

**The Chinese herb, Danshen (*Radix Salviae miltiorrhizae*), induces salivary fluid secretion**

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## **Summary**

[INTRODUCTION] Xerostomia is mainly caused by salivary gland hypofunction. The incidence of salivary gland hypofunction in the population over 65 years old is 30-40%. However, the common current treatments for salivary gland hypofunction by parasympathomimetic drugs are accompanied by systemic side effects. Danshen has been clinically used to relieve dry mouth in Chinese medicine. However, only a few reports addressed the direct effect of Danshen on the salivary glands. Murakami et al. (2009) examined 20 Chinese herbs, focusing on their capability to promote salivary fluid secretion in the submandibular glands of rats. The results of that study showed that Danshen not only promotes during muscarinic stimulation with carbamylcholine, but it also induces salivary fluid secretion without the addition of any other stimulants.

[PURPOSE] In this study, Danshen was chosen as a candidate for the relief of xerostomia, and the capability of Danshen to induce salivary fluid secretion and its mechanism were examined.

[METHODS] The submandibular gland was isolated from the rat and vascularly perfused, and also acinar cells were isolated to measure the  $\text{Ca}^{2+}$  influx. The excretory duct was cannulated and the secreted volume was measured by electronic balance. Arterio-venous difference of partial oxygen pressure was measured as an indicator of oxygen consumption. Arterio-venous pressure difference was also measured as indicator of increase in microcirculation.

[RESULTS & DISCUSSION] Although Danshen induced salivary fluid secretion in the submandibular glands, the time course of that secretion differed from fluid secretion induced by carbamylcholine. There was a latency associated with the fluid secretion induced by Danshen, followed by gradual increase in the secretion until it reached its

highest value, and thereafter there was a slow decline to a near zero level. These characteristics suggested that the mechanism for Danshen-induced salivary fluid secretion could be different from that induced by carbamylcholine. Carbamylcholine activates the  $M_3$  receptor to release  $IP_3$  and quickly releases  $Ca^{2+}$  from the intracellular calcium stores. The elevation of intracellular  $Ca^{2+}$  level induces chloride release and quick osmosis, resulting in an onset of fluid secretion. Furthermore, the  $\alpha_1$  adrenergic and neurokinin A receptors use the same signalling sequence after binding the individual receptors. Therefore,  $IP_3$ -store  $Ca^{2+}$  release signalling may not be involved in secretion induced by Danshen, but rather, there may be a distinct signalling process. During Danshen stimulation, the additional application of either ouabain (inhibitor of  $Na^+/K^+$  ATPase) or bumetanide (inhibitor of NKCC1), inhibited the oxygen consumption and suppressed the fluid secretion by more than 90%. These results indicated that Danshen activates  $Na^+/K^+$  ATPase and NKCC1 to maintain  $Cl^-$  release and  $K^+$  release for fluid secretion. Next, we examined the involvement of the main membrane receptors,  $M_3$  muscarinic and  $\alpha_1$  adrenergic receptors. Neither atropine nor phentolamine inhibited the fluid secretion induced by Danshen. Accordingly, Danshen does not bind with  $M_3$  nor  $\alpha_1$  receptors. An increase in  $[Ca^{2+}]_i$  is essential for the activation of the luminal  $Cl^-$  and basolateral  $K^+$  channels. The nominal removal of extracellular  $Ca^{2+}$  and chelating of intracellular  $Ca^{2+}$  by BAPTA-AM totally abolished the fluid secretion induced by Danshen, suggesting the involvement of  $Ca^{2+}$  in the activation of these channels. However the fluorescent  $Ca^{2+}$  indicators could not show the changes in the  $[Ca^{2+}]$  due to the dark color of Danshen and its extract, Salvianolic acid B. The quick drop seen in the arterio-venous pressure difference suggests an increase in the microcirculation due to Danshen. However, the paracellular fluid

secretion indicated by the fluorescent dye was smaller than that induced by carbamylcholine, suggesting that the transcellular fluid secretion was dominant in the whole fluid secretion during Danshen stimulation.

[CONCLUSION] The present findings support the use of Danshen in the treatment of xerostomia, and we consider that DS is a promising secretagogue which could avoid the systemic side effects induced by the recent muscarinic drugs such as cevimeline, pilocarpine and so on.

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## **Acknowledgements**

## **I. Background.**

***I-1. Xerostomia and problems in the present treatments.*** Saliva is important to maintain the oral environment. It moistens and washes the surface of the oral cavity, and facilitates swallowing and chewing food as lubricant. The subjective feeling of dry mouth is called xerostomia and mainly caused by salivary gland hypofunction (SGH). SGH is a condition in which non-stimulated or stimulated salivary flow is significantly reduced due to many reasons such as aging, radiation therapy, medications, and Sjögren's syndrome. The incidence of SGH in the population over 65 years old is 30-40% (Sreebny, 2010). Xerostomia reduces the quality of life (QOL) (Atkinson et al., 2005; Guggenheimer et al., 2003). The current treatment for xerostomia includes the administration of parasympathomimetic drugs or artificial saliva (Ge and Lin, 2008). Parasympathomimetic drugs, including pilocarpine and cevimeline, activate muscarinic receptors on the salivary glands to stimulate fluid secretion. However, the muscarinic receptors exist in many organs of the body. So this medication is accompanied by systemic side effects, for example, nausea, diarrhea and other adverse gastrointestinal reactions (Joraku et al., 2005; Miao et al., 2007). Therefore, most patients tend not to take these drugs, but rather use artificial saliva. However, artificial saliva also has disadvantages. It is difficult to be taken when the mouth is used continuously, such as during speech. Therefore, there is a clinical requirement for salivary fluid promoters with fewer side effects.

***I-2. Danshen (DS).*** Murakami et al. (2009) examined 20 Chinese herbs on their capability to promote salivary fluid secretion using isolated and vascularly perfused submandibular glands (SMG) of rats. They found 15 herbs that could promote salivary fluid secretion during muscarinic stimulation with carbamylcholine (carbachol, CCh).

Among those 15 herbs, Danshen was found not only to promote salivary fluid secretion, but also induce it.

Danshen (DS) is a representative Chinese herb in the category of the agents that promote blood circulation and eliminate stasis (Wu, 2008). DS is mainly used for coronary atherosclerotic disease of heart. The main pharmacological functions of DS have been reported as 1) dilatation of the blood vessels, 2) promotion of microcirculation, 3) anti-coagulant activity, 4) antithrombotic activity, and 5) anti-inflammation. Among the components of the DS extracts, Salvianolic acids, the phenolic acids and diterpenequinone compounds of the tanshinone type are equally effective (Du et al., 2004).

DS has been clinically used to relieve dry mouth in Chinese medicine. However, only a few reports have addressed the direct effect of DS on salivary glands (Jiang et al., 2005) and no studies have been conducted to examine the mechanisms related to the induction of salivary secretion. The present study was planned and conducted to confirm the salivary induction due to DS and examine the possible mechanisms involved in DS-induced salivary fluid secretion.

***I-3. Experimental plan to assess the ability of Chinese herbs to induce salivary fluid secretion.*** In order to measure fluid secretion in response to Chinese herbs, we employed isolated and arterially perfused rat salivary glands, because this is only the method that makes it possible to measure fluid secretion, except for a method employing *in vivo* gland. The *in vivo* gland is an ideal model for clinical application, but with this method, we cannot avoid influences from neural and hormonal effects. A previous study showed that the five kinds of Chinese herbs, which were known as medicine for xerostomia, could not promote salivary fluid secretion in isolated and

perfused glands (Murakami et al., 2009a). In addition, because the epithelial structure is maintained in the isolated perfused SMG, this method makes it possible to observe not only transcellular fluid secretion, but also paracellular fluid secretion (Murakami et al., 2009b).

Transcellular fluid secretion is based on  $\text{Cl}^-$  release through the  $\text{Ca}^{2+}$  dependent- $\text{Cl}^-$  channel (TMEM16A) across the luminal plasma membrane (Romanenko et al., 2010). The junctional flow of  $\text{Na}^+$  is followed by a requirement for electroneutrality to increase luminal osmolarity. The osmotic gradient allows osmosis through aquaporine 5 on the luminal membrane and junctional water flow. For  $\text{Cl}^-$  entry across the basolateral membrane, the  $\text{Na}^+/\text{K}^+/2\text{Cl}^-$  cotransporter (NKCC1) is driven by the  $\text{Na}^+$  electrochemical gradient, which is maintained by  $\text{Na}^+/\text{K}^+$  ATPase. Because the double antiport system, including anion exchanger and  $\text{Na}^+/\text{H}^+$  exchanger, has a minimal contribution for  $\text{Cl}^-$  entry during  $\text{HCO}_3^-$ -free perfusion, the experimental system can be simplified by avoiding  $\text{HCO}_3^-$  utilization.

