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Characterization of Central- and Peripheral-Type Benzodiazepine Receptors in Rat Salivary Glands

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ABSTRACT. Benzodiazepines have been shown to inhibit salivary secretion from the rat salivary gland. This action is mediated by specific benzodiazepine binding sites in the glands. The presence and characteristics of central- and peripheral-type benzodiazepine receptors in rat parotid and submandibular glands were examined employing [^{3}H]Ro15-1788 and [^{3}H]PK11195 as radioligands. [^{3}H]Ro15-1788 and [^{3}H]PK11195 bound with high affinity for both salivary glands ([^{3}H]Ro15-1788: 24.5 and 37.4 nM, [^{3}H]PK11195: 1.37 and 1.88 nM, for parotid and submandibular glands, respectively). [^{3}H]Ro15-1788 binding sites occupied only 0.22 to 0.43% of the total binding for benzodiazepine receptors in the glands. The rank order of the competing potency of [^{3}H]Ro15-1788 binding (Ro15-1788 = clonazepam > diazepam > flunitrazepam > PK11195 > Ro5-4864) and [^{3}H]PK11195 binding (Ro5-4864 = PK11195 > diazepam = flunitrazepam > clonazepam) demonstrated that [^{3}H]Ro15-1788 and [^{3}H]PK11195 binding sites were characteristic of the central and peripheral type, respectively. These studies show that both central- and peripheral-type benzodiazepine receptors exist in rat parotid and submandibular glands. BIOCHEM PHARMACOL **55**;2:209–214, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. central-type benzodiazepine receptor; peripheral-type benzodiazepine receptor; [^{3}H]Ro15-1788; [^{3}H]PK11195; parotid gland; submandibular gland

BDZs† cause a decrease in salivary secretion from rat salivary glands *in vivo*. Kawaguchi and co-workers [1] have reported that the actions of central- and peripheral-type BDZs may play a role in the inhibition of salivary secretion *in vivo* and permit $^{36}Cl^-$ influx into rat parotid acinar cells *in vitro*. These findings suggest that central- and peripheral-type BDZs act directly on respective binding sites in the salivary glands, and thus induce a decrease in saliva secretion.

BDZs such as diazepam and clonazepam have several actions, as anxiolytics, anticonvulsants, hypnotics, and muscle relaxants. These actions on the central nervous system have been shown to be mediated by central-type BDZ receptors, which are functionally coupled to $GABA_A$ receptors and chloride channels [2, 3]. It has been reported that the central-type BDZ receptor exists in peripheral tissues, such as the adrenal gland [4] and the pancreas [5]. The peripheral-type BDZ receptor is distinguished from the central-type receptor by the following criteria: (1) it is not coupled to the $GABA_A$ receptor [6, 7], (2) it binds Ro5-4864 with high affinity, but it binds clonazepam and Ro15-1788, a selective ligand for the central-type BDZ receptor, with low affinity [7, 8], and (3) it binds PK11195, unrelated to the BDZs, with high affinity [9].

The aim of the present study was to characterize the BDZ receptors in rat parotid and submandibular gland membranes. Anholt and De Souza and coworkers have demonstrated the existence of the peripheral-type BDZ receptor in rat parotid and submandibular glands by using [^{3}H]Ro5-4864 [10, 11]. However, the existence of central-type receptor or the minute characteristics of peripheral-type receptor in both glands have not been demonstrated sufficiently. The present report provides the first demonstration that both central- and peripheral-type BDZ receptors exist in rat parotid and submandibular glands.

MATERIALS AND METHODS

Materials

[*N-methyl-*^{3}H]Ro15-1788 (sp. act. 75.3 Ci/mmol) and [*N-methyl-*^{3}H]PK11195 (sp. act. 85.9 Ci/mmol) were purchased from Dupont/New England Nuclear. The following drugs were donated: Ro15-1788 and flunitrazepam (Nippon Roche), Ro5-4864 (Hoffmann–La Roche), clonazepam (Sumitomo Pharmaceuticals), zolpidem (Fujisawa Pharmaceuticals), and CL218,872 (Lederle Laboratories). PK11195 and β-CCE were obtained from Research Biochemical Inc. Diazepam was from Wako Pure Chemical Industries. The other reagents were of analytical grade.

