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Modulation of benzodiazepine receptor, adrenoceptor and muscarinic receptor by diazepam in rat parotid gland

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ABSTRACT

This study investigated the influence of diazepam on the binding characteristics of adrenoceptor, muscarinic and benzodiazepine receptors in rat parotid gland membrane using a radioligand binding assay. At a concentration of $>10^{-6}$ M, diazepam competed with [^{3}H]dihydroalprenolol for β-adrenoceptor, but not [^{3}H]prazosin for α-adrenoceptor or [^{3}H]quinuclidinyl benzilate for muscarinic receptor. Continuous administration of diazepam at doses of 0.4 mg/kg/day, i.p. for 7 days in rat significantly decreased pilocarpine (4.0 mg/kg, i.p.)-induced parotid salivary flow. Diazepam also produced a significant increase in the dissociation constant (Kd) value for [^{3}H]dihydroalprenolol binding, but no change in the maximal binding capacity (Bmax) value, and a decrease in the Kd value for [^{3}H]diazepam binding to benzodiazepine receptors, but no change in the Kd or Bmax values for [^{3}H]prazosin or [^{3}H]quinuclidinyl benzilate binding. These results suggest that continuous administration of diazepam modifies affinity for β-adrenoceptor and benzodiazepine receptor binding sites in parotid gland membrane and that changes in these binding sites may be closely related to diazepam-induced suppression of salivary secretion.

1. Introduction

Secretory response in salivary gland is controlled by both sympathetic and parasympathetic innervations (Baum, 1987). Mammalian salivary gland carries a number of receptors for secretory response located on the basolateral membrane of epithelial cells (Kawaguchi and Yamagishi, 1995). α- and β-adrenoceptors and M_3-muscarinic receptors play a major role in the regulation of fluid and protein secretion in salivary gland (Ito et al., 1981, 1982; Dai et al., 1991).

The clinical effects of benzodiazepines such as diazepam are mediated by benzodiazepine receptors. These receptors are classified as central-type, which are linked to the $GABA_A$ receptor-chloride channel complex, and peripheral-type, which are not (Braestrup and Squires, 1977; Möhler and Okada, 1977; Verma and Snyder, 1989). Furthermore, chronic administration of benzodiazepines induces several side effects such as drug dependence, amnesia, anorexia and xerostomia (Sreebny and Schwartz, 1986). Xerostomia is caused by a reduction in salivary secretion and is a critical factor in the development of dental caries, periodontitis and stomatitis. Xerostomia has also been studied in relation to diabetic stress and the GABAergic system in salivary gland (Izumi et al., 2008; Kosuge et al., 2009). In earlier *in vivo* and *in vitro* studies, we reported that acute administration of benzodiazepines at high doses suppressed salivary response in rat (Kawaguchi et al., 1995). Benzodiazepines at a clinical dose in human inhibited both amylase release from parotid gland cells and phosphoinositide turnover, which regulates salivary secretion in these cells; furthermore, the inhibitory response of parotid gland cells was blocked by antagonists of benzodiazepine receptors (Okubo and Kawaguchi, 1998, 2010; Kujirai et al., 2002). We also reported that both central- and peripheral-type benzodiazepine receptors were found in salivary glands of rat and human, as well as brain (Yamagishi and Kawaguchi, 1998; Yamagishi et al., 2000). In a receptor binding assay, the ligand for the peripheral-type benzodiazepine receptor bound to the salivary gland membranes of both rat and human with high affinity at nanomolar level (Yamagishi et al., 2000). These findings indicate that benzodiazepines not only suppress the central nervous system, but also act directly on salivary gland to inhibit secretory response. They also suggest that the affinity of benzodiazepine receptors in rat salivary gland is likely similar to that in human gland. However, it is not yet clear as to whether benzodiazepines affect other major receptors related to salivary secretion.

The aim of this study was to investigate the effect of diazepam on the radioligand binding properties of α- and β-adrenoceptors and muscarinic receptors in salivary gland. We also investigated the effect of continuous administration of diazepam on the binding characteristics of benzodiazepine receptors in salivary gland in rat. Our goal was to elucidate the relationship between benzodiazepines and adrenoceptors in salivary gland.

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2. Materials and methods

2.1. Chemicals

[^{3}H]Prazosin (77.9 Ci/mmol), [^{3}H]dihydroalprenolol hydrochloride (100.5 Ci/mmol), [^{3}H]quinuclidinyl benzilate (43.5 Ci/mmol) and [^{3}H]diazepam (83.0 Ci/mmol) were purchased from NEN (Boston, MA, USA). Flunitrazepam was donated by Nippon Roche (Kamakura, Japan). Ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo(1,5-α)(1,4)-benzodiazepine-3-carboxylate (Ro 15-1788) and 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepine-2-one (Ro 5-4864) were obtained from Hoffman-La Roche (Basel, Switzerland). Prazosin hydrochloride, rauwolscine hydrochloride, atenolol, salbutamol sulfate, pirenzepine dihydrochloride monohydrate, 2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane hydrochloride (WB4101), diazepam, propranolol hydrochloride, atropine sulfate, dimethyl sulfoxide and pilocarpine hydrochloride were obtained from Wako Pure Chemical (Osaka, Japan). 4-Diphenylacetoxy-N-methylpiperidine methbromide (4-DAMP) was obtained from Research Biochemical International (Natick, MA, USA). Methoctramine tetrahydrochloride and protease inhibitors were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). All other reagents used were of analytical grade. Each drug solution was freshly prepared before the experiment. For the receptor binding assay, drugs were dissolved in 99.5% ethanol or distilled water and then diluted. The final concentration of ethanol (<1%) had no effect on the assay.