The experimental system for the assessment of new secretagogues including Chinese herbs was established as followings. 1) Fluid secretion measured by the use of computer-aided electronic balance in the isolated perfused SMG. 2) Oxygen consumption measured by arterio-venously placed oxygen electrodes. Inhibition of fluid secretion and oxygen consumption by ouabain for assessment of the activity of  $\text{Na}^+/\text{K}^+$  ATPase. 3) Inhibition of fluid secretion by bumetanide for assessment of NKCC1 for the fluid secretion. 4) Use of atropine and phentolamine to assess the involvement of muscarinic and  $\alpha_1$ -adrenergic receptors stimulated by new secretagogues. 5) The requirement of extracellular  $\text{Ca}^{2+}$ . 6) Measurement of intracellular  $\text{Ca}^{2+}$ . 7) The possibility of paracellular fluid secretion.

Using experimental plan shown above, this study was conducted to investigate salivary fluid secretion induced by DS. In the final experiment, measurement of intracellular  $\text{Ca}^{2+}$  stood like a solid stone wall due to the intrinsic color of DS.

## **II. Materials and Methods.**

**II-1. Isolation and Perfusion of SMG.** Adult Wistar male rats (9 weeks, 240-280 g) were purchased from Japan SLC, Inc. (Wistar/ST) and given a standard pellet diet and water *ad libitum*. The rats were anesthetized by intraperitoneal injection of pentobarbital sodium at a dose of 30 mg/kg body weight (Somnopenyl, Kyoritsu Seiyaku Co. Japan) after induction with 3 vol% isoflurane (Abbot). The SMGs were surgically isolated as previously described (Murakami et al., 1990). Briefly, the attached sublingual gland and sublingual duct were removed after ligation of the feeding arteries, and draining of the vein. The main duct of the SMG was cannulated with a fluorine-fiber tube (0.3 mm I.D.  $\times$  0.5 mm O.D., EXLON, Iwase Co. Ltd., Japan) for sampling. The artery distal to the glandular branch was cannulated with a stainless steel catheter connected to the infusion line for perfusion. Then, the vein from the gland was cut free.

**II-2. Perfusion of the gland.** The isolated SMGs were placed in a humidified chamber at 37°C and perfused arterially with the aid of a peristaltic pump (Cole-Palmer, 7553-10, USA) at a rate of 1.8 mL/min to supply enough oxygen even without a specific oxygen carrier during the secretory period. Immediately before the excretory duct cannulation, the fluorine-fiber tube was filled with distilled water. The other end of the ductal cannula was placed under water in a small cup on an electronic balance with a minimum digital readout of 0.1 mg (Shimadzu AEG-220, Kyoto, Japan), avoiding any contact with the side and bottom of the cup.

After a control perfusion of buffered salt solution for 20 min, the weight of the

secreted saliva was recorded for 5 min prior to the start of the stimulation.

Carbamylcholine chloride (CCh, Sigma, MO, USA) was applied at 0.2  $\mu\text{mol/L}$  for 5 min as a control to test the vigor of the gland, and in these experiments, the fluid secretion of CCh at 4.95 - 5 min was set as 100% to normalize the individual variation. CCh was removed by washing another 5 min using perfusion fluid. The DS solution was continually perfused for 60 min.

**II-3. Preparation of the perfusion fluid.** The perfusion fluid was a salt solution with the following composition (in mmol/L):  $\text{Na}^+$  140.0,  $\text{K}^+$  4.3,  $\text{Ca}^{2+}$  1.0,  $\text{Mg}^{2+}$  1.0,  $\text{Cl}^-$  148.3, and glucose 5.0. The solution was buffered at pH 7.4 with N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES, 10 mmol/L) and gassed with 100%  $\text{O}_2$  at 37°C. Salts, glucose, HEPES, CCh and phentolamine were purchased from Sigma (MO, USA).

**II-4. Preparation of the DS solution.** The granular extract from the root of the DS was provided by Tian Jiang Pharmaceutical Co. Ltd., Jiang Yin, China. Each gram of this DS granule was concentrated and equivalent to 5 g of the crude DS. The DS stock solution was prepared as follows. 1) Two gram of DS were dissolved in the perfusion fluid by ultrasonic concussion for 5 min. The precipitate was removed twice after each centrifugation at 4,000 rpm for 5 min. The clear supernatant was obtained and its pH was adjusted to 7.4 by sodium hydroxide solution (1 mol/L, Nacalai Tesque, Inc., Kyoto, Japan). 2) The neutral solution was prepared to 0.5 g/mL (each concentration in this study refers to crude DS, rather than the extract of DS) in a buffer solution. This solution was filtered through a filter with a pore size of 0.22  $\mu\text{m}$  (Sterivex-GV, Millipore, MA, USA) to obtain the DS stock solution. Finally, before using, the stock solution was diluted with the perfusate, and prepared into concentrations of 1, 3, 5, 25

and 50 g/L.

**II-5. Salivary fluid secretion.** The cumulative weight was automatically measured every 3 s and the data was transferred to a spreadsheet file in a computer. The fluid secretion rate was calculated from the time differentiation of the cumulative volume of saliva, assuming a saliva specific gravity of 1.0.

**II-6. Oxygen consumption.** The partial oxygen pressures of the perfusate and the effluent were measured polarographically at 37°C by the Dissolved Oxygen Measuring System (Model 203, Instech Laboratories, PA, USA). The artery and the vein of each of the isolated SMGs were cannulated and arterially perfused with perfusion fluid.

Clark-type electrodes were placed serially on the arterial and venous sides of the perfusion line. The data were stored in every 15 s by computer. The calibration of the system was performed during perfusion with distilled water 100% equilibrated with air. The partial pressure of oxygen was obtained as the value at 100% air saturation at 37°C and 1 atm. The rate of oxygen consumption was expressed by changes in the arterio-venous (A-V) difference of oxygen pressure from the resting level. Oxygen consumption during DS-induced salivary fluid secretion was measured in the same time course as the salivary fluid secretion induced by 5 g/L DS. Oxygen consumption during DS perfusion with other drugs was simultaneously measured with the fluid secretion.

**II-7. Effects of ouabain, bumetanide, atropine and phentolamine.** While atropine (1 µmol/L; Nacalai Tesque, Inc., Japan) or phentolamine (5 µmol/L; Sigma, MO, USA) was continually perfused and 10 min later, DS (5 g/L) solution was added in addition to atropine or phentolamine, and continually perfused for 50 min. The salivary fluid secretion and oxygen consumption were recorded simultaneously during experiments that involved ouabain and bumetanide. In addition to the first experiment, ouabain (1

mmol/L; Merck KGaA, Germany) or bumetanide (100  $\mu\text{mol/L}$ ; Sankyo, Japan), were respectively added at 10 min after the DS was administrated and removed at 15 min.

***II-8. Removal of extracellular and chelation of intracellular  $\text{Ca}^{2+}$ .*** The measurement of the salivary fluid secretion and the perfusion of the SMGs were almost the same as in the first experiment, except that  $\text{Ca}^{2+}$  in the perfusate was removed nominally without any chelating agent before the administration of CCh or DS, in order to examine the contribution of extracellular  $\text{Ca}^{2+}$  on DS-induced salivary fluid secretion.

BAPTA-AM (Dojin-Do, Japan) was employed to chelate the intracellular  $\text{Ca}^{2+}$  of the SMG cells. The measurement of the salivary fluid secretion was the same as the first experiment. Initially CCh (0.2  $\mu\text{mol/L}$ ) was added in the perfusion for 5 min as control stimulation. After CCh was washed for 5 min by perfusion without CCh, the BAPTA-AM was added in the perfusion for 20 min at 100  $\mu\text{mol/L}$  in the  $\text{Ca}^{2+}$ -free perfusate. Then, the DS was continuously perfused for 40 min in  $\text{Ca}^{2+}$ -free perfusate.

***II-9. Measurement of the intracellular  $\text{Ca}^{2+}$  concentration using dispersed acinar cells from rat SMGs.***

***II-9-1. Solutions.*** We used a Krebs-Ringer-bicarbonate (KRB) solution, which was prepared by replacing 25 mmol/L of  $\text{Cl}^-$  by  $\text{HCO}_3^-$  and gassed with a gas mixture of 5%  $\text{CO}_2$  and 95%  $\text{O}_2$  for the isolation of the acinar cells. Then the  $\text{CaCl}_2$  was removed from the perfusate solution ( $\text{Ca}^{2+}$  nominally zero), resulting in a decrement of  $\text{Cl}^-$  by 2 mmol/L.

Salvianolic acid B (SAB), which is a main component of DS, could induce fluid secretion by the SMG with a similar pattern. SAB was purchased from Shaanxi Sciphar Biotechnology Co., Ltd., China (DS111217). Because the dark color of the DS granules masked the fluorescent signal of  $\text{Ca}^{2+}$  indicators (see appendix 1), then SAB was used

instead of the DS granules in order to reduce the masking effect. The SAB was dissolved in the perfusion solution and the pH was adjusted to 7.4. The precipitate was removed by centrifugation, and the supernatant was prepared into SAB solutions of 150, 200, and 250  $\mu\text{g}/\text{mL}$ . The SAB content in DS granule was measured as 2.53% by HPLC purification (unpublished data). Then the DS concentration (5 g/L) was equivalent to 126.5  $\mu\text{g}/\text{mL}$ . The same concentration range of SAB was used for isolated SMG cells than that of DS used for the perfused gland.