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† *Abbreviations:* BDZ, benzodiazepine; β-CCE, (ethyl-9*H*-pyrido[3,4-b]indole-3-carboxylate); CL218,872, (3-methyl-6-[3-trifluoromethyl)phenyl]-1,2,4-triazolo[4,3-b]pyridazine); $GABA_A$, γ-aminobutyric $acid_A$; PK11195, [1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide]; Ro5-4864, [7-chloro-1,3-dihydro-1-methyl-5-(*p*-chlorophenyl)-2*H*-1,4-benzodiazepine-2-one]; and Ro15-1788, [ethyl- 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo(1,5-a)(1,4)-benzodiazepine-3 carboxylate].

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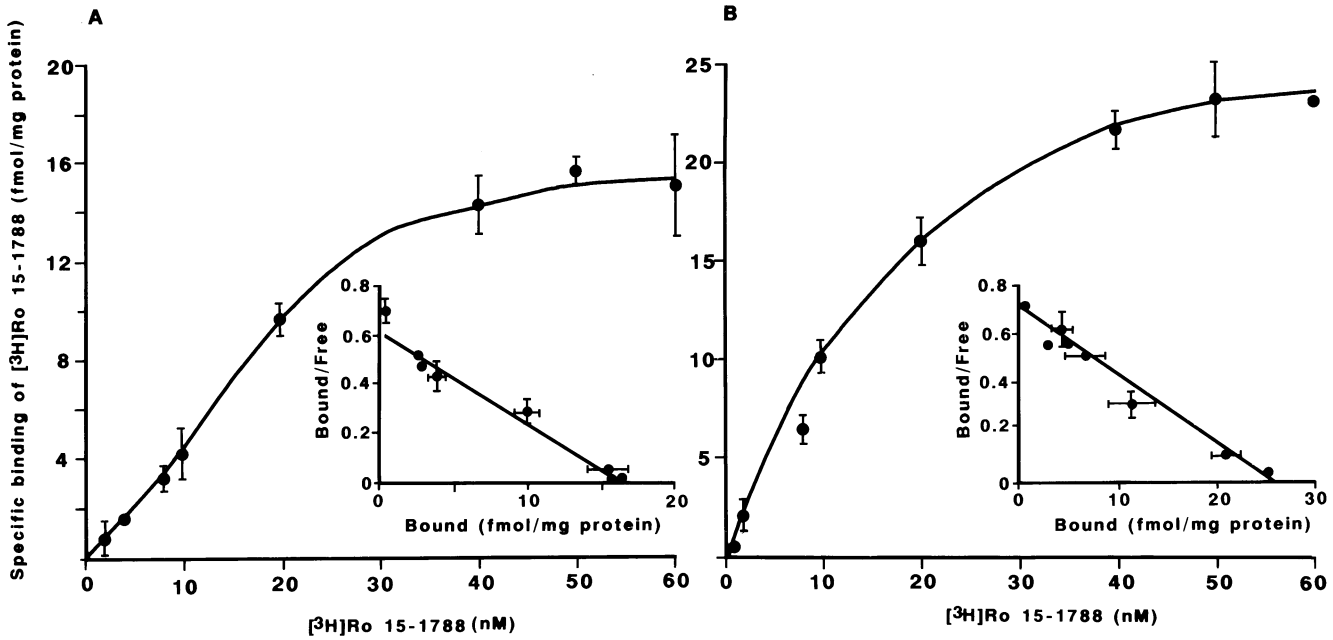

FIG. 1. Saturation isotherm and Scatchard analysis (inset) of [^{3}H]Ro15-1788 binding to rat parotid (A) and submandibular (B) gland membranes. Membranes were incubated with [^{3}H]Ro15-1788 for 60 min at 4°. Specific binding represents the difference between total binding and nonspecific binding in the presence of 100 μM unlabeled Ro15-1788. Results are the means ± SD of four independent experiments measured in triplicate.

