brain. Slide-mounted brain sections were incubated with [^{3}H]DAA1106 (1 nM) in 50 mM HEPES buffer (pH 7.4) for 60 min at room temperature. (a) Longitudinal planes; (b) transverse planes.

(Fig. 5a,b). The distribution of [^{3}H]DAA1106 binding sites in the rat brain is consistent with that for [^{3}H]PK11195 binding sites (data not shown).

4. Discussion

We obtained evidence that [^{3}H]DAA1106 is a potent and specific ligand for peripheral benzodiazepine receptors. The binding of [^{3}H]DAA1106 was time dependent and reached a plateau at 90 min at 4°C. [^{3}H]DAA1106 bound to the mitochondrial fraction in a saturable manner, and Scatchard plot analysis showed a single class of binding sites. The K_d value of [^{3}H]DAA1106 obtained from Scatchard plot analysis was 0.12 nM. The binding of [^{3}H]DAA1106 was inhibited by several peripheral benzodiazepine receptor ligands in an order similar to that observed for [^{3}H]PK11195, with DAA1106 being the most potent inhibitor of [^{3}H]DAA1106 binding. These results show that [^{3}H]DAA1106 has the highest affinity for peripheral benzodiazepine receptors among the peripheral benzodiazepine receptor ligands hitherto identified.

To determine whether [^{3}H]DAA1106 specifically binds to peripheral benzodiazepine receptors, numerous neurotransmitter-related ligands were tested for their effects on [^{3}H]DAA1106 binding. None of the neurotransmitter-related ligands affected [^{3}H]DAA1106 binding, even at the highest concentration (10 μM) used. Even a ligand which binds to the central benzodiazepine receptor/GABA$_A$ complex, such as clonazepam, had no significant effect on [^{3}H]DAA1106 binding. Thus, [^{3}H]DAA1106 is a potent and selective ligand for peripheral benzodiazepine receptors.

Although peripheral benzodiazepine receptors were first identified in peripheral tissues, it became clear that the density of peripheral benzodiazepine receptors can equal or exceed the density of central benzodiazepine receptors in regions of the brain, and that peripheral benzodiazepine receptors mediate physiological responses in the central nervous system (Anholt et al., 1985). The regional distribution of [^{3}H]DAA1106 binding in the rat brain, determined in autoradiographic and biochemical studies, revealed that the high density of [^{3}H]DAA1106 binding is localized in the olfactory bulb and structures related to the secretion of cerebrospinal fluid (choroid plexus), followed by the cerebellum and cerebral cortex. These results are consistent with findings obtained with [^{3}H]PK11195 as a peripheral benzodiazepine receptor ligand (Benavides et al., 1983).

The peripheral benzodiazepine receptor has been reported to be composed of at least three different protein

subunits, 18-, 30- and 32-kDa proteins (Parola et al., 1993). The binding domain for isoquinoline derivatives such as PK11195 was demonstrated to be on the 18-kDa protein (Doble et al., 1987; Riond et al., 1991), while the benzodiazepine-binding domain is thought to reside on a protein subunit distinct from that for isoquinoline (Garnier et al., 1994). Because DAA1106 is not classified as isoquinoline and benzodiazepine derivatives, there is a possibility that DAA1106 has its own binding sites. Thus, it is intriguing to postulate that the binding site for [^{3}H]DAA1106 is distinct from those for both isoquinoline and benzodiazepines, and that [^{3}H]DAA1106 could possibly be used for further elucidation of the molecular characteristics of peripheral benzodiazepine receptors, including novel binding sites, and possibly novel subunits.

It is reported that the binding of [^{3}H]Ro5-4864 is temperature dependent, while that of [^{3}H]PK11195 is not (Le Fur et al., 1983). In a preliminary study, the inhibitory effect of both DAA1106 and PK11195 on [^{3}H]DAA1106 binding was practically the same at 4° and 25°C, while the inhibitory effect of Ro5-4864 was 20 times lower at 25° than at 4°C, showing that the binding properties of DAA1106 and PK1195 are thermodynamically similar.

The peripheral benzodiazepine receptors of most nonrodent species have only moderate-to-low affinity for benzodiazepines such as Ro5-4864 and diazepam (Parola et al., 1993). Unlike the species variation in the ligand affinity observed for benzodiazepines, isoquinoline derivatives have a high affinity in all species tested and are diagnostic for the receptor (Parola et al., 1993). In the present study, DAA1106 had substantially the same affinity for mitochondrial preparations from both rat and rhesus monkey, as PK11195 did, albeit the affinity of DAA1106 was slightly lower in the monkey. This result suggests that the binding sites for DAA1106 do not show species variation and that DAA1106 may have a high affinity for human peripheral benzodiazepine receptors.

Peripheral benzodiazepine receptors have been postulated to be involved in the pathology and/or etiology of certain central nervous system disorders such as anxiety (Romeo et al., 1992), although the effect of peripheral benzodiazepine receptor ligands is still controversial. FGIN-(1-27), a selective agonist for peripheral benzodiazepine receptors with nanomolar affinity (Romeo et al., 1992), has been reported to have anxiolytic-like effects in an elevated plus maze without side effects such as sedation, ataxia and muscle relaxation (Auta et al., 1993; Romeo et al., 1993). Thus, drugs which potently and selectively stimulate peripheral benzodiazepine receptors could be effective anxiolytics without the side effects sometimes seen with classical benzodiazepines. In in vivo studies, DAA1106 had anxiolytic effects in the light/dark exploration test in mice and elevated plus-maze test in rats, without affecting locomotor activity or hexobarbital-induced sleeping time (Okuyama et al., 1999). Moreover, by using ex vivo binding techniques, it was demonstrated that DAA1106 occupied peripheral benzodiazepine receptors when administered intraperitoneally, which suggests that DAA1106 does act on peripheral benzodiazepine receptors in vivo (unpublished data). As DAA1106 bound to peripheral benzodiazepine receptors selectively and potently in the rhesus monkey as well as in the rat, DAA1106 could be tested for clinical use. Furthermore, since DAA1106 is the most potent ligand for peripheral benzodiazepine receptors reported to date and occupies peripheral benzodiazepine receptors in the brain, DAA1106 could be used as a ligand for positron emission tomography.

In conclusion, [^{3}H]DAA1106 is a potent and selective ligand for peripheral benzodiazepine receptors with pharmacological properties similar to those of [^{3}H]PK11195 and may prove useful for clarifying the physiological significance and molecular properties of peripheral benzodiazepine receptors. Since DAA1106 binds with high affinity in the rhesus monkey, [^{3}H]DAA1106 can be tested for the treatment of subjects with anxiety-like disorders.

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