***II-9-2. Isolation of the SMG cells*** (Sakai et al., 2002). Two SMGs were isolated from one rat, placed on thick wax layer in a petri dish, and minced using a single-edge razor. Then the mixture was moved to a 15 mL centrifuge tube, 5 mL of perfusate was added, and in a vortex well, centrifuged at 1,000 rpm for 1 min at room temperature. After discarded the supernatant, the minced SMGs were suspended with 5 mL of the KRB solution with a 0.5% BSA fraction V (Sigma, MO, USA), collagenase type II (Worthington Biochemical Corporation, 0.2 mg/mL) and hyaluronidase (Sigma, MO, USA, 0.2 mg/mL) in a 25 mL centrifuge tube. After cutting and pipetting 10 times, the tube was placed into a water bath and was incubated for 45 min at 37°C. The tissue mixture was dispersed at 10 min intervals by cutting and pipetting. Then, the tissue mixture was slowly filtered through nylon mesh and gently put on 5 ml of the KRB solution with a 4% BSA fraction V. After centrifuging the filtrate at 500 rpm for 5 min, the supernatant was discarded.  $\text{Ca}^{2+}$  indicator (Indo-1 AM (Sigma-Aldrich, USA),  $\text{Ca}^{2+}$ TM-2 AM (Goryo Chemicals, Japan) or Fura-2 AM (Dojin-Do, Japan) was added to the cell suspension. The tube was covered with aluminum foil and incubated for another 30 min with gentle shaking at 160 rpm. Then it was washed by spin-down at 500 rpm for 3 min and the supernatant was discarded. Next, 2 mL of perfusate was added for

incubation and the mixture was shaken again for 3 min. After centrifuging the mixture again, the cell suspension was prepared by gently mixing the sediments with trypsin inhibitor (Sigma-Aldrich, MO, USA, 1 mg/5 mL) solution (Krebs-Ringer-HEPES solution [(in mmol/L) 120 NaCl, 5 KCl, 1 MgSO<sub>4</sub>, 0.96 NaH<sub>2</sub>PO<sub>4</sub>, 0.2% glucose, 20 HEPES (pH 7.4), and 1 CaCl<sub>2</sub>] contained 0.2 mg/mL trypsin inhibitor and 0.1% BSA).

**II-9-3. Measurement of the Ca<sup>2+</sup> uptake.** The fluorescence of Fura-2-loaded cells was measured with a CAF-110 spectrofluorometer (Nihon Bunko, Japan) with excitation at 340 nm and 380 nm and emission at 500 nm. The data were recorded in computer with LabChart software (AD Instrument, Australia).

The Ca<sup>2+</sup> influx in the isolated submandibular cells was measured with the <sup>45</sup>Ca radioactive tracer method as described by Yang et al. (2006). <sup>45</sup>CaCl was obtained from Perkin-Elmer (MA, USA). The cells were prepared as described in the Methods section, and separated in three tubes with a volume of 1.8 mL in each tube. Then 4 µL of tracer was poured into each tube (final radioactivity was 74 kBq/mL, i.e. 2 µCi/mL).

In the first tube, 0.2 mL of KRB solution was added to obtain a control solution. In the second tube, 0.2 mL of the vehicle with 2 µL of 1 mM ionomycin (final 1 µM) was added to obtain a positive control. In the third tube, 0.2 mL of the vehicle with 2.5 g/L of SAB (final ratio 250 µg/mL) was added for the SAB stimulation. The cell suspensions were incubated with 95% O<sub>2</sub>/ 5%CO<sub>2</sub> gas mixture at 37°C during the experiment. At 0, 10, 20 and 30 min, 0.5 mL of the suspensions were extracted from each of the tubes. The extracted samples were centrifuged at 5,000 rpm (2,000 g) for 1 min and the supernatant was discarded. The extracted samples were washed 3 times by KRB solution. Thereafter 100 µL of 0.5 N NaOH was added to solubilize the cells. Finally, 2 mL of liquid scintillation cocktail (Ultima Gold, Perkin Elmer, MA, USA)

was added and the radioactivity of each sample was counted by the liquid scintillation counter (Beckman, LS 6000TA).

***II-10. Increase in microcirculation as measured by A-V pressure difference.*** Both the artery and the vein of the isolated gland were cannulated and vascularly perfused with perfusion fluid. Blood pressure transducers (MLT0670, AD Instruments, NSW, Australia) were placed on the arterial and venous sides of the perfusion system to measure the pressure difference between perfusate and effluent. The values were recorded in computer and the pressure differences between the artery and the venous sides of the SMG were calculated (POWER Lab, AD Instrument, NSW, Australia).

***II-11. Assessment of the paracellular transport using LY secretion as an indicator.***

The isolated SMGs were vascularly perfused with a perfusate containing 50 mg/L Lucifer Yellow K salt (LY, mw=521.56, L1177, Molecular Probe, OR, USA). A reserve sample of LY buffer was kept for establishing the standard curve after the gland perfusion. During the stimulation, the secreted saliva was collected every minute. The samples were weighed and immediately labeled as fluid secretion. After the addition of 100  $\mu$ L of distilled water, the samples were mixed well and 100  $\mu$ L of the mixed sample was shifted to the microplate reader (DTX800, Beckman Coulter, CA, USA). The calibration curve was obtained by a diluted series of the LY solution as 0.1, 0.05, 0.025, 0.0125, 0.00625, 0.003125, 0.0015625, 0.00078625, and 0.000390625 of the perfusate. To avoid a time lag considering the small secretion, the catheter for sampling was shortened as much as possible and the sampling time was corrected by the volume of the catheter lumen.

**II-12. Statistics.** The values for the salivary fluid flow rates were presented as means  $\pm$  SE. N was the number of glands. Statistically significant differences between the values were determined by student's t-test or one way ANOVA, *P* values below 0.05 were regarded as statistically significant.

### **III. Results.**

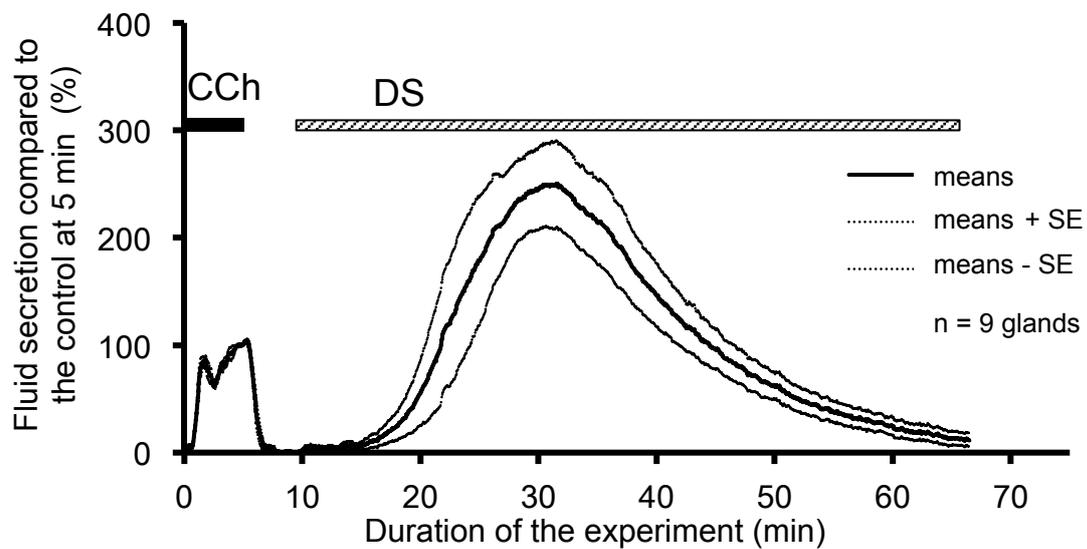
#### **III-1. Characteristics of the Danshen-induced salivary fluid secretion.**

**III-1-1. Time course of the DS induced salivary fluid secretion.** A low dose of CCh (0.2  $\mu\text{mol/L}$ ) was used as control stimulation to normalize the variation of fluid secretion shown by individual glands. All values for the fluid secretory rate during the experiment were normalized to the fluid secretion at 5 min of the control CCh stimulation. Addition of the CCh immediately induced salivary fluid secretion. In contrast, after the addition of the DS, the fluid secretion did not start immediately, i.e., latency was observed, as shown in Figure 1-1.

