Methamphetamine-withdrawal Stress Activates PACAP-DBI Pathway in Rat Salivary Gland, Resulting in Inhibition of Salivary Secretion

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The purpose of this study was to investigate activation of inhibitory regulation pathways by methamphetamine (METH)-withdrawal stress in rat salivary gland. Our previous study showed that METH-withdrawal stress activated steroid biosynthesis and that pregnenolone produced during the early stage of this process inhibited salivary secretion. However, how this type of stress inhibits salivary secretion and the activation pathway of steroid biosynthesis in salivary gland remain to be clarified. In the present study, using an in vivo cannulation method, METH-withdrawal stress decreased salivary secretion and increased expression of diazepam-binding inhibitor (DBI), an endogenous peripheral-type benzodiazepine receptor (PBR) agonist; Western blot and RT-PCR also showed increased expression of DBI mRNA in parotid, submandibular, and sublingual gland. In addition, METH-withdrawal stress also elicited an increase in pituitary adenylate cyclase–activating polypeptide (PACAP) and PBR mRNA, which is associated with DBI activity. These results suggest that METH-withdrawal stress activates a PACAP-DBI pathway in salivary gland, enhancing steroid genesis and inhibiting secretion.

Key words: ’Salivary secretion’ ’Methamphetamine withdrawal stress’ ’Diazepam binding inhibitor’ ’Pituitary adenylate cyclase-activating polypeptide’ ’pregnenolone’

INTRODUCTION

In earlier studies, we showed that gamma-aminobutyric acid A receptor (GABA_A,R) and benzodiazepine receptors mediated inhibitory mechanisms in salivary gland [6–8, 15, 16]. We also demonstrated that stress activated steroid biosynthesis in salivary gland using a rat model of stress induced by methamphetamine (METH)-withdrawal and that pregnenolone (PRG) produced during the early stage of this process inhibited salivary secretion through the GABA_A receptor using rat submandibular gland perfusion method [13]. However, how this type of stress inhibits salivary secretion and the activation pathway of steroid biosynthesis in salivary gland remain to be clarified.

Diazepam-binding inhibitor (DBI) is an endogenous agonist of benzodiazepine receptor [17]. Binding of DBI to peripheral type benzodiazepine receptor (PBR) in mitochondria produced neurosteroids such as PRG, which can modulate GABA_A receptor activity [11]. Several studies showed that repetitive administration of morphine [3, 9], ethanol [4], nicotine [5], or METH [20] increased DBI mRNA in the central nervous system (CNS). Expression of DBI is regulated at the transcriptional level through pituitary adenylate cyclase–activating polypeptide (PACAP) [12, 20]. Previously, we reported that these pathways also existed in salivary gland and that they correlated with inhibition of salivary secretion [21]. Stress induced by METH withdrawal may activate steroid biosynthesis via a PACAP-DBI pathway in salivary gland, resulting in inhibition of salivary secretion. The purpose of the present study was to investigate inhibition of salivary secretion using an in vivo cannulation method. In addition, the effect of METH-withdrawal stress on expression of DBI and on PACAP or PBR, substances related to DBI activity, was also examined.

MATERIALS AND METHODS

Animals

Male Wistar strain rats weighing 200–250 g each were purchased from Japan SLC, Inc. (Hamamatsu, Japan). They were kept for one week prior to the experiments (constant room temperature, 23 ± 2°C; humidity, 55% ± 5%; 12-hr light/dark cycle, with lighting from 6:00 am to 6:00 pm; maintained on commercial laboratory chow and tap water). The animals were treated in accordance with the Guiding Principles for the Care and Use of Laboratory Animals of Tokyo Dental College, and were approved by the Animal Experimentation Committee of Tokyo Dental College.

Materials

Methamphetamine hydrochloride was supplied by Dainippon Sumitomo Pharma, Osaka, Japan. Pilocarpine, carbachol chloride (CCh), and steroids were purchased from Sigma St. Louis, MO, USA. All other reagents used were of the highest grade commercially available.

Preparation of METH withdrawal-stress rat

Induction of stress by METH withdrawal was...
performed as described in our previous study [13]. Briefly, the rats received intraperitoneal injection of METH (1 mg/kg) twice a day for 2 weeks. One week after terminating administration, salivary secretion was investigated and parotid gland (PG), submandibular gland (SMG), sublingual gland (SLG), and cerebral cortex (Cx) harvested for quantitative RT-PCR. The control group received physiological saline instead of METH.