Drugs were dissolved in 99.5% ethanol or distilled water and diluted. The final concentration of ethanol (<1%) had no effect on the receptor binding assay.

Membrane Preparation

Male Wistar strain rats (SLC, Inc.), weighing 200–250 g, were used for this experiment. Tissues of the parotid gland, submandibular gland, cerebral cortex, kidney, and adrenal gland were quickly dissected free of fat and connective tissues. Only in the adrenal gland, among these tissues, are the localizations of the central- and peripheral-type BDZ receptors limited to the medulla and cortex, respectively [10, 12]. In our experiment, the medulla of the adrenal gland was separated from the cortex for the exclusive assay of central- and peripheral-type receptors using the radioligands [^{3}H]Ro15-1788 or [^{3}H]PK11195. Samples were homogenized in 10 vol. of ice-cold 0.32 M sucrose (except for the adrenal gland, which was homogenized in 20 vol.) containing protease inhibitors (aprotinin, antipain, pepstatin A, and leupeptin) using a Brinkmann Polytron at a speed setting of 5 for 1 min (except for the adrenal gland: 30 sec). The homogenates were centrifuged at 1,000 × *g* for 10 min. Then the supernatants were centrifuged at 48,000 × *g* for 20 min. The resulting pellets were resuspended in 10 vol. of ice-cold 50 mM Tris–HCl buffer, pH 7.4, and then centrifuged at 48,000 × *g* for 20 min. This wash step was repeated two times. The final pellets were resuspended in ice-cold 50 mM Tris–HCl buffer, pH 7.4. Proteins were determined according to the procedure of Lowry *et al.* [13], using BSA as the standard.

Radioligand Binding Assays

Membranes (0.05 to 2.0 mg protein/mL) were suspended in 50 mM Tris–HCl, pH 7.4, in a final volume of 0.5 mL with [^{3}H]Ro15-1788 (1–60 nM, final concentration) or [^{3}H]PK11195 (1–30 nM, final concentration) in triplicate. The samples were incubated for 1 hr at 4°. After incubation, the reaction was terminated by rapid filtration under vacuum through a Whatman GF/B glass fiber filter using a Brandel M-24S filtering manifold (Brandel Instruments), followed by two 5-mL washes with the same ice-cold buffer. Radioactivity in the filters was determined in 6 mL of Scintisol (Dojin) by a liquid scintillation counter (Aroka). Specific binding of [^{3}H]Ro15-1788 or [^{3}H]PK11195 was defined as the amount of radioactivity displaced by unlabeled Ro15-1788 (10^{-4} M) or PK11195 (10^{-6} M), respectively. All experiments were repeated at least four times, and the membrane preparations for each experiment were independent. The K_i values of the drugs were determined by using at least eleven concentrations of inhibitors. The K_d, B_{max}, and K_i values were determined by the LIGAND program [14] on a Toshiba Dynabook EZ computer. Results are expressed as means ± SD.

RESULTS

Saturation Experiments of [^{3}H]Ro15-1788 and [^{3}H]PK11195 Binding

Specific binding of [^{3}H]Ro15-1788 (20 nM) to rat parotid and submandibular gland membranes increased linearly with protein concentrations up to 1.2 mg/assay, reached a

TABLE 1. Binding constants of [^{3}H]Ro15-1788 and [^{3}H]PK11195 binding to rat parotid gland, submandibular gland, adrenal gland, kidney, and cerebral cortex membranes