2.2. Animals

Male Wistar rats weighing 200–250 g each were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The animals were kept at a constant room temperature (21 ± 2 °C) under a 12-h light-dark cycle (lights on between 6:00 am and 6:00 pm) and maintained on commercial laboratory chow and tap water for at least 7 days before being used. All efforts were made to minimize the number of animals used and suffering. All experiments complied with the Guidelines for the Treatment of Experimental Animals approved by The Japanese Pharmacological Society and Tokyo Dental College.

2.3. Administration of diazepam

Diazepam at doses of 0.2 or 2 mg/kg/day was intraperitoneally administered twice daily (total volume 0.4 or 4 mg/kg/day) at a 12-h interval for 7 days in rats. The low dose of diazepam was determined from the therapeutic dose in human, but was not a high dose. Diazepam was dissolved in dimethyl sulfoxide and control rats were given an equivalent volume of dimethyl sulfoxide (0.1 ml/100 g body weight) at the same interval.

2.4. Collection of parotid saliva

Parotid saliva was collected as described previously (Kawaguchi et al., 2000). Briefly, the drug-treated and control rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), secured in a supine position and a tracheal tube inserted to support respiration. The tapered end of each capillary cannula (PE-50; Clay-Adams, Becton Dickinson, MD, USA) was inserted into the parotid duct exposed on the buccal surface using a microscope and the other end was inserted into a 0.5-ml microtube. Saliva secreted after pilocarpine (4 mg/kg, i.p.) administration was collected every 30 min for 1 h.

2.5. Preparation of membranes

Membranes were prepared as described previously (Yamagishi and Kawaguchi, 1998). Briefly, the rats were anesthetized with ether and parotid gland quickly excised, taking care that the tissue was removed free of fat and connective tissues. The gland was homogenized in 10 volume of ice-cold 0.32 M sucrose containing protease inhibitors (aprotinin, 15 μg/ml; antipain, 5 μg/ml; pepstatin A, 5 μg/ml; and leupeptine, 5 μg/ml) with the Brinkmann Polytron at a speed setting of 5 for 1 min. The homogenate was centrifuged at 1000 × *g* for 10 min. The supernatant was then centrifuged at 48,000 × *g* for 20 min. The resulting pellet was re-suspended in 10 volume of 50 mM Tris/HCl buffer (pH 7.4) and re-centrifuged at 48,000 × *g* for 20 min. The wash step was repeated twice. The final pellet was re-suspended in the same buffer and used as the membrane source for the receptor binding assay. All preparative steps were performed at 4 °C. Protein concentration was measured with the BCA Protein Assay Reagent Kit (Perce, Rockford, IL, USA).

2.6. Receptor binding assay

The assay was carried out under optimum conditions for the competitive compounds and temperatures and durations of incubation for each receptor binding assay based on earlier reports (Dashwood, 1982; Dai et al., 1991; Landi et al., 1992; Yamagishi and Kawaguchi, 1998). The assay conditions are shown in Table 1. Parotid membrane (approximately 0.2 mg protein) was suspended in 50 mM Tris/HCl buffer (pH 7.4) with radioligands and unlabeled compounds to a final volume of 0.5 ml. After incubation, the reaction was terminated by rapid vacuum filtration through a Whatman GF/B glass fiber filter using the Brandel M-24S filtering manifold (Brandel Instruments, Gaithersburg, MD, USA). The filter was then washed twice with 5 ml of the same ice-cold buffer. Radioactivity in the filter was measured in 6 ml Scintisol (Dojin, Kumamoto, Japan) using a liquid scintillation counter (Packard, Meriden, CT, USA). The dissociation constant (Kd) and maximal binding capacity (Bmax) for each ligand binding to the membranes were obtained from the results of

Table 1
Conditions for receptor binding assay.

Target receptor	Benzodiazepine receptor	α-adrenoceptor	β-adrenoceptor	Muscarinic receptor
Radioligand	[^{3}H]diazepam	[^{3}H]prazosin	[^{3}H]DHA	[^{3}H]QNB
Radioligand concentration for saturation experiment	0.1–50 nM	0.4–10 nM	0.5–20 nM	0.1–10 nM
Unlabeled compound for saturation experiment (concentration)	Diazepam (1 μM)	Prazosin (2 μM)	Propranolol (10 μM)	Atropine (10 μM)
Radioligand concentration for competition experiment	2 nM	0.1 nM	1 nM	0.1 nM
Unlabeled compound for competition experiment	Diazepam, Flunitrazepam Ro 15-1788, Ro 5-4864	Diazepam, Rauwolscine WB4101, Prazosin	Diazepam, Atenolol Salbutamol Propranolol	Diazepam, Methoctramine 4-DAMP, Pirenzepine
Temperature	4 °C	25 °C	37 °C	37 °C
Incubation time	20 min	20 min	10 min	90 min

DHA, dihydroalprenolol; QNB, quinuclidinyl benzilate; Ro 15-1788, ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo(1,5-α)(1,4)-benzodiazepine-3-carboxylate; Ro 5-4864, 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepine-2-one; WB4101, 2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane hydrochloride; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methbromide.