Full Paper

Rat Submandibular Gland Perfusion Method for Clarifying Inhibitory Regulation of GABA<sub>A</sub> Receptor

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Abstract. GABA is an inhibitory transmitter found in rat salivary gland. However, the inhibitory potential of GABA on salivary secretion is unclear. Using an in vivo cannulation method, intraperitoneal administration of GABA was ineffective in the absence of gabaculine, a GABA transaminase inhibitor, on pilocarpine-induced salivary secretion, suggesting that GABA was rendered metabolically inactive before reaching the salivary gland. We hypothesized that the action of a drug on the salivary glands could be measured directly using a submandibular gland perfusion system. The submandibular gland artery, veins, and duct were cannulated in situ so that physiological functions such as innervation would not be compromised. Hank’s balanced salt solution (pH 7.4) was perfused at a rate of 0.5 ml/min together with 1 µM carbachol (CCh) over a 5-min period every 30 min. Amount of secreted saliva showed no change to the recurrent addition of CCh to the perfusate. GABA or muscimol dose-dependently inhibited CCh-induced salivary secretion. This effect was blocked by bicuculline, a GABA<sub>A</sub>-receptor (GABA<sub>A</sub>-R) antagonist, and enhanced by clonazepam, a central-type benzodiazepine-receptor agonist. These results suggest that salivary secretion is suppressed by GABA<sub>A</sub>-R in rat salivary gland and that the perfusion method used was effective in clarifying inhibitory regulation of GABA<sub>A</sub>-R.

Keywords: submandibular gland, perfusion system, xerostomia, γ-aminobutyric acid, salivary secretion

Introduction

The evidence for a peripheral GABAergic system in several tissues outside the mammalian brain was provided by some advanced studies in the 1990’s (1–4). Their studies led cumulative investigations on the response to GABA and GABA agonists in a series of peripheral organs and a number of suggestive studies on the modulator role for GABA in the heart (5), the lung (6), and in the regulation of the peristaltic reflex (7) and adrenal catecholamine secretion (8). Since 1995, we have shown that inhibitory mechanisms in rat salivary gland were regulated by a GABA<sub>A</sub> receptor (GABA<sub>A</sub>-R)-chloride ion channel complex: it was demonstrated that GABA and its biosynthetic and metabolic enzyme existed in rat salivary gland (9, 10); and GABA and benzodiazepines accelerated <sup>36</sup>Cl<sup>-</sup> influx (11) and inhibited amylase release (12) in rat parotid gland acinar cells in vitro. These results suggest that GABA inhibits cellular function in the salivary gland. However, in preliminary studies, we found that lower doses of exogenous GABA similar to the physiological levels administered by an in vivo cannulation method did not inhibit salivary secretion. Therefore, the inhibitory potential of GABA on salivary secretion remains to be clarified.

We have used an in vivo cannulation method to clarify the inhibitory effects of intraperitoneal administration (i.p.) of drugs such as benzodiazepines on pilocarpine-induced salivary secretion in parotid, submandibular, and sublingual gland ducts (13, 14). Although this method allowed saliva to be collected from three major salivary glands simultaneously and without compromising physiological function, there were still some problems in studying GABA<sub>A</sub>-R. Systemic administration (e.g.,

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intraperitoneal injection) of GABA may cause it to be metabolized into other substances before reaching the target organ, so that it will be hard to estimate a correct response of GABA to physiological demands. Furthermore, general anesthetics such as pentobarbital influence the effect of GABA. Pentobarbital exerts a stable anesthetic effect over a long period of time. However, binding sites for barbiturates on GABA_A-R form a complex with Cl^- channels. Barbiturates regulate Cl^- permeability, as do benzodiazepines and steroids (15, 16). Therefore, in this study, we designed a submandibular gland perfusion system to investigate the action of GABA on salivary secretion.