Tissue	[^{3}H]Ro15-1788		[^{3}H]PK11195		Density ratio [A/(A + B) × 100%]
	K_d (nM)	B_{max} (A) (fmol/mg protein)	K_d (nM)	B_{max} (B) (fmol/mg protein)	
Parotid gland	24.5 ± 4.9	16.7 ± 1.8	1.37 ± 0.22	3,882 ± 706	0.43
Submandibular gland	37.4 ± 1.8	25.5 ± 3.8	1.88 ± 0.35	11,353 ± 339	0.22
Adrenal gland	28.3 ± 2.0*	54.0 ± 7.2*	2.04 ± 0.12†	21,044 ± 635†	0.26
Kidney	ND‡	ND	1.06 ± 0.01	4,690 ± 591	ND
Cerebral cortex	0.87 ± 0.15	1,457 ± 136	2.20 ± 0.39	404 ± 64	78.3

Values are means ± SD of four independent experiments measured in triplicate.
* Shown in adrenal medulla.
† Shown in adrenal cortex.
‡ Not determined.

steady state within 30 min of incubation at 4°, and was maintained for at least 120 min (data not shown). Figure 1 represents the saturation curves and Scatchard analysis of [^{3}H]Ro15-1788 binding to parotid and submandibular gland membranes. Specific binding of [^{3}H]Ro15-1788 to rat parotid or submandibular gland membrane was saturated at 40–50 nM, whereas nonspecific binding increased linearly with the concentration of [^{3}H]Ro15-1788, maintaining 70–80% of the total binding in both glands. Scatchard analysis from the saturation curves yielded a single population of non-interacting binding sites to both gland membranes (Fig. 1), as well as to adrenal medulla and cerebral cortex (Table 1); the Hill coefficients of [^{3}H]Ro15-1788 binding in both gland membranes were close to 1.00 (0.97 ± 0.06 and 1.03 ± 0.10 for parotid and submandibular gland, respectively). Table 1 shows the binding constants (K_d and B_{max} values) of [^{3}H]Ro15-1788 to the rat parotid gland, submandibular gland, adrenal gland, and cerebral cortex. [^{3}H]Ro15-1788 bound with high affinity (nanomolar range) to both salivary gland membranes. The densities of [^{3}H]Ro15-1788 binding sites in rat parotid and submandibular glands were 1.2 and 1.8% of that in the cerebral cortex, and 30 and 47% of that in the adrenal medulla, respectively (Table 1).

Specific binding of [^{3}H]PK11195 (1 nM) to rat parotid and submandibular gland membranes increased linearly with protein concentrations up to 1.0 mg/assay, reached a steady state within 20 min of incubation at 4°, and was maintained for at least 120 min (data not shown). Figure 2 shows the saturation curves and Scatchard analysis of

