Introduction

In an earlier study using whole-body autoradiography with \(^{3}H\)diazepam, benzodiazepine binding sites were reported to be distributed not only in the brain, but also in salivary gland (1), strongly suggesting the existence of benzodiazepine-associated receptors in parotid gland (PG), submandibular gland (MG), and sublingual gland (LG).

During the past decade, we have accumulated evidence that benzodiazepine-induced xerostomia was mediated through linking of central-type benzodiazepine receptors (CBR) to \(\gamma\)-aminobutyric acid A receptors (GABA\(_A\)-R) containing chloride channels (GABA\(_A\)/CBR) and peripheral-type benzodiazepine receptors (PBR) on mitochondrial membrane in rat salivary gland (2, 3). Yamagishi et al. demonstrated the specificity of the \(K_d\) and \(B_{max}\) values in CBR and PBR in PG and MG in rats (4, 5). Kujirai et al. showed that benzodiazepine receptors were involved in suppression of inositol 1,4,5-trisphosphate (IP\(_3\)) production (6). Furthermore, Okubo et al. showed that PBR, CBR, and GABA\(_A\)-R were involved in suppression of amylase release (7, 8). These findings support the hypothesis that benzodiazepines specifically inhibit salivary secretion through both CBR and PBR. These receptors hold a specific endogenous ligand, diazepam-binding inhibitor (DBI), which is characterized by approximately 10-kDa polypeptides and is distributed throughout both the central nervous system and peripheral organs (9 – 12).

DBI was found to be an inverse agonist of CBR (13), as well as an endogenous agonist of PBR in mitochondria (14). Binding of DBI to PBR leads to the formation of neurosteroids such as pregnenolone (PRG), which can modulate CBR activity (15, 16). Expression of DBI is regulated at the transcriptional level through pituitary adenylate cyclase–activating polypeptide (PACAP) (17, 18). Several studies demonstrated that repetitive administration of morphine (19, 20), ethanol (21), nicotine (22, 23), or methamphetamine (24) increased DBI mRNA in...
the cerebral cortex (Cx) of mice and rats.

In recent studies, we have demonstrated that repetitive, but not single, administration of diazepam (DZP) at low doses (0.4 mg/kg) for more than 7 days markedly inhibited saliva secretion (in submission) and that perfusion of MG with PRG, a major neurosteroid produced by binding of DBI with its receptor, in rat suppressed carbachol-induced salivary secretion (25). Based on these observations, we hypothesized that DBI was involved in xerostomia in adult patients receiving long-term administration of clinical dosages of DZP or other benzodiazepines. To determine the relationship between DBI and salivary secretion, we investigated the effects of repetitive administration of low-dose DZP for 14 days on expression of DBI mRNA and peptide in salivary gland in rats.

**Materials and Methods**

*Animals and materials*

All the present animal experiments were performed in strict accordance with the Guidelines for the Treatment of Experimental Animals approved by The Japanese Pharmacological Society and Tokyo Dental College. Male Wistar rats (Clea Japan, Tokyo) weighing 170 – 270 g each were group-housed in laboratory cages and kept in a temperature-controlled room (21 ± 2°C) under a 12-h light/dark cycle (lights on at 6:00) with food and water freely available. DZP was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). PCR primers and the SUPERSCRIPT First-Strand Synthesis System for RT-PCR containing reverse transcriptase (RTase) (Superscript™ III, RNase H−), dithiothreitol (DTT), oligo(dT)12–18 primer, deoxynucleosidetriphosphate (dNTP), RNase H, DNase I, and RNase inhibitor were purchased from Invitrogen (San Diego, CA, USA). NucleoSpin RNAII was obtained from MACHEREYNAGEL GmbH&Co. (Duren, Germany). The DyNAmo SYBER green qPCR Kit was obtained from Finnzymes (Espoo, Finland), and the DNA 1000 Lab Chips Kit (Agilent Technologies, Palo Alto, CA, USA) was obtained from Takara Bio Co. (Tokyo).