**Collection of saliva from PG, SMG, and SLG by in vivo cannulation method**

Saliva was collected by an in vivo cannulation method as described previously [22]. 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 was inserted into the parotid duct, sublingual papillae, and sublingual openings to obtain saliva from PG, SMG, SLG, 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 hr by stimulation with pilocarpine (1.0 mg/kg, i.p.).

**Extraction of total RNA and real time quantitative RT-PCR**

The salivary glands and brain were removed to allow PG, SMG, SLG, and Cx to be harvested for gene analysis by RT-PCR. Total RNA was treated with DNase 1 and first-strand cDNA synthesized using Oligo(dT)_{18-20} primers and Superscript™III RNase H-reverse transcriptase. Gene expression of DBI, PACAP, and PBR was determined using the beta actin (accession number NM_031144) gene as an internal control and primers specific for DBI mRNA (accession number NM_031853) (Left: 5'-GGAGATTAATGTGCTGCTC-3', Right: 5'-GAATCTACGTACTCTCCTCGGTC-3') to collect saliva, the capillary end of a polyethylene tube was inserted into the submandibular duct toward the glandular body. The tapered end was placed inside a 0.5-ml microtube inserted to support respiration. The tapered end of the capillary cannula was inserted into the parotid duct, sublingual papillae, and sublingual openings to obtain saliva from PG, SMG, SLG, 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 hr by stimulation with pilocarpine (1.0 mg/kg, i.p.).

**Western blot analysis for DBI**

For Western blot analysis of DBI, Cx, PG, SMG, or SLG was homogenized in RIPA Buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate-Na, 0.1% 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 × g for 15 min at 4°C, the resulting supernatants were boiled in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer for 10 min at 70°C. The proteins were subjected to SDS-PAGE using 4-12% NuPage Bis-Tris gels with MES Running Buffer (Invitrogen, San Diego, CA, USA). Proteins were electrically transferred onto polyvinylidene fluoride membranes (Immobilon P, Nippon Millipore; Tokyo, Japan) at 20 V for 1.5 hr. Membranes were blocked for 1 hr at room temperature with 0.5% bovine serum albumin in Tris-buffered saline (TBS). The membrane was washed in TBS containing 0.05% Tween-20 (T-TBS) then incubated overnight at 4°C in primary antibody consisting of anti-DBI (1:10000; sc-30190, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-beta-actin (1:5000; A1978, Sigma Aldrich Japan), followed by horseradish peroxidase–linked secondary antibody. Signal detection was carried out using Pierce Western Blotting Substrate Plus (Thermo Scientific, Tokyo Japan). The western blot band images were captured using a CCD camera system, Cool Saver (ATTO, Tokyo Japan). The specific bands were then analyzed for density using a CS Analyzer (ATTO). Beta-Actin protein expression was quantified to normalize the amount of DBI protein in each sample.

**Collection of saliva by rat submandibular gland perfusion method**

Saliva was collected by the rat SMG perfusion method as described previously [13]. The submandibular artery was isolated and a 29-G needle with a round tip inserted through a small incision. The artery was then perfused with Hank’s balanced salt solution containing 1.27 mM CaCl₂, 0.81 mM MgSO₄, and 30 mM HEPES, pH 7.4 (HBSS-H) at 0.5 ml/min using the Perista minipump SJ-1220 (ATTO, Tokyo Japan). Carbachol chloride (CCh), a muscarinic agonist and salivary stimulant, was administered over a 5-min period every 30 min during perfusion. Pretreatment with steroids was carried out 5 min before CCh stimulation. To collect saliva, the capillary end of a polyethylene tube (IMAMURA, Tokyo Japan) was inserted 3 mm into the submandibular duct toward the glandular body. Saliva was collected into microtubes and net weight determined. To correct for individual differences, these data were expressed as a percentage of secretion induced by CCh alone in each animal.

**Statistical analysis**

The data are expressed as the mean ± S.E.M. 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.