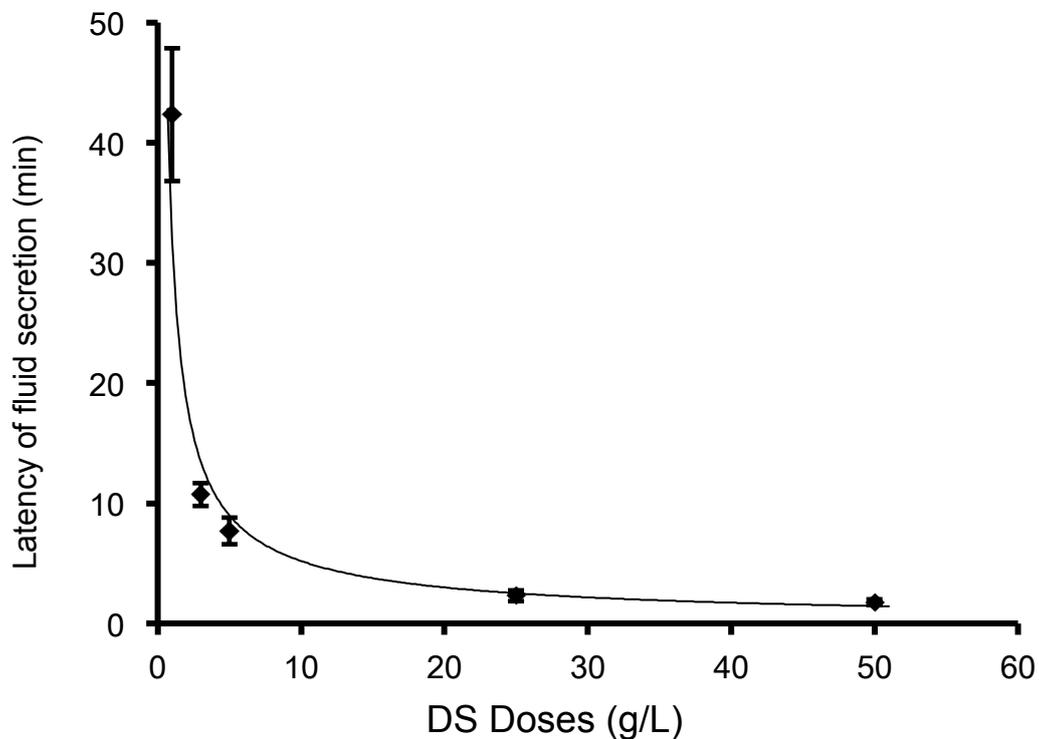
The DS-induced secretory process had two stages. Initially the fluid secretion increased gradually to reach the highest secretion ( $64.9 \pm 10.7 \mu\text{L/g-min}$ ,  $250.1 \pm 40.3\%$  of the CCh control at 5 min). Thereafter, the fluid secretion declined gradually to the plateau value ( $3.9 \pm 1.4 \mu\text{L/g-min}$ ,  $12.0 \pm 5.1\%$  of the CCh control) at 60 min as shown in Figure 1-1. After this decline, the gland did not start another fluid secretion process even during prolonged perfusion with DS.

**III-1-2. The latency of the salivary fluid secretion decreased with high doses of DS perfusion.** The latency is the time period required for the SMG to start fluid secretion, that is, the time from the application of DS perfusion to the start of fluid secretion. The

latency of the salivary secretion was observed at different doses of DS, as shown in Figure 1-2. The latency of the salivary fluid secretion (mean  $\pm$  SE) decreased with higher doses of DS perfusion:  $42.3 \pm 5.5$  min at 1 g/L DS (n = 6),  $10.7 \pm 1.0$  min at 3 g/L (n = 9),  $7.7 \pm 1.1$  min at 5 g/L (n = 9),  $1.3 \pm 0.5$  min at 25 g/L (n = 6) and  $1.8 \pm 0.3$  min at 50 g/L (n = 8), respectively (n = the number of glands employed for each dose).

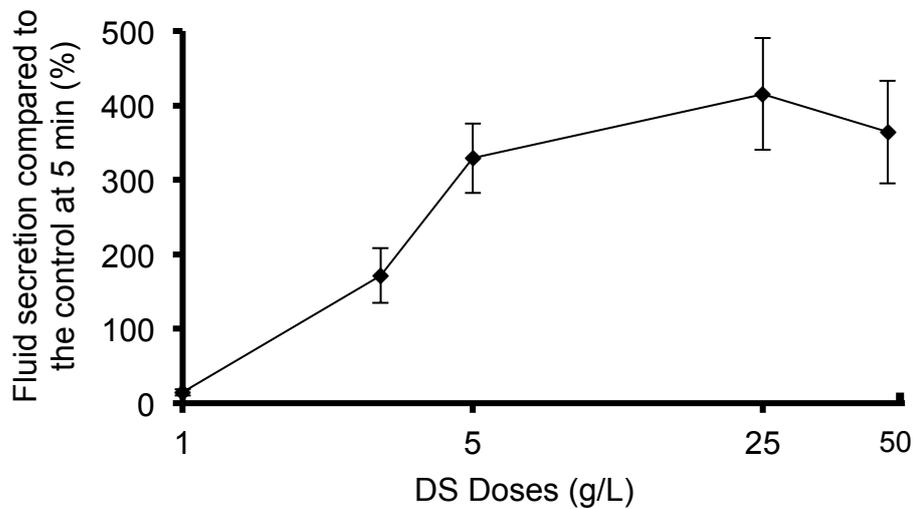


**Figure 1-1. Time course of the DS-induced salivary fluid secretion.** After control stimulation with  $0.2 \mu\text{mol/L}$  CCh for 5 min, the DS (5 g/L) was introduced at 10 min and perfused for 60 min. The bold and dotted lines show, respectively, the average values and the means  $\pm$  SE of the salivary fluid secretion of 9 glands.



**Figure 1-2. The latency of different doses of DS perfusion.** The latency of the fluid secretion was shown as mean  $\pm$  SE for different DS doses. The latency reduced as the dose of DS increased.

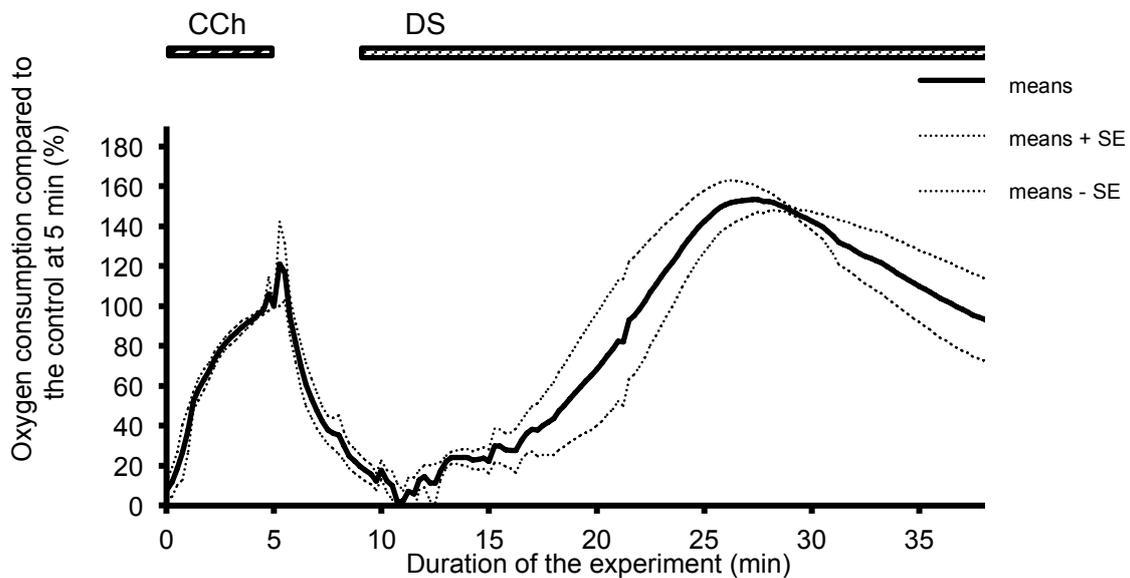
**III-1-3. Highest secretory rate of the DS-induced salivary fluid secretion.** The fluid secretion induced by DS perfusion was demonstrated in a dose-dependent manner within the range of 1-50 g/L. DS perfusion in the rat SMGs at doses of 1, 3, 5, 25 and 50 g/L induced the highest fluid secretion of  $14.7 \pm 4.3\%$  of the CCh control fluid secretion, with the various doses showing fluid secretion values of  $171.5 \pm 36.7\%$ ,  $325.2 \pm 46.4\%$ ,  $415.3 \pm 66.8\%$ , and  $364.0 \pm 68.7\%$ , respectively, as shown in Figure 1-3. There was no significant difference among the groups with 5, 25 and 50 g/L. The median effective dose ( $ED_{50}$ ) of DS was 3.5 g/L, calculated by the straight line of regression method ( $Y = 77.6X - 62.4$ ,  $R^2 = 1$ ).



**Figure 1-3. Dose response of the highest DS-induced fluid secretion.** The highest fluid secretion was shown as mean  $\pm$  SE and the doses of DS employed were 1, 3, 5, 25 and 50 g/L, respectively. The fluid secretion increased from 1 g/L to 5 g/L, and reached a plateau level at the higher doses.