**Administration of DZP**

DZP was dissolved in dimethyl sulfoxide (DMSO). DZP was administered at 10 mg/kg as an effective dose for maximally suppressing salivary flow vs. at 0.4 mg/kg as the maximal daily dose in clinical use. Rats were injected intraperitoneally (i.p.) with 10 or 0.4 mg/kg DZP as a single administration or with 0.4 mg/kg DZP once-daily (either at 12:00 PM or at 6:00 PM) for 14 days as repetitive administration. The control group was injected with DMSO. Animals were randomly allocated to a group receiving DMSO or an experimental group receiving DZP injections. The salivary glands and brain were removed and separated into PG, MG, LG, or Cx at 2 or 16 h after the final administration for analysis of expression of mRNA and peptide (Fig. 1). Rats were sacrificed between 10:00 AM and 2:00 PM.

**Real time quantitative RT-PCR**

After treatment of total RNA with DNase I, first-strand cDNA was synthesized using Oligo(dT)12–18 primers and Superscript™ III RNase H− reverse transcriptase. Gene expression of DBI, PACAP, and PBR was determined by using the β-actin gene (GenBank accession number NM_031144) as an internal control and primers specific for DBI mRNA (accession number NM_031853) (upper primer, ACG CTC TGG AAC TTG ATT GC; lower primer, CAG TTG GCT GAG TCT TGA GG; product size, 138 base pairs), PACAP mRNA (NM_016989) (upper primer, TGT CCG CCA GGA AGT ACC; lower

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**Fig. 1.** Time schedule of administration of 0.4 mg/kg DZP. N.D.: Not determined.
primer, CCG AGT GGC GTT TGG TAA; product size, 105 base pairs), and PBR mRNA (NM_012515) (upper primer, ACA CTG GTC AGC TGG CTC TGA A; lower primer, CAG GCC AGG TAA GGA TAC AGC AA; product size, 175 base pairs). The cDNA was amplified by real-time quantitative PCR using the DyNAmo SYBER green qPCR Kit (Finnzymes) on the DNA Engine Opticon 2 System (Bio-Rad Laboratories; Hercules, CA, USA), running 48 cycles of the following protocol: 15 min predenaturation at 95°C, 10-s denaturation at 94°C, 20-s annealing at 60°C for \(\beta\)-actin or 58.6°C for DBI, PACAP, and PBR, followed by a 30-s extension at 72°C. The PCR products were separated with the Agilent 2100 Bioanalyzer (Agilent Technologies), which utilizes chip-based nucleic acid separation technology. Furthermore, identification of the amplified PCR products of the DBI and \(\beta\)-actin cDNAs was performed by dye terminator cycle sequencing.

**Western blot analysis for DBI**

Tissues were washed with ice-cold phosphate buffered saline (PBS)\(^{-}\) and then stored at \(-80^\circ\mathrm{C}\). The tissues were homogenized in 500 \(\mu\)l ice-cold lysis buffer [150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 50 mM Tris-HCl (pH 8.0)] containing complete mini protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany) and incubated for 30 min on ice. After centrifuging at 14,000 \(\times\) g for 15 min at 4°C, the resulting supernatants were heated in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer for 10 min at 70°C. The proteins (10 \(\mu\)g) were subjected to SDS-PAGE using 4% – 12% Bis-Tris-SDS gels (Invitrogen) for separation of DBI and \(\beta\)-actin, followed by electrophoretic transfer onto a polyvinylidene fluoride (PVDF) membrane (Hybond™-LFP; GE-Healthcare, Buckinghamshire, UK) at 20 V for 1.5 h. Membranes were blocked with 0.5% bovine serum albumin (BSA) for 1 h at room temperature and blotted with rabbit polyclonal antibodies specific for DBI (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-30190), followed by horseradish peroxidase–linked secondary antibody. The ECL plus Western blotting detection system (GE Healthcare) was used for detection.