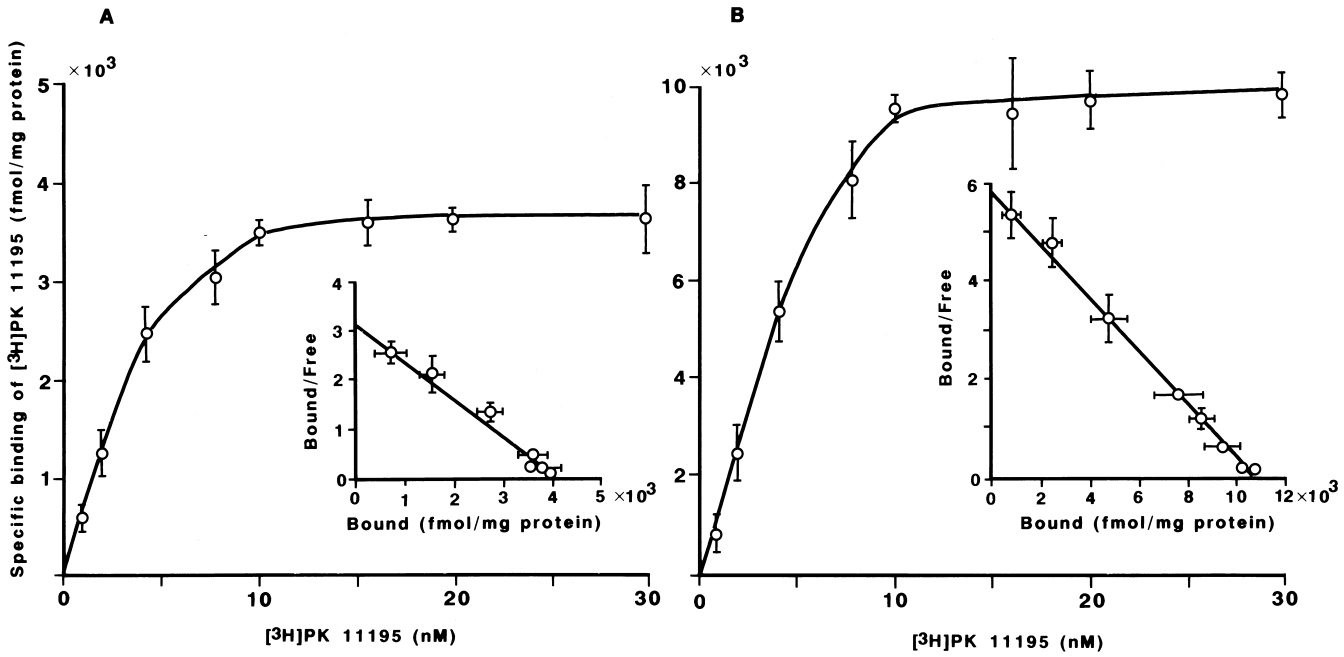

FIG. 2. Saturation isotherm and Scatchard analysis (inset) of [^{3}H]PK11195 binding to rat parotid (A) and submandibular (B) gland membranes. Membranes were incubated with [^{3}H]PK11195 for 60 min at 4°. Specific binding represents the difference between total binding and nonspecific binding in the presence of 1 μM unlabeled PK11195. Results are the means ± SD of four independent experiments measured in triplicate.

TABLE 2. Inhibition of [^{3}H]Ro15-1788 binding to rat parotid and submandibular gland membranes

Inhibitors	K_i (nM)	
	Parotid gland	Submandibular gland
Ro15-1788	13.9 ± 2.3	34.8 ± 4.6
Clonazepam	18.6 ± 3.0	33.4 ± 1.7
Diazepam	180 ± 31	193 ± 12
Zolpidem	622 ± 35	767 ± 93
CL218,872	644 ± 39	3,860 ± 62
Flunitrazepam	3,136 ± 700	3,450 ± 480
β-CCE	5,800 ± 1,520	2,920 ± 227
PK11195	16,200 ± 2,700	39,200 ± 1,700
Ro5-4864	55,900 ± 3,500	42,900 ± 1,400

Each K_i value was calculated from the equation $K_i = IC_{50}/(1 + [L]/K_d)$. The IC_{50} value was the concentration of inhibitor producing 50% inhibition in the presence of 20 nM [^{3}H]Ro15-1788 and inhibitors (10^{-3} to 10^{-10} M). The K_d value was obtained from Table 1. [L] was the radioligand concentration. Values are means ± SD of four independent experiments measured in triplicate.

TABLE 3. Inhibition of [^{3}H]PK11195 binding to rat parotid and submandibular gland membranes

Inhibitors	K_i (nM)	
	Parotid gland	Submandibular gland
Ro5-4864	0.41 ± 0.06	1.24 ± 0.06
PK11195	0.54 ± 0.17	1.41 ± 0.35
Diazepam	12.0 ± 2.5	30.8 ± 1.2
Flunitrazepam	14.9 ± 2.6	40.4 ± 4.1
Dipyridamole	87.9 ± 27	162 ± 34
Zolpidem	187 ± 45	616 ± 26
Clonazepam	73,300 ± 15,300	20,200 ± 5,800
CL218,872	53,160 ± 5,710	66,870 ± 24,200
Ro15-1788	Inactive at 10^{-4} M	Inactive at 10^{-4} M