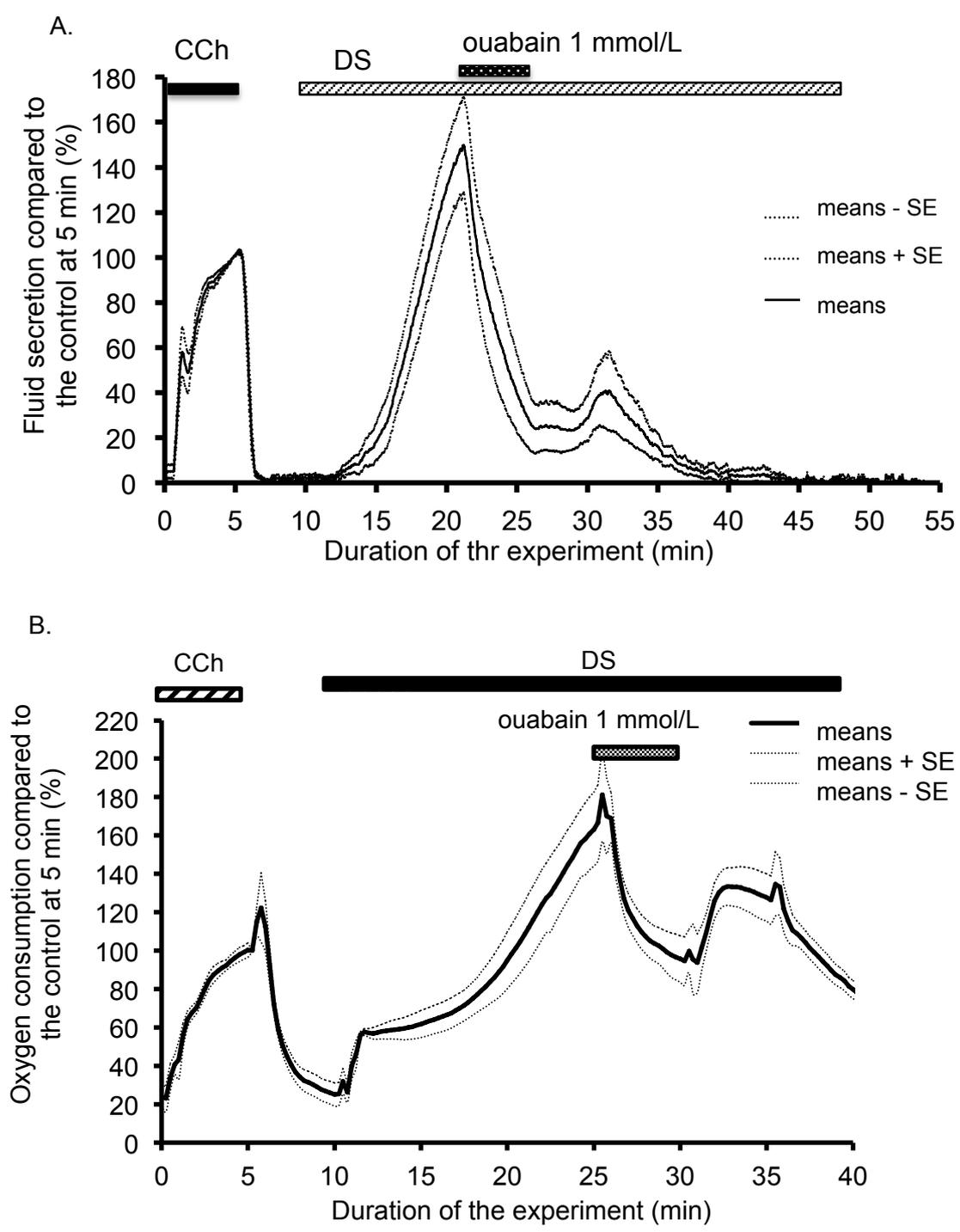
***III-2. Contribution of  $Na^+/K^+/2Cl^-$  cotransporter and  $Na^+/K^+$  ATPase to the DS-induced fluid secretion.***

***III-2-1. Oxygen consumption during the DS-induced salivary fluid secretion.*** The oxygen consumption of the isolated SMGs increased rapidly after the administration of DS without any latency. Corresponding to the induction of the fluid secretion, the oxygen consumption increased and reached the highest value at 27.25 min ( $46.0 \pm 4.0$  mmHg,  $153.5 \pm 7.4\%$  of the control stimulation with CCh), following by a gradually decrease as shown in Figure 2-1.



**Figure 2-1. Time course of the A-V difference of PO<sub>2</sub> during the DS perfusion.** CCh stimulation was conducted for 5 min. as a control measure. At 10 min., DS was added and perfused for 30 min. This figure shows the average values (bold line, n=5 glands) and the standard error of the means (means ± SE, dotted lines).

**III-2-2. Ouabain decreased fluid secretion and oxygen consumption.** Ouabain, an inhibitor of Na<sup>+</sup> / K<sup>+</sup> ATPase, was applied to assess the activity of the primary active transport during DS stimulation. The fluid secretion was measured by 1 mmol/L ouabain during stimulation with 5 g/L DS stimulation. The fluid secretion decreased to a plateau level of 24.3 ± 1.1% at 26-29 min. The remaining fluid secretion during inhibition by ouabain was compared with the maximum fluid secretion induced by the same dosage of DS (250.1 ± 40.3%, at 5 g/L). Ouabain significantly ( $P < 0.05$ ) decreased the DS-induced fluid secretion by 90% of the highest value. After the removal of ouabain, the fluid secretion showed a slight recovery, and thereafter gradually decreased to reach the zero-level, as shown in Figure 2-2 A.



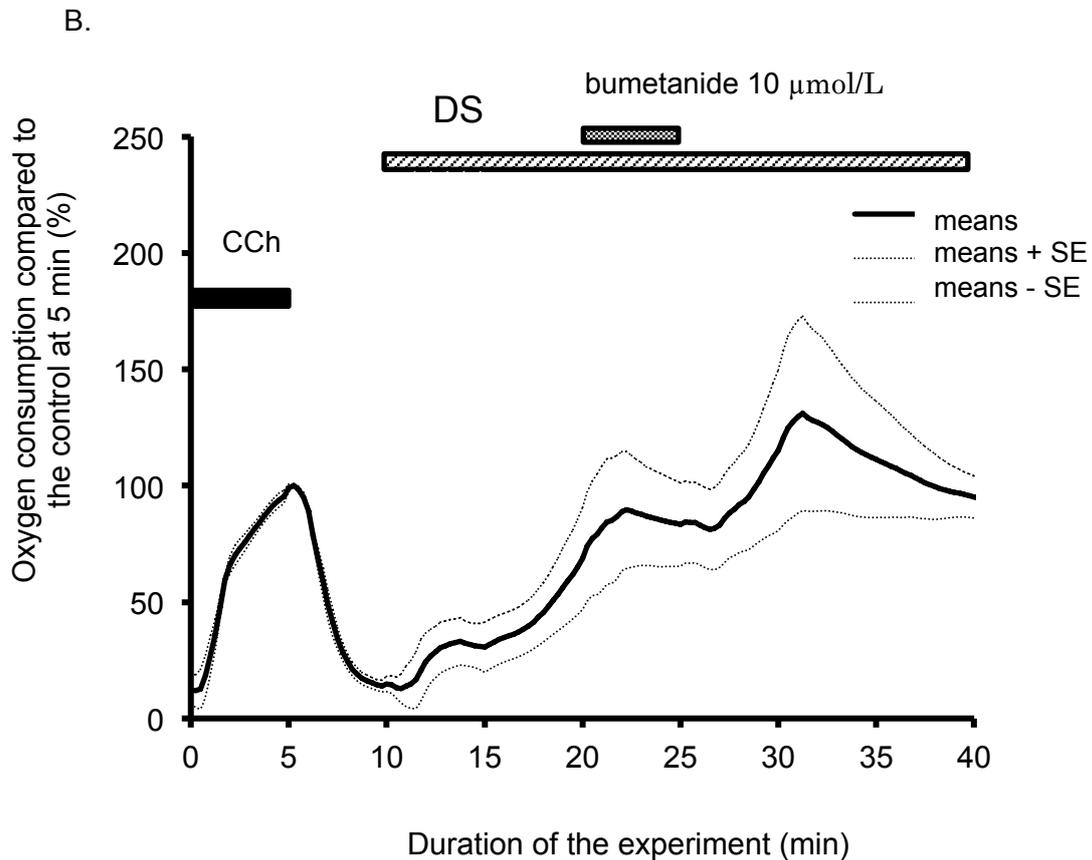
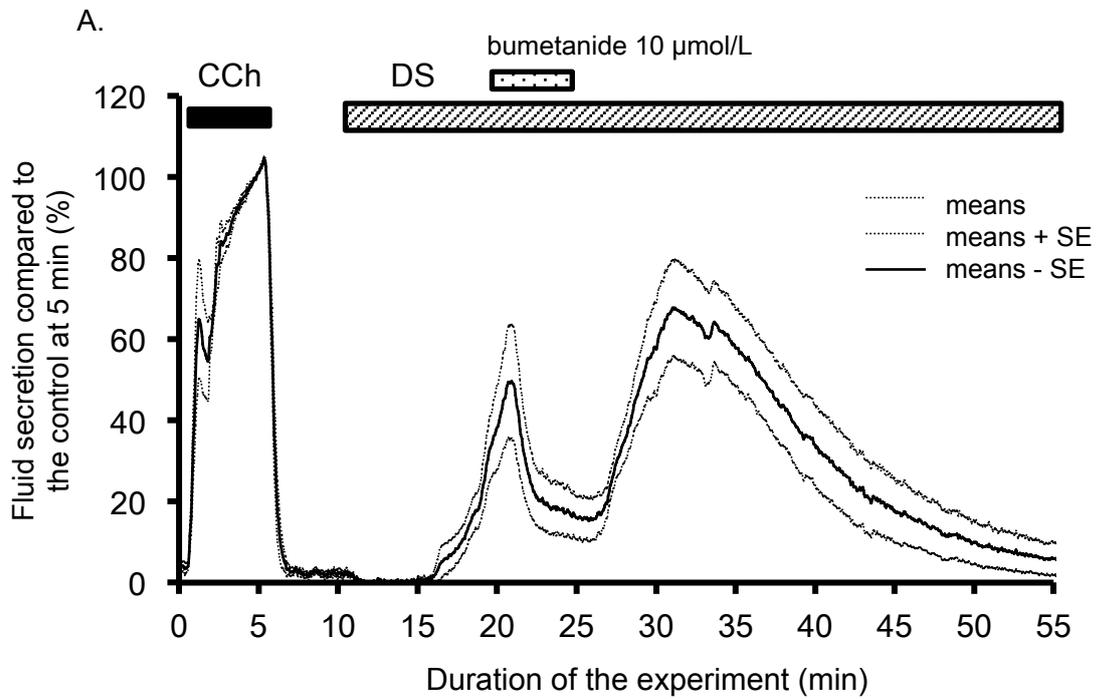
**Figure 2-2. Inactivation of Na<sup>+</sup>/K<sup>+</sup> ATPase by ouabain. A. DS-induced salivary fluid secretion.** CCh stimulation was conducted for 5 min as control measure. At 20 min ouabain was added for 5 min, and the DS-induced fluid secretion decreased significantly ( $P < 0.05$ ). The figure shows the average (bold line, n=5 glands) and the