Some groups have employed an isolated submandibular gland perfusion system (17, 18). In contrast, our goal was to construct an in situ perfusion system so that physiological functions such as innervation would not be compromised. In previous studies, our group showed that benzodiazepines enhanced the inhibitory regulation of salivary secretion by GABA_A-R (11, 12). Central-type benzodiazepine-receptor (CBR) agonists exert allosteric action on the GABA_A-R–chloride channel complex (19–21). These findings suggest that the CBR agonist is inactive in the absence of GABA. In addition, we previously reported that when both the sympathetic and parasympathetic nerves were denervated, the immunoreactivity of GABA was dramatically depressed in the submandibular gland (9). These results suggest that it is necessary to allow submandibular gland innervation to continue when investigating the effects of benzodiazepines using a perfusion system.

The purpose of this study was to establish an in situ submandibular perfusion method for GABA in order to clarify its effect at physiological-level doses on inhibition of salivary secretion through GABA_A-R.

**Materials and Methods**

**Animals**

Male, Wistar-strain rats weighing approximately 250 g each were purchased from Japan SLC, Inc. (Hamamatsu). They were kept at a constant room temperature (23°C ± 2°C; humidity, 55% ± 5%) under a 12-h light–dark cycle (light, 6:00 am – 6:00 pm) and maintained on commercial laboratory chow and tap water for one week before the experiments. All the animals were treated in accordance with the Guiding Principles for the Care and Use of Laboratory Animals established at Tokyo Dental College.

**Materials**

Carbachol (carbamylecholine chloride: CCh), pilocarpine, GABA, muscimol, bicuculline clonazepam, flumazenil, and N-[2-hydroxyethyl] piperazine-N'.[2-ethanesulfonic acid] (HEPES) were purchased from Sigma, St. Louis, MO, USA. Hank’s balanced salt solution (HBSS) without Ca^2+, Mg^2+, or HCO_3^- was obtained from Gibco BRL, Grand Island, NY, USA. Lidocaine (3% xylocaine) was purchased from AstraZeneca, Osaka. Dimethyl sulfoxide (DMSO) was obtained from Nacalai Tesque, Kyoto. All other reagents used were of the highest grade commercially available. The CCh was dissolved in distilled water. Hank’s balanced salt solution containing 1.27 mM CaCl_2, 0.81 mM MgSO_4, and 30 mM HEPES, pH 7.4 (HBSS-H) were used as perfusates.

**Perfusion of rat submandibular gland**

Figure 1 shows the rat submandibular perfusion model used in this study. The rats were anesthetized with pentobarbital sodium (50 mg/kg) and secured in the supine position. A median incision was made to allow visualization of the skin of the neck, trachea, and submandibular gland. A tracheal tube was then inserted to ensure an airway and support respiration. The submandibular artery, vein, and duct were then cannulated under a stereomicroscope.

Submandibular artery cannulation: The submandibular artery (glandular branches of facial artery) was carefully separated from the surrounding tissue to avoid damaging the nerves. The artery was then dilated with 3% lidocaine for easy insertion of the cannula (Fig. 1a). The artery was ligated to stop the flow of blood and a cut of 1/3 the periductal length made on the mesial side to provide an insertion point. A 29G needle from a syringe (Insulin Syringe with Needle, Myjector®, Terumo, Tokyo) was inserted into the vein in the direction of the submandibular gland and fixed with a ligature. In this study, perfusate from the vein cannula was collected to verify that the system was working properly.

Submandibular vein cannulation: After cannulation of the artery, the submandibular vein was cannulated while distributing fluid. After ligation on the mesial side, the submandibular vein was immediately cut to the insertion point of the cannula. The cannula itself was an extended polyethylene tube (N0.4; Imamura, Tokyo). While inserting the cannula through the artery toward the glandular body, perfusate was distributed from the point of the needle. After cannulation, the artery and cannula were fixed with a ligature and the tube and surrounding skin were fixed with instant adhesive.

Submandibular duct cannulation: Two parallel ducts
running anterior to the submandibular and sublingual glands were made visible by denuding the covering membrane and separating them. Only the submandibular gland duct was cannulated. The submandibular duct was carefully separated from the surrounding tissues to avoid damaging the nerves. A cut was made at 1/3 of the periductal length with a 29G needle. A polyethylene tube (No.4, Imamura) extended by heating and tapered to a tip of 0.2 mm in diameter was used as the cannula. After cannulation, the cannula and duct were fixed with a ligature. After surgery, the operative field was washed in physiological saline and covered with a wet cotton wool pad to prevent drying. When over 90% of the perfusate had flowed out of the venous cannula, it was considered that the perfusion system was functioning properly.