Each K_i value was calculated from the equation $K_i = IC_{50}/(1 + [L]/K_d)$. The IC_{50} value was the concentration of inhibitor producing 50% inhibition in the presence of 1 nM [^{3}H]PK11195 and inhibitors (10^{-3} to 10^{-10} M). The K_d value was obtained from Table 1. [L] was the radioligand concentration. Values are means ± SD of four independent experiments measured in triplicate.

[^{3}H]PK11195 binding to rat parotid and submandibular gland membranes. Specific binding of [^{3}H]PK11195 to parotid or submandibular gland membrane was saturated at 10–15 nM, whereas nonspecific binding increased linearly with the concentration of [^{3}H]PK11195 while maintaining less than 10% of the total binding. Scatchard analysis from the saturation curves yielded a single population of non-interacting binding sites to both gland membranes (Fig. 2); the Hill coefficients of [^{3}H]PK11195 binding were close or equal to 1.00 (1.00 ± 0.10 and 0.98 ± 0.07 for parotid gland and submandibular gland, respectively). [^{3}H]PK11195 bound with high affinity (nanomolar range) to both salivary gland membranes. The densities of [^{3}H]PK11195 binding to parotid and submandibular gland membranes were 19 and 54%, respectively, of the adrenal cortex, in which the highest density was observed (Table 1).

Pharmacological Characterization of [^{3}H]Ro15-1788 and [^{3}H]PK11195 Binding

The pharmacological specificity of [^{3}H]Ro15-1788 and [^{3}H]PK11195 binding sites in rat parotid and submandibular glands was analyzed with competition curves using at least eleven concentrations of BDZs and non-BDZ drugs (Tables 2 and 3). Ro15-1788 and clonazepam potently inhibited [^{3}H]Ro15-1788 binding to both salivary gland membranes at nanomolar concentrations. Diazepam moderately inhibited [^{3}H]Ro15-1788 binding, and the other inhibitors tested were effective in the following order: zolpidem > CL218,872, flunitrazepam, β-CCE > PK11195 > Ro5-4864 (Table 2). On the other hand, PK11195 and Ro5-4864 potently inhibited [^{3}H]PK11195 binding to both salivary gland membranes at low nanomolar concentrations. Diazepam and flunitrazepam inhibited [^{3}H]PK11195 binding strongly, and zolpidem inhibited it moderately. Clonazepam and CL218,872 were much less effective in the inhibition of the binding to both glands. These results suggest that characteristic and specific binding sites for [^{3}H]Ro15-1788 and [^{3}H]PK11195 exist in rat parotid and submandibular glands.

DISCUSSION

Braestrup and Squires [8] originally proposed that central- and peripheral-type BDZ receptors were distributed in the brain and peripheral organs such as kidney, respectively. These receptors were indicated by the potencies of selective ligands for each receptor in displacing [^{3}H]diazepam binding. Their study was enforced by Anholt *et al.* [10], who demonstrated by autoradiography and receptor binding assays the binding of the selective peripheral-type radioligand [^{3}H]Ro5-4864 in several peripheral organs, including the rat salivary gland. We have extended their findings by precisely examining the properties of BDZ receptors in the rat salivary gland with [^{3}H]Ro15-1788 and [^{3}H]PK11195 as selective radioligands for central-type and peripheral-type BDZ receptors, respectively.