**Disclosure statement**
The authors declare that they had no conflicts of interest in regard to this study.

**RESULTS**

Inhibitory effect of methamphetamine (METH)-withdrawal stress on salivary secretion using in vivo cannulation method

Pilocarpine (1.0 mg/kg)-induced salivary secretion from PG, SMG, and SLG was 115.4 ± 6.7 µl/1 hr, 106.0 ± 8.6 µl/1 hr, and 47.6 ± 6.3 µl/1 hr, respectively (Fig.1). Stress induced by METH withdrawal resulted in a significant reduction in salivary secretion from PG, SMG, and SLG to 63.6 ± 12.1 µl/1 hr, 56.5 ± 10.7 µl/1 hr, and 25.8 ± 3.2 µl/1 hr, respectively. Methamphetamine withdrawal stress induced an approximately 45% decrease in secretion in all types of salivary gland tested. No difference was observed in level of inhibition between each type of gland.

Effect of METH-withdrawal stress on expression of DBI, PACAP, and PBR mRNA in salivary glands and Cerebral cortex using real time quantitative RT-PCR

DBI, PACAP, and PBR mRNA expression in salivary glands and Cx was determined by real time quantitative RT-PCR (Fig.2). DBI and PBR mRNA were expressed in Cx, PG, SMG, and SLG, with the level in each type of tissue showing a significant increase by METH-withdrawal stress. DBI mRNA levels increased to 17.8-, 20.1-, and 20.0-fold, PACAP mRNA levels to 2.0-, 7.0-, 4.0-, and 3.9-fold, PBR mRNA levels to 2.4-, 4.0-, 2.8-, and 2.2-fold of those in the control group in Cx, PG, SMG, and SLG, respectively, by METH-withdrawal stress.

Effect of METH-withdrawal stress on peptide levels of DBI in salivary glands and cerebral cortex using western blot analysis

Expression of DBI and PACAP protein was assessed by Western blot analysis. Bands indicating DBI (10 kD) and β-actin (42 kD) in SMG, SLG, PG, and Cx in METH-withdrawal stress and control rats are shown in Fig.3. a. Semi-quantitative analysis was performed according to density of DBI bands and normalized to the density of the β-actin bands. The relative amounts of DBI protein in Cx, PG, SMG, and SLG were 0.65, 0.67, 0.73, and 0.84, respectively (Fig.3.b). Following METH-withdrawal stress, production of DBI protein in Cx, PG, SMG, and SLG significantly increased to 1.5-1.9-fold that in the control group. Cx, 1.5-fold increase; PG, 1.6-fold increase; SMG, 1.5-fold increase; and SLG, 1.9-fold increase.

Effects of steroids on CCh-induced salivary secretion in rat submandibular gland perfusion system

We demonstrated the inhibitory effects of various steroids on salivary secretion, using a rat submandibular gland perfusion method. Allopregnanolone (AP), testosterone (TES), hydrocortisone (HC), progesterone (PRO), estradiol (EST) and PRG showed inhibition of CCh-induced salivary secretion with an inhibition-ratio of 72.4%, 73.5%, 67.5%, 65.5%, 73.8%, and 68.8%, respectively (Fig.4).

**DISCUSSION**

Clinical research has shown that chronic METH abuse can result in withdrawal symptoms such as anxiety and induce xerostomia [2, 19]. In this study, METH-withdrawal stress significantly decreased pilocarpine-induced salivary secretion in rat PG, SMG, and SLG (Fig.1). In our previous study, METH-withdrawal stress activated production of CYP11A1, a key enzyme in early-stage steroid biosynthesis, in PG, SMG, and SLG [13]. Pregnenolone produced by CYP11A1 inhibited salivary secretion in an in situ rat submandibular perfusion model [13] (Fig.4). In addition, PRG enhanced the inhibitory effects of muscimol, a GABA_A-R agonist, on salivary secretion. These inhibitory effects were blocked by bicuculline, a GABA_A-R antagonist [13]. These results suggested that stress-produced steroid hormones acted as autocrines, inhibiting salivary secretion by enhancing GABA_A-R regulation in salivary gland.