Perfusate and drug application: HCO$_3$-free HBSS-H was used as the perfusate. The artery was perfused with perfusate after bubbling 95% O$_2$ / 5% CO$_2$ gas and warming at 37°C using the Perista mini-pump SJ-1220 (ATTO, Tokyo). The perfusion rate was adjusted to 0.125 – 2.0 ml/min. Replacement of the perfusate with other drugs was carried out by changing the flow channel using a T-shaped stopcock. Concentration of CCh as a stimulant of salivary secretion was set at from 50 μM to 0.01 μM. Atropine was administered 5 min before stimulation with CCh. After applying each drug, the perfusate was replaced with no additives.
Measurement of salivary flow rate: After commencing CCh stimulation, the secreted submandibular saliva was collected every 5 min into microtubes (0.5 ml) and net weight determined using an electronic balance (RL-U20FD: Shimazu, Kyoto). These results were represented by volume (μl) calculated using gravity at 1.0. Saliva was collected in a 0.1-ml polypropylene tube of pre-determined weight.

*In vivo cannulation study of inhibitory effect of GABA on pilocarpine-induced salivary secretion*

Saliva was collected 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 submandibular papillae to obtain saliva from the submandibular gland, while the other end was placed inside a 0.5-ml microtube to collect secreted saliva. Pilocarpine (1.0 mg/kg, i.p.)-induced saliva was collected every 15 min for 60 min. After the first 60 min of saliva collection, GABA (200 mg/kg, i.p.) and gabaculine (50 mg/kg, i.p.) were administered for 60 min. Pilocarpine was then administered again for 60 min and saliva collected every 15 min. Data were calculated as the ratio of the first to the second collection of saliva.

**Statistical analyses**

The data are expressed as the mean ± S.E.M. of 3 – 5 experiments. Significant differences between two groups were determined with the t-test in the case of equal and unequal variance. A P-value of less than 0.01 was considered to be statistically significant. To determine the effect of dose, significant differences were determined with the Dunnett test.

**Results**

**Effect of continuous 60-min replacement media containing CCh on salivary secretion using the perfusion system**

Figure 2 shows time-dependent change in 1 μM CCh–induced salivary secretion every 5 min for 60 min when the perfusion rate was set to 0.5 ml/min. A stable volume of approximately 16 μl/5-min salivary secretion was obtained for the first 20 min after commencement of stimulation. Secretion gradually decreased, however, after this point. At 40 min after commencement of stimulation, secretion was approximately 8 μl/5 min.

**Effect of periodic CCh stimulation on salivary secretion using the perfusion system**

CCh at 1 μM was administered over a 5-min period every 30 min at a perfusion rate of 0.5 ml/min (Fig. 3a). Rapid salivary secretion was obtained during 5-min CCh stimulation, which immediately decreased when stimulation ceased. Amount of secretion and secretory pattern did not change through each successive stimulation. A constant rate in total amount of secretion over 30 min was observed during each of the 4 rounds of stimulation, yielding 21.7 ± 0.8, 21.5 ± 1.9, 21.3 ± 2.3, and 21.9 ± 2.0 μl/30 min.

Change in salivary secretion was examined with or without 5 min of 1 μM CCh stimulation when the perfusion rate was set to 0.125, 0.25, 0.5, 1.0, or 2.0 ml/min (Fig. 3b). No secretion without CCh stimulation could be obtained, except at a high perfusion rate. The maximal secretion without CCh was only 2.5 μl/30 min at a 2.0 ml/min perfusion rate. On the other hand, secretion with CCh stimulation increased perfusion rate-dependently. With CCh stimulation at a perfusion rate of 0.125, 0.25, 0.5, 1.0, and 2.0 ml/min, secretion was 3.3 ± 0.7, 14.4 ± 1.9, 24.6 ± 2.8, 36.5 ± 4.0, and 47.2 ± 2.0 μl/30 min, respectively.