**Collection of saliva from PG, MG, and LG**

Saliva was collected as described previously (26). Briefly, the rats were secured in a supine position, anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and a tracheal tube inserted to support respiration. The tapered end of a capillary cannula (size No.4; Imamura, Tokyo) was inserted into the parotid duct, sublingual papillae, and sublingual openings to obtain saliva from PG, MG, and LG, respectively, while the other end was placed inside a 0.5-ml microtube to collect secreted saliva. Saliva was collected every 15 min over 1 h by stimulation with pilocarpine (1.0 mg/kg, i.p.), which was the same dose as that used in a previous report (27). At 2 or 16 h after the final administration of DZP, 1.0 mg/kg pilocarpine was injected.

**Statistical analyses**

The results are given as the mean with standard error of the mean (S.E.M.). Statistical analyses were conducted by using computer software (Prism, version 5.0c; GraphPad Software, San Diego, CA, USA) for comparison across the experimental conditions. Statistical comparisons were performed using a one-way analysis of variance (ANOVA) followed by the Dunnett’s post-hoc test or the Mann-Whitney test. A value of \(P < 0.05\) was considered to be statistically significant.

**Results**

**Suppression of secretion of saliva amplified/induced by repetitive administration of 0.4 mg/kg DZP**

Stimulation with 1.0 mg/kg pilocarpine increased salivary secretion to a maximum at 30 min, after which it returned to initial levels within 1 h.

Although single administration of 0.4 mg/kg DZP did not suppress pilocarpine-induced salivary secretion, single administration of 10 mg/kg DZP induced a significant reduction in salivary secretion in PG, MG, and LG (Fig. 2). Salivary secretion decreased to 43%, 41%, and 44% of that of the controls in PG, MG, and LG, respectively, at 2 h after administration of 10 mg/kg DZP.

Repetitive administration of 0.4 mg/kg DZP induced a
significant reduction in salivary secretion in PG, MG, and LG (Fig. 3). Salivary secretion decreased to 42%, 53%, and 46% of that of the controls in PG, MG, and LG, respectively, at 2 h after the final administration of DZP. Furthermore, at 16 h after the final administration, salivary secretion decreased to 61%, 63%, and 56% of that of the control in PG, MG, and LG, respectively.

Repetitive administration, but not single administration, of 0.4 mg/kg DZP increased both mRNA and peptide expression levels of DBI, PACAP, and PBR

Single administration of 0.4 mg/kg DZP did not affect the mRNA levels of DBI, PACAP, or PBR in PG, MG, LG, or Cx, either at 2 or 16 h after medication with DZP (Fig. 4: A – C). Similarly, Western blot analysis revealed no change in DBI peptide levels (Fig. 5: A and B). A higher dose of DZP at 10 mg/kg resulted in a significant increase in DBI mRNA levels, reaching 1.4- to 2.0-fold of that in the controls in all regions, although PACAP and PBR mRNA levels showed no change (Fig. 6: A – C).

![Fig. 3. Inhibitory effect at 2 or 16 h after repetitive administration of 0.4 mg/kg DZP on pilocarpine-induced salivary secretion in PG, MG, and LG. Results represent the mean with S.E.M. of data. Significantly different from values in group treated with DMSO by the Mann-Whitney test, *P < 0.05. PG (DMSO, n = 4; 2 h, n = 4; 16 h, n = 5), MG (n = 4, n = 4, n = 4, respectively), LG (n = 4, n = 5, n = 4, respectively).](image)

![Fig. 4. The mRNA expression of DBI (A), PACAP (B), and PBR (C) in PG, MG, LG, and Cx in rats at 2 or 16 h after single administration of 0.4 mg/kg DZP. Results represent the mean with S.E.M. of data. Significantly different from values in the group treated with DMSO by Dunnett’s post-hoc test following one-way analysis of variance (ANOVA) test. Cx (DMSO, n = 5; 2 h, n = 5; 16 h, n = 5), PG (n = 5, n = 4, n = 4, respectively), MG (n = 4, n = 4, n = 4, respectively), LG (n = 5, n = 5, n = 4, respectively).](image)
**Fig. 5.** Peptide levels of DBI in PG, MG, LG, and Cx in rats at 2 h (A) or 16 h (B) after single administration of 0.4 mg/kg DZP. Results represent the mean with S.E.M. of data from five rats. Significantly different from values in the group treated with DMSO by the Mann-Whitney test.