Production of PRG as a neurosteroid was promoted
expression of DBI, PBR, and PACAP mRNA [21]. The diazepam inhibited salivary secretion and enhanced addition, TNF-α activated receptor-
-and PACAP mRNA by METH-withdrawal stress (Fig.2).

activation of PACAP type I receptor (PAC1-R) [12, 20].

regulation of the CBR-GABA pathway is also involved in production of DBI. METH-withdrawal stress may enhance DBI activity via METH-withdrawal stress was suggested to inhibit regulation of the CBR-GABA_2-R complex in CNS and induce anxiety [20]. If this theory also holds true for salivary gland, DBI would increase salivary secretion via GABA_2-R. However, our data show that salivary secretion decreased by METH-withdrawal stress or repetitive administration of diazepam, which increases DBI. This means that DBI has a more potent effect on PBR than CBR in salivary gland. This may be because PBR exists in greater quantities in salivary gland. The results of the present study showed that the level of PBR mRNA in salivary gland was higher than that in Cx. This result was supported by the receptor binding assay, which showed that the Kd value of [3H] PK11195 was similar in Cx and salivary gland, but that that of Bmax was higher in salivary gland than that in Cx [29].

The inhibitory effect of METH-withdrawal stress on salivary secretion was greater than that of steroids or GABA_2 agonist. It is possible that METH-withdrawal stress inhibits salivary secretion not only via steroid biosynthesis, but also some other pathway. It is known that PBR regulates Ca\(^{2+}\) efflux from mitochondria and apoptosis [1]. Further study is required to elucidate PBR-regulated Ca\(^{2+}\) efflux from mitochondria in rat salivary gland. In addition, METH-withdrawal stress may influence other organs. An earlier study showed that steroidogenic enzyme CYP11A1, but not 3β-HSD or CYP17a, was found in salivary gland by RT-PCR and Western blot [15]. These results suggest that PRG is the only convincing candidate for steroid production in salivary gland. However, Fig.4 shows that some steroid hormones which are specifically produced in each organ such as adrenal gland, testis, ovary or ovarian follicle, inhibit salivary secretion, not only PRG. These results indicate that these steroids move hematogenously to the salivary gland, where they affect secretion. Further study is needed to determine the levels of these steroids in blood plasma.

Fig. 5 shows a schema of the pathways activated by METH-withdrawal stress in salivary gland based on

by DBI via mitochondrial PBR in CNS [5]. In addition, DBI expression was regulated by PACAP through the activation of PACAP type I receptor (PAC1-R) [12, 20]. Recently, we showed that repetitive administration of diazepam inhibited salivary secretion and enhanced expression of DBI, PBR, and PACAP mRNA [21]. The protein level of DBI was consistent with its mRNA level in salivary gland. These results suggest that DBI production may result in an increase in the suppressive effect of diazepam on salivary secretion. In the present study, an increase was observed in DBI, PBR, and PACAP mRNA by METH-withdrawal stress (Fig.2). Levels of DBI protein also showed an increase with METH-withdrawal stress (Fig.3). We believe that this marked change in PACAP mRNA levels would have been sufficient to drive DBI production. However, the PACAP mRNA was significantly lower in each type of salivary gland than in Cx, indicating that some other pathway is also involved in production of DBI. METH-withdrawal stress was reported to enhance production of DBI by activation of peroxisome proliferator-activated receptor-γ (PPAR-γ) in CNS [18,19]. In addition, TNFα activated expression of PBR [18]. METH-withdrawal stress may enhance DBI activity via multiple pathways in salivary glands.

It is known that DBI acts not only as a PBR agonist, but also as an inverse agonist of central-type benzodiazepine receptor (CBR) (Fig.5). Increased DBI by METH-withdrawal stress was suggested to inhibit regulation of the CBR-GABA_2-R complex in CNS and induce anxiety [20]. If this theory also holds true for salivary gland, DBI would increase salivary secretion via GABA_2-R. However, our data show that salivary secretion decreased by METH-withdrawal stress or repetitive administration of diazepam, which increases DBI. This means that DBI has a more potent effect on PBR than CBR in salivary gland. This may be because PBR exists in greater quantities in salivary gland. The results of the present study showed that the level of PBR mRNA in salivary gland was higher than that in Cx. This result was supported by the receptor binding assay, which showed that the Kd value of [3H] PK11195 was similar in Cx and salivary gland, but that that of Bmax was higher in salivary gland than that in Cx [29].

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In conclusion, our present results suggest that METH-withdrawal stress activates steroid biosynthesis via a PACAP-DBI pathway in salivary gland, resulting in inhibition of salivary secretion.

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REFERENCES


Fig. 5  Inhibitory mechanism of METH-withdrawal stress in rat salivary gland cell

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