standard error of mean (mean  $\pm$  SE, dotted lines). **B. Oxygen consumption during DS-stimulation.** At 25 min ouabain was added for 5 min, and the oxygen consumption decreased significantly ( $P < 0.05$ ). This figure shows the average values (bold line, n=5 glands) and the standard error of means (means  $\pm$  SE, dotted lines).

The addition of ouabain decreased the oxygen consumption to  $93.6 \pm 15.3\%$  of control by CCh ( $153.5 \pm 7.4\%$  in the sole DS group). After the removal of the ouabain, the oxygen consumption partially recovered, and then gradually decreased, as shown in Figure 2-2 B.

**III-2-3. Bumetanide decreased fluid secretion and oxygen consumption.** Bumetanide was used to block the NKCC1 cotransporter. Bumetanide ( $10 \mu\text{mol/L}$ ) was administrated at 10 min from the start of the DS administration, and then removed at 15 min. Bumetanide significantly decreased the DS-induced fluid secretion to a plateau level of  $17.2 \pm 0.7\%$  at 23-26 min. After the removal of the bumetanide, the salivary fluid secretion increased again to the peak. Thereafter the fluid secretion gradually decreased to the zero-level. The decreased level of fluid secretion was significantly lower than the maximum fluid secretion of the DS-induced salivary fluid secretion. The residual fluid secretion during inhibition by bumetanide was  $17.3 \pm 6.5 \mu\text{L/g-min}$  at 27 min from the start of the experiment. The inhibition of salivary fluid secretion was 91.2% of the maximum secretion induced by DS. This finding indicates NKCC1 is activated during DS induced fluid secretion (Figure 2-3 A).

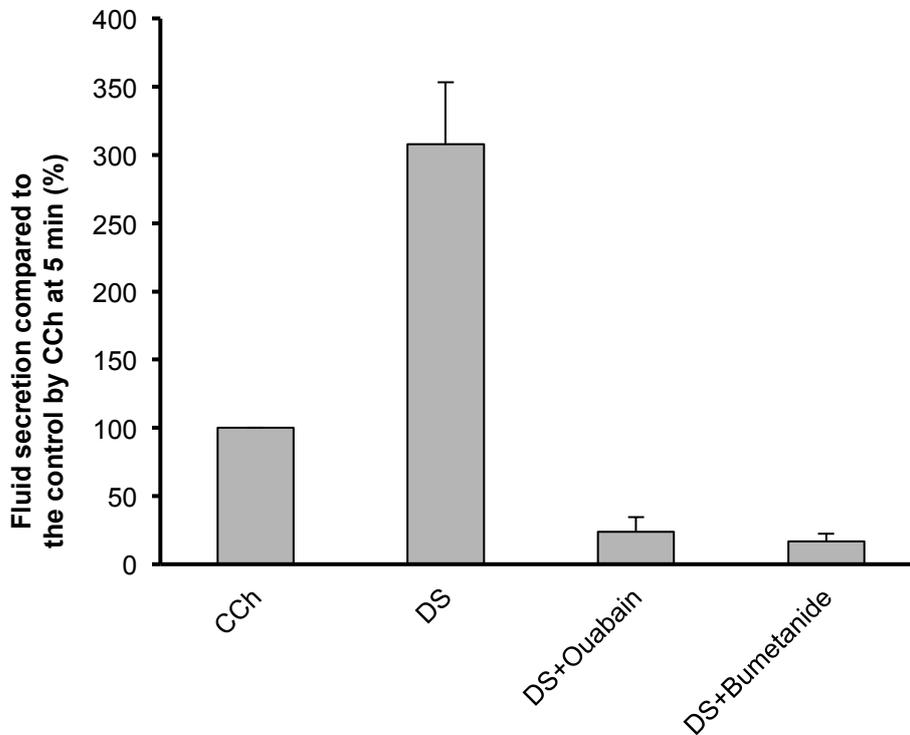
The increase in oxygen consumption by DS was stopped by bumetanide and stayed at the plateau level of  $86.1 \pm 22.1\%$  at 21-25 min. After removal of bumetanide, the oxygen consumption increased again to the peak (Figure 2-3 B).



**Figure 2-3. A. The effects of bumetanide on DS-induced salivary fluid secretion.** Initially, CCh was added for 5 min as a control measure. At 20 min bumetanide was added for 5 min, and the fluid secretion decreased significantly to a plateau inhibited level ( $P < 0.05$ ). This figure shows the average values (bold line,  $n=6$  glands) and the

standard error of the means (means  $\pm$  SE, dotted lines). **B. The effects of bumetanide on DS-induced on DS-induced oxygen consumption.** The oxygen consumption was shown by A-V difference of partial oxygen pressure (mmHg). Initially, CCh was added for 5 min as a control measure. At 20 min bumetanide was added for 5 min.

**III-2-4. Comparison of the inhibition between ouabain and bumetanide.** Ouabain inhibition of Na<sup>+</sup>/K<sup>+</sup> ATPase decreases both the ATP hydrolysis and the driving force of Na<sup>+</sup> entry and K<sup>+</sup> release. As a result, the oxygen consumption decreases, the Cl<sup>-</sup> entry via the cotransporters and antiporters are decreased, and then fluid secretion will be reduced. On the other hand, bumetanide blocks only NKCC1. The DS stimulation induced a salivary fluid secretion, accumulating to  $1428.0 \pm 152.8$   $\mu$ L/g for 60 min. The maximal fluid secretion due to DS (5 g/L) was 2.5 times larger than that of CCh (0.2  $\mu$ mol/L). Bumetanide (10  $\mu$ mol/L) decreased the fluid secretion rate to  $17.2 \pm 0.7\%$  of the CCh control. Because the highest DS fluid secretion was  $250.1 \pm 40.3\%$  of the CCh control, bumetanide inhibited the highest fluid secretion by 93% of the highest DS-induced fluid secretion. In contrast, ouabain decreased the fluid secretion rate to  $24.3 \pm 1.2\%$  of the CCh control ( $P < 0.01$ ), which means that ouabain inhibited the fluid secretion by 90% of the highest DS-induced fluid secretion. The inhibition by bumetanide was significantly larger than that due to ouabain ( $P < 0.05$ ) as shown in Figure 2-4.