Figure 3c shows the dose–response curve of CCh on salivary secretion at a 0.5 ml/min perfusion rate for 5 min. Amount of secretion represents total secretion at 30 min after commencement of stimulation. At concentrations ranging from 0.1 to 5 μM, CCh significantly increased salivary secretion in a concentration-dependent manner. Secretion of 40.0 ± 6.6 μl was obtained with 5 μM CCh. However, secretion showed little change with more than 10 μM CCh stimulation.

**Inhibitory effect of GABA with or without gabaculine on salivary secretion, in vivo study**

The attenuation ratio of the first to second collection
of saliva induced by pilocarpine alone was 74.8±4.4% (Fig. 4). This ratio changed to 67.1±3.1% and 51.1±4.3% by pretreatment with GABA with and without gabaculine, respectively. The results showed that GABA was ineffective in the absence of gabaculine on pilocarpine-induced salivary secretion in rat submandibular gland, in vivo.

**Effect of GABA\(_A\)-R agonists and antagonist on salivary secretion using perfusion system**

CCh (10\(^{-6}\) M) was loaded over a 5-min period every 30 min. The indicated concentration of GABA with or without bicucullin was then administered over a 5-min period prior to CCh administration and saliva collected every 5 min (Fig. 5a). Dose-dependent inhibition of CCh-induced salivary secretion by administration of GABA or muscimol was observed using this perfusion system (Fig. 5b). The maximum decrease was obtained with 5 \(\mu\)M GABA and muscimol, at an inhibition ratio of 31%
and 33% of CCh-induced salivary secretion, respectively. These effects were blocked by bicuculline (Fig. 5c).

**Effect of central type benzodiazepine agonists on salivary secretion using perfusion system**

Clonazepam, CBR agonist, inhibited CCh-induced salivary secretion (Fig. 6). This inhibition was blocked with flumazenil, CBR antagonist, and enhanced with muscimol.

**Discussion**

We have devised an improved procedure for perfusion of rat submandibular gland. This method allowed the inhibitory effect of exogenous GABA on salivary secretion through GABA$_A$-R to be directly evaluated.

Administration of GABA by an in vivo cannulation method (i.p.) showed no effect on pilocarpine-induced salivary secretion in the submandibular gland (Fig. 4). When administered in combination with gabaculine, which acts as a GABA transaminase inhibitor (23, 24),
GABA inhibited salivary secretion. This means that GABA was rendered metabolically inactive before reaching the salivary gland. GABA transaminase, a catabolic enzyme of GABA, is found in peripheral tissues such as liver, kidney, plasma, and pancreas (25, 26). In addition, GABA receptors have been found in a number of peripheral tissues (1 – 4). It is possible that this drug affects the salivary gland indirectly via the action of other organs. Administration via the submandibular gland artery allows the direct action of various drugs on the salivary gland and their inhibitory effect on salivary secretion to be observed.

In the present study, salivary flow rate showed a gradual decrease after continuous stimulation for the first 15 min with 1 μM CCh using our submandibular gland perfusion system (Fig. 2). This result was similar to that observed in an earlier study (27). This finding may be explained by a reduction in receptor sensitivity or a decrease in cellular activity. Therefore, in the present study, we decided to perform another series of experiments employing periodic stimulation and provision of sufficient time for recovery in order to maintain cellular activity.