**Fig. 6.** The mRNA expression of DBI (A), PACAP (B), and PBR (C) in PG, MG, LG, and Cx in rats at 2 or 16 h after single administration of 10 mg/kg DZP. Results represent the mean with S.E.M. of data. Significantly different from values in the group treated with DMSO by Dunnett’s post-hoc test following one-way ANOVA; *P < 0.05, **P < 0.01. Cx (DMSO, n = 5; 2 h, n = 5; 16 h, n = 4), PG (n = 4, n = 5, n = 5, respectively), MG (n = 4, n = 5, n = 5, respectively), LG (n = 5, n = 5, n = 4, respectively).
Repetitive administration of 0.4 mg/kg DZP resulted in a marked increase in mRNA levels of DBI, PACAP, and PBR to 1.6- to 4.0-fold of that in the controls in all regions at 2 h after injection of DZP (Fig. 7: A – C). However, at 16 h, all mRNA levels in all regions had returned to normal levels. Western blot analysis revealed that all peptide levels were enhanced in the same manner with increase in mRNA levels at 2 h, showing a marked increase of 1.4- to 2.0-fold of that in the controls at 16 h after the final injection of DZP (Fig. 8: A and B).

Discussion

Our present study has shown clearly that repetitive administration of DZP decreased secretion of saliva and increased expression of DBI and PACAP mRNA and DBI peptide in rat salivary gland. This suggests that repetitive administration of DZP increases DBI peptide levels in salivary gland through increase in expression of PACAP mRNA. These results are supported by several lines of evidence. First, upregulation of DBI mRNA expression may increase DBI peptide levels after repetitive administration of DZP, as DBI peptide levels are regulated at the transcriptional level (17). Second, a sequence homologous to the cyclic AMP response element was found in the 5′-flanking region of the PACAP gene (28). Third, administration of DZP causes phosphorylation of cyclic AMP response element binding protein (CREB) and induction of downstream CREB-regulated genes (29). Fourth, an increase in PACAP levels upregulates DBI mRNA expression through activation of PACAP-selective receptors (PAC1-R) located in astrocytes (18). A substantial amount of PAC1-R mRNA was expressed in PG, MG, and LG, as well as in Cx in rats (data not shown). Further study is needed to identify which cell types in salivary gland, acinar cells or duct cells, secrete PACAP and express PAC1-R.

To the authors’ knowledge, this is the first study to demonstrate that substantial amounts of DBI mRNA and peptides are expressed in PG, MG, and LG, as well as in Cx of rats by quantitative analysis. Moreover, these results demonstrate that repetitive administration of DZP enhances DBI peptide expression, which is related to an increase in mRNA expression. These results agree well with those of Ferrarese et al. suggesting that DBI peptide levels are regulated at the transcriptional level (17).

**Fig. 7.** The mRNA expression of DBI (A), PACAP (B), and PBR (C) in PG, MG, LG, and Cx in rats at 2 or 16 h after repetitive administration of 0.4 mg/kg DZP. Results represent the mean with S.E.M. of data. Significantly different from values in the group treated with DMSO by Dunnett’s post-hoc test following one-way ANOVA; *P < 0.05, **P < 0.01. Cx (DMSO, n = 5; 2 h, n = 5; 16 h, n = 5), PG (n = 5, n = 4, n = 4, respectively), MG (n = 4, n = 4, n = 4, respectively), LG (n = 5, n = 5, n = 4, respectively).
Effects of Diazepam on DBI Production

Single administration of 10 mg/kg DZP increased expression of DBI mRNA without increasing expression of PACAP mRNA. This observation does not agree with that from repetitive administration of 0.4 mg/kg DZP. These results after single administration of DZP suggest that expression of DBI mRNA is regulated by mechanisms other than increase in PACAP. Further study is needed to clarify the other mechanisms involved in such increases in expression of DBI mRNA.