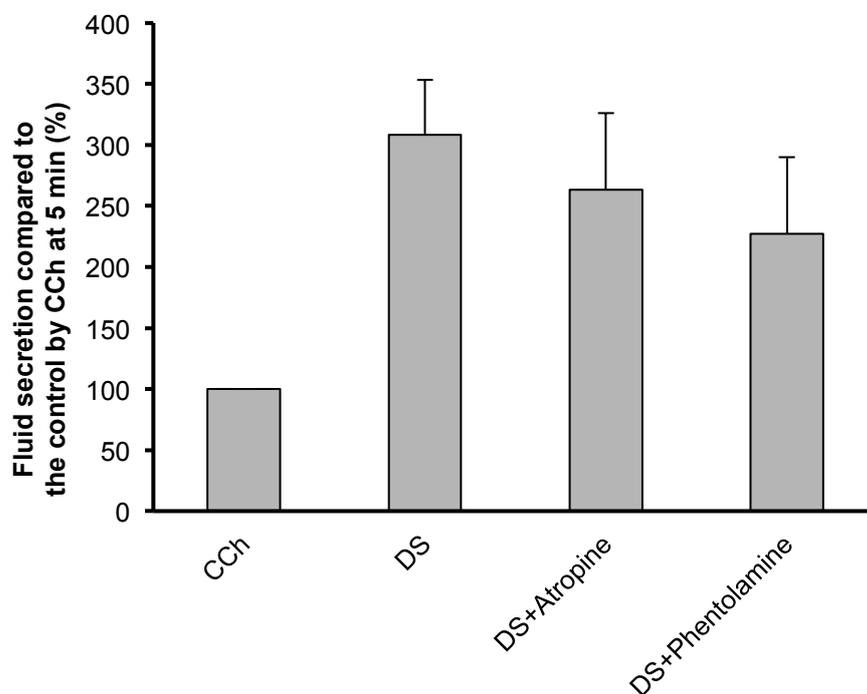


**Figure 2-4. The residual DS-induced fluid secretion during inhibition due to ouabain or bumetanide.** This figure shows the standard error of the means (means  $\pm$  SE) of the salivary fluid secretion. Ouabain (the inhibitor of  $\text{Na}^+/\text{K}^+$  ATPase ) and bumetanide (the inhibitor of NKCC1) decreased the DS-induced fluid secretion due to the DS (n = 9). The DS column indicates the maximum response of the fluid secretion due to DS (5 g/L).

The oxygen consumption was measured as an arterio-venous difference in the partial oxygen pressure (A-V difference in  $\text{PO}_2$ , mmHg). Although this value includes the oxygen that escaped from the gland surface and the perfusion line, the A-V  $\text{PO}_2$  difference was normalized (%) by the value at 5 min during CCh stimulation. The AV

difference in PO<sub>2</sub> was 93.6 ± 2.3 mmHg at 5 min from the start of the CCh stimulation. DS increased the A-V PO<sub>2</sub> difference to the highest value, 153.5 ± 7.4% of CCh, at 27 min from the start of the experiment, as shown in Figure 2-1. Ouabain (1 mmol/L) was added about 25 min for 5 min, as shown in Figure 2-2 B. Ouabain decreased the oxygen consumption to 97.1 ± 4.0% of the CCh control at 29-31min. Bumetanide (10 μmol/L) decreased the oxygen consumption to 86.1 ± 22.1% (at 21-25 min) of the CCh control. Therefore, as a minimal estimation, ouabain inhibited the oxygen consumption by 37% and bumetanide inhibited it by 44%.

**III-3. Participation of muscarinic or α<sub>1</sub> adrenergic receptors in DS-induced fluid secretion.** Figure 3 shows the highest secretion rate during perfusion with DS, DS with atropine, and DS with phentolamine, which were shown as % of fluid secretion rate due to CCh at 5 min. When atropine (1 μmol/L) or phentolamine (5 μmol/L) was applied separately on the DS perfusion, the salivary fluid secretion decreased slightly, but not significantly, to 221 ± 60% (n = 7) and 200 ± 66% (n = 14), respectively (DS induced a secretion at 250 ± 40, n = 9). The latency and the time course of the fluid secretion remained the same. The inhibition ratio of atropine and phentolamine was 12% and 20%, respectively, without any statistically significance differences ( $P > 0.5$ ).

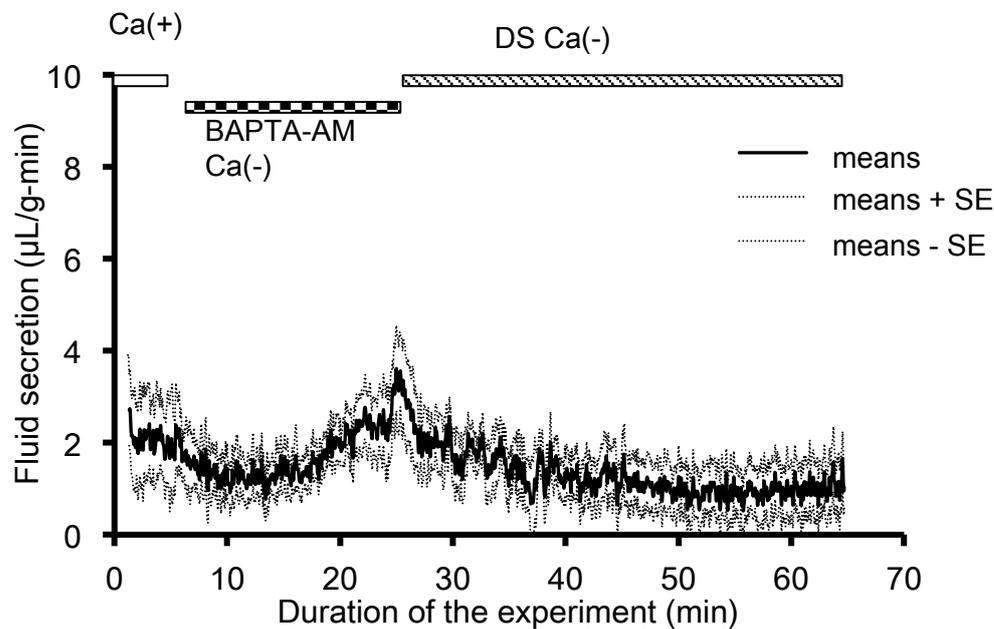


**Figure 3. Effects of atropine and phentolamine on the DS stimulation.** The standard error of means (means  $\pm$  SE) of the highest fluid secretion was shown in the DS only group (n = 9), the DS with atropine (n = 7), and the DS with phentolamine (n = 14). The number of the glands employed was shown as N. The values were expressed as percentage of the fluid secretion rate at 5 min with CCh as control. Among the 3 groups, there was no significant difference ( $P > 0.5$ )

**III-4. Requirement of extracellular  $Ca^{2+}$  for DS-induced fluid secretion.** As for physiological stimulation necessary to induce salivary fluid secretion, the increase in cytosolic  $Ca^{2+}$  level ( $[Ca^{2+}]_i$ ) is necessary for activation of  $K^+$  channels on the basolateral membrane and  $Cl^-$  channel on the luminal membrane. After the initial release of  $Ca^{2+}$  from cytosolic stores, the elevated  $[Ca^{2+}]_i$  is maintained by the balance

of entry and extrusion of cytosolic  $\text{Ca}^{2+}$ . In order to examine the  $\text{Ca}^{2+}$  entry during DS stimulation, the  $\text{Ca}^{2+}$  in the perfusate was removed nominally without any chelating agent. During  $\text{Ca}^{2+}$ -free perfusion, CCh (0.2  $\mu\text{mol/L}$ ) was perfused from 5 min to 10 min, followed by perfusion with DS (25 g/L) for 15 min. DS and CCh could hardly induce any fluid secretion. During DS perfusion, the highest fluid secretion rate was only  $1.8 \pm 0.8 \mu\text{L/g-min}$  at 8.65 min ( $n = 8$ ) (while during perfusion with DS and  $\text{Ca}^{2+}$ , the fluid secretory rate was  $101.9 \pm 17.2 \mu\text{L/g-min}$  at 8.65 min,  $n = 6$ ,  $P < 0.01$ ). These results indicated that the extracellular  $\text{Ca}^{2+}$  was essential for DS to induce salivary fluid secretion.

The requirement for cytosolic  $\text{Ca}^{2+}$  was also tested by application of the cytosolic  $\text{Ca}^{2+}$  chelating agent, BAPTA-AM. By perfusion with BAPTA-AM (100  $\mu\text{mol/L}$ ) in the  $\text{Ca}^{2+}$ -free perfusate for 20 min,  $\text{Ca}^{2+}$  could be chelated in the cytosol of SMG cells. Then the perfusion was changed to the  $\text{Ca}^{2+}$ -free perfusate with DS. The gland tested did not induce any fluid secretion due to DS, except for the initial quick and small increase in the fluid secretion. The salivary fluid secretion was significantly lower during perfusion with BAPTA-AM than the base secretion during control perfusion with  $\text{Ca}^{2+}$  before the experiment ( $P < 0.001$ ) as shown in Figure 4.