Bleeding from the submandibular gland artery was approximately 0.2 ml/min with cannulation toward the heart. The circulation volume in tissue as calculated using a Doppler blood flow meter was 0.09 ± 0.02 ml/min (data not shown). This suggests that physiological blood flow through the artery to the submandibular gland in the rat is approximately 0.1 ml/min. It is desirable to approximate the perfusion rate to the physiological blood flow rate. With a 0.125-ml/min perfusion rate, which is close to the blood flow rate, however, 1 μM CCh induced too little salivary secretion for accurate measurement. Other study groups set the perfusion rate to 2 ml/min (28). A perfusion rate of over 2 ml/min produces an increase in secretion, but the salivary glands become hypertrophied and a small amount of saliva is observed during perfusion, even without stimulation (Fig. 3b). In this experiment, a 0.5-ml/min perfusion rate yielded stable salivary secretion and the burden on the submandibular gland was minimized. Figure 3c shows that CCh increased the secretion rate in a concentration-dependent manner. The concentration of CCh should be as low as possible. Less than 0.1 μM CCh, however, is insufficient to produce enough salivary secretion for measurement. Stable secretion by 0.5 or 1 μM CCh was obtained using the present experimental system. Under the present experimental conditions, no change was observed in the amount of salivary secretion or secretory pattern over several rounds of CCh administration (Fig. 3a).

In the in vivo study, high doses of GABA and gabaculine (i.p.) were needed to inhibit salivary secretion (Fig. 4). Using this perfusion system, GABA in the absence of gabaculine produced dose-dependent inhibition of CCh-induced salivary secretion (Fig. 5b). This inhibition was caused at a physiological concentration (10−6 M) close to that required to enhance Cl− influx in rat parotid acinar cells in vitro (11). Previously, we demonstrated that GABA and its biosynthetic and metabolic enzyme existed in rat salivary gland (10). However, we believe that the perfusion pathway used in the present study would be too short to allow GABA to be metabolized. Muscimol, a selective GABA-A-R agonist, also inhibited secretion. The inhibitory effects of GABA or muscimol were blocked by bicuculline (Fig. 5c) and enhanced with clonazepam, a CBR agonist (Fig. 6). This suggests that salivary secretion is suppressed by GABA-A-R in rat submandibular gland.

Our previous study showed that GABA increased 36Cl− influx and decreased 36Cl− efflux in rat parotid acinar cells in vitro (11). Cl− is an indicator of water movement in secretory gland cells. GABA induced suppression of Cl− efflux in Ca2+-dependent Cl− channels in the luminal membrane of salivary gland, indicating that the inhibition of water movement from basal to luminal sites causes salivary production. On the other hand, the results of the present study directly demonstrate the inhibitory effect of GABA on salivary secretion.

Clonazepam alone inhibited CCh-induced secretion (Fig. 6). This inhibitory effect was blocked by flumazenil, a CBR antagonist, indicating the presence of GABA, as autonomic innervation remained. Further study is necessary, however, to measure the quantity of GABA in the submandibular gland.

The present in situ submandibular gland perfusion system, therefore, offers some advantages over other in vivo cannulation methods or in vitro perfusion systems in clarifying the inhibitory regulation of GABA-A-R (Table 1). By using an in vivo cannulation method, it was possible to collect saliva from the parotid, submandibular, and sublingual glands without compromising physiological function. However, the in vivo cannulation method does not allow the direct action of drugs on salivary gland to be examined as the metabolism, circulation, and other organs are all affected. On the other hand, the perfusion method allows administration by a route completely isolated from the circulatory system, thus allowing the direct action of exogenous GABA on salivary secretion through GABA-A-R to be accurately assessed. In addition, in situ submandibular gland perfusion allows innervation of the autonomic nerves to be maintained.

Steroid hormones undergo various enzyme-induced changes in various types of tissue, and it is possible that
the present submandibular gland perfusion system would allow investigation of their effect on salivary secretion. In a recent study using the present experimental system, pregnenolone, which is produced during the early stages of steroid biosynthesis, inhibited salivary secretion (29). In addition, pregnenolone enhanced the inhibitory effects of muscimol, which were blocked by bicuculline. These results suggest that, similarly to benzodiazepines, steroids act as an allosteric agonist of GABA_A-R, enhancing its inhibitory function in the salivary gland. It is known that many substances regulate the function of the GABA_A-R/CBR/Cl\(^{-}\) channel complex. Further study using the present experimental technique should help provide valuable data on how the GABA_A-R/CBR/Cl\(^{-}\) channel complex inhibits salivary secretion in rat salivary gland.

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**Table 1.** Some advantages of the in situ submandibular gland perfusion system

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