During the past decade, some investigators have accumulated evidence that central-type BDZ receptors are also located in peripheral organs, including the adrenal gland [4, 11, 12], pancreas [5], and retina [15]. Our present study has shown clearly that the central-type BDZ receptors are located in the rat parotid and submandibular gland. [^{3}H]Ro15-1788 binding sites in both salivary glands displayed high affinity, with nanomolar levels of K_d values. These values are substantially similar to that observed in adrenal medulla by Kataoka *et al.* [12] (Table 1). The density of this binding site was ~60- to ~90-fold less than that counted in the brain and ~200- to 400-fold less than peripheral-type ligand binding sites in both glands (Table 1). Moreover, [^{3}H]Ro15-1788 binding was reduced remarkably by lower levels of unlabeled Ro15-1788 or clonazepam, which have been shown to have high affinities for central-type BDZ receptor, whereas it was not modified by submicromolar concentrations of selective peripheral-type BDZ

receptor ligands (Table 2). These findings strongly support the hypothesis that BDZ receptors in both salivary glands include small numbers of central-type BDZ receptors.

Scatchard analysis (Table 1) demonstrated that the K_d values of [^{3}H]Ro15-1788 binding in these salivary glands were 28- to 43-fold higher than that of the binding in the cerebral cortex. These differences suggest that central-type BDZ receptors in parotid and submandibular glands include subunits with a structure that is slightly changed from those in the cerebral cortex. Additional studies on subunits of central-type BDZ receptors in salivary glands and the cerebral cortex will be essential for clarifying their molecular biological properties.

The central-type BDZ receptor includes two subtypes classified as BZ1 and BZ2 by their differences in affinity for some BDZ derivatives. BZ1 is defined as a binding site with high affinity for β-carbolines, triazolopyridazines, and imidazopyridines, whereas BZ2 has low affinity for these ligands [16, 17]. In bovine adrenal medulla, the central-type BDZ receptor was shown to be a BZ1-type by immunoblotting [4]. In our experiments using rat salivary glands, [^{3}H]Ro15-1788 binding was not replaced by lower concentrations of β-CCE, zolpidem, and CL218,872 (Table 2). These findings suggest that, in the rat salivary gland, the major subtype of central-type BDZ receptor is BZ2. Our findings demonstrated that not only large amounts of peripheral-type BDZ receptors but also small amounts of central-type receptors exist in rat salivary glands. The central-type BDZ receptors in both salivary glands have high affinity for [^{3}H]Ro15-1788, and their densities represented 30–50% of that in the rat adrenal gland (Table 1).

On the peripheral-type BDZ receptor, several studies have suggested that the site of BDZs (Ro5-4864) partially overlaps or is allosterically coupled to the binding domain of isoquinoline derivatives like PK11195. Their detailed findings were as follows: (1) the differential modulation of [^{3}H]Ro5-4864 and [^{3}H]PK11195 bindings by arachidonate, diethylpyrocarbonate, and thiol reagents in the rat kidney [18], and (2) the differential potency of protoporphyrin IX in displacing [^{3}H]Ro5-4864 and [^{3}H]PK11195 binding sites in rat vas deferens [19].

In the study of the peripheral-type BDZ receptor, the binding of PK11195 was saturable, reversible, and temperature and time dependent. From the saturation binding studies, we have obtained a K_d value similar to that observed in adrenal cortex by Anholt *et al.* [10] (Table 1), and that in other peripheral tissues [20, 21], with a Hill coefficient of ~1.00 (data not shown). These binding sites had high affinity for Ro5-4864 but were hardly displaced by the central-type specific ligands clonazepam, CL218,872, and Ro15-1788. Moreover, Ro5-4864 was not able to competitively displace [^{3}H]PK11195 from its binding sites, as derived from the value of the slope of the Hill plot (n_H ~0.60 to ~0.70) (data not shown). Our findings suggest that these binding sites in both salivary glands as well as in other peripheral tissues [18, 19] have either negative cooperativity or two differential domains for binding the isoquinoline PK11195 and the BDZ Ro5-4864.

In conclusion, the present results indicate the presence of central- and peripheral-type BDZ receptors with high affinity for [^{3}H]Ro15-1788 and [^{3}H]PK11195, respectively, in cell membrane preparations of rat salivary glands.

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