DBI, an endogenous agonist of PBR, stimulates neurosteroid synthesis in mitochondria. Neurosteroids are produced at the early stage of steroid biosynthesis. PRG, a major precursor of steroid hormones formed from cholesterol by the cytochrome P450 side-chain cleavage

![Fig. 8. Peptide levels of DBI in PG, MG, LG, and Cx in rats at 2 h (A) or 16 h (B) after repetitive administration of 0.4 mg/kg DZP. Results represent the mean with S.E.M. of data from five rats. Significantly different from values in the group treated with DMSO by the Mann-Whitney test; *P < 0.05, **P < 0.01.]

![Fig. 9. Pathways of action of DBI in salivary gland. An increase in PACAP upregulates DBI mRNA expression through activation of PAC1-R. DBI, an endogenous agonist of PBR, stimulates neurosteroid synthesis through PBR in mitochondria. PRG, a neurosteroid and a first metabolite formed from cholesterol by CYP11A1, acts as an allosteric agonist of GABA_A-R. Therefore, overexpression of PRG enhances GABA_A-R function, resulting in an increase in intracellular Cl^- levels, followed by termination of Ca^{2+} release from intracellular calcium stores and suppression of Cl^- efflux throughout Ca^{2+}-dependent Cl^- channels in apical membrane of acinar cells. These events lead to a decline in transcellular movement of water, causing a decrease in salivary secretion.]


enzyme (P450sec: CYP11A1), acts as an allosteric agonist of GABA_A-R (30, 31). From our recent study, it was speculated that steroid biosynthesis by CYP11A1 occurred in salivary gland and that salivary secretion induced by perfusion of rat MG with carbachol chloride was inhibited by PRG (25). Expression of PRG enhances GABA_A-R function, resulting in an increase in intracellular Cl^- levels, followed by termination of Ca^{2+} release from intracellular calcium stores and suppression of Cl^- efflux throughout Ca^{2+}-dependent Cl^- channels in the apical membrane of acinar cells. These events lead to a decrease in transcellular movement of water, causing a decrease in salivary secretion (Fig. 9).

The present observations suggest that activation of PBR in salivary gland potentiates the suppressive effect of DZP on saliva secretion. Several lines of evidence support this possibility. First, expression of PBR mRNA in salivary gland was more than 10-fold greater than that in Cx. This result agrees with that of our previous study, in which it was demonstrated that the B_{max} of PBR in salivary gland was 10- to 30-fold greater than that in Cx (4). The B_{max} of CBR in salivary gland was 60- to 90-fold less than that in Cx using [3H]Ro15-1788, a CBR antagonist (4). Second, the affinity of [3H]Ro15-1788 in salivary gland was 30- to 40-fold less than that in Cx (4). Third, DZP acts through both CBR and PBR binding sites in salivary gland (2). Fourth, the selective PBR antagonist PK11195 markedly reduced [3H]thymidine incorporation in rat astrocytes induced by low-dose triakontatetraneuropeptide (TTN), a DBI-derived peptide. In contrast, the selective CBR antagonist flumazenil did not significantly modify the effect of TTN (32). Further research is, however, needed to elucidate whether the inhibition of salivary secretion by repetitive administration of DZP is attenuated by simultaneous treatment with a peripheral-type benzodiazepine antagonist.

In conclusion, repetitive administration, but not single administration, of 0.4 mg/kg DZP increased DBI, PACAP, and PBR mRNA and DBI peptide in rat salivary gland, probably resulting in an increase in the suppressive effect of DZP on saliva secretion. Further study is needed to determine whether repetitive administration of DZP increases neurosteroids such as PRG together with augmenting DBI levels in salivary gland.

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References