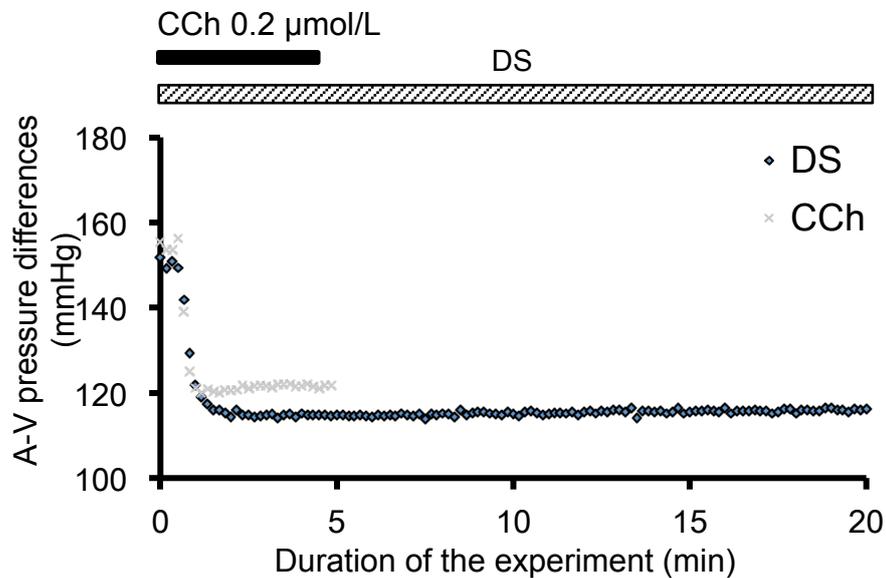
**Figure 4. Chelation of cytosolic  $\text{Ca}^{2+}$  with BAPTA-AM.** CCh ( $0.2 \mu\text{mol/L}$ ) and DS ( $5 \text{ g/L}$ ) were perfused respectively in this condition, however, neither of these secretagogues induced any significant amount of salivary fluid secretion.

**III-5. The effect of SAB on uptake of  $\text{Ca}^{2+}$ .** SAB is a main effective component of DS and induces a similar fluid secretion pattern to that by DS. To reduce the masking effect of DS on the fluorescence by  $\text{Ca}^{2+}$  indicators, SAB was used for the experiment using isolated SMG cells. However, the measurement of intracellular  $\text{Ca}^{2+}$  by fluorescent  $\text{Ca}^{2+}$  indicators (Indo-1, CaTM-2, and Fura-2.) were not succeeded due to masking of the fluorescence of  $\text{Ca}^{2+}$  indicators even by the SAB, as described in Appendix 1. Instead, the radioisotope  $^{45}\text{Ca}$  was applied to examine the uptake of  $\text{Ca}^{2+}$  by the isolated SMG cells, as shown in Appendix II.

**III-6. Paracellular fluid transport during the DS perfusion.** Recently the transcellular fluid secretion by the parotid salivary gland was measured by dilution of luminal LY

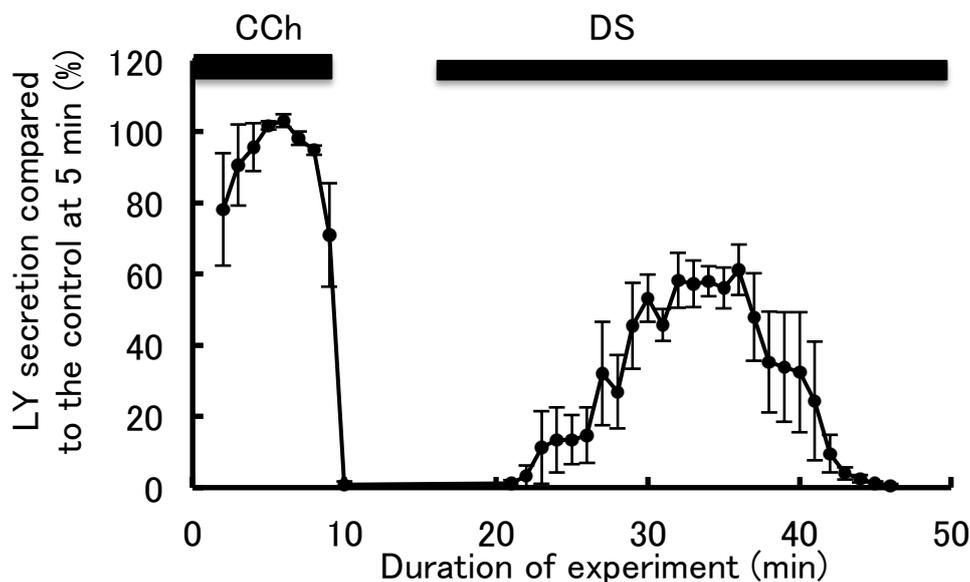
upon CCh stimulation (Segawa et al., 2002). Compared to the whole secretion by the gland, the paracellular fluid secretion was more than 60% of the whole secretion. In addition, the microcirculation is dilatated in order to supply enough oxygen and fluid to the secretory end pieces, thus the local hydrostatic pressure increases to facilitate paracellular transport. The dilataion of the microcirculation can be monitored by A-V pressure difference. Based on this evidences, the paracellular transport was assessed by measurement of LY.

***III-6-1. Increase in microcirculation measured by A-V pressure difference.*** Decrease in the A-V pressure difference is reflexed by increases in the total area of the vascular bed and increases in the microcirculation in the acinar area. The A-V pressure difference was immediately decreased by DS, and reached a plateau level after 1 min. Compared to CCh (0.2  $\mu\text{mol/L}$ ) at 4 to 5 min, the decrement of A-V pressure difference due to DS (5 g/L) was significantly higher than that due to CCh ( $38 \pm 3$  mmHg by DS and  $30 \pm 3$  mmHg by CCh,  $n = 7$ ,  $P < 0.05$ ).



**Figure 5-1. A-V pressure differences during DS (black) or CCh (gray) perfusion.**

**III-6-2. Increase in paracellular transport measured by LY secretion.** LY secretion at 5 min from the start of CCh stimulation was set as 100%. DS was added at 15 min. Thereafter the LY secretion increased to the highest value of  $61.2 \pm 7.1\%$  at 30 min. Then the LY secretion decreased to almost zero at 25 min from the start of the DS perfusion. The LY secretion was significantly different between the CCh and DS stimulation ( $P < 0.05$ ,  $n = 6$ ), indicating that DS could increase the paracellular permeability for fluid secretion. The time course of LY secretion was in parallel with the total fluid secretion during the DS-stimulation. However, the LY secretion was decreased to 60% of that due to CCh, whereas the highest fluid secretion due to DS was 250% of the CCh-induced secretion. This finding suggests that DS induces more transcellular fluid secretion than paracellular fluid secretion.



**Figure 5-2. LY secretion during CCh or DS perfusion.** CCh was perfused for 10 min as a control measure (by 0.2  $\mu\text{mol/L}$ ) and DS was perfused from 15 min to 40 min from the start of the experiment. This figure shows the average values of the LY secretion (bold line,  $n=6$  glands) and the standard error of the means (means  $\pm$  SE).

#### ***IV. Discussion.***

The application of traditionally used Chinese herbs to clinical medicine is increasing especially for chronic diseases all around the world. However, the information on the mechanisms of their pharmaceutical action is limited. Collaborative experiments conducted by the Nanjing Medical College (China) and the National Institute for Physiological Sciences (Japan) screened the Chinese herbs that promote salivary fluid secretion in the isolated perfused rat salivary glands (Murakami et al., 2009a). During the collaborative work, the methods for screening the effective Chinese herbs were developed. As a result, it was discovered that Danshen (DS) induced salivary fluid secretion without other added stimulants. The present work was planned

to clarify the mechanism by which DS induces salivary fluid secretion. During this study, a number of different methodologies required to pursue the mechanisms involved in salivary secretion were combined. This set of methodologies will be useful for future studies in the search for new drugs with unknown mechanisms for salivary fluid secretion.

***IV-1. DS.*** DS is obtained from the dried root of *Salvia miltiorrhiza*, a native plant in China and Japan. In 1934, Nakao and Fukushima first isolated the tanshinones from Danshen. According to the traditional usage, Danshen is reduced into a water decoction, which contains more hydrophilic components. Therefore, the phenolic acids from Danshen have been extracted since the 1980s, and were called the Salvianolic acids (Li et al., 1984; Ai and Li, 1988, 1992). In the present study, we used Ringer's solution to dissolve DS, so most phenolic acids and a small amount of tanshinone were dissolved in our DS solution. The plant, *Salvia miltiorrhiza*, was briefly mentioned in Mabberley's Plant-Book (2009), with a short comment on its use locally for heart conditions. However, DS is famous as a blood-activating drug in the field of Traditional Chinese Medicine (TCM). In TCM, the "blood-activating" means a treatment of the symptoms caused by the reduction of fluid secretion, such as dry mouth, dry eyes and so on (Jiang et al., 2002; Zhang et al., 2013). While the TCM reports have not revealed any mechanism for production of saliva, it is well known in the field of Physiology that salivary fluid secretion is mainly induced by activation of the muscarinic receptors on salivary acinar cells. Acetylcholine (ACh) is released from parasympathetic nerve endings and binds with this receptor, then links with elevation of cytosolic  $Ca^{2+}$  levels. The elevation of cytosolic  $Ca^{2+}$  levels activates the  $Cl^-$  channel to release  $Cl^-$  into lumen.

The mechanisms in which DS induces salivary fluid secretion may be hidden within this sequential mechanism.

During the screening Chinese herb, they used a moderate concentration of CCh, 0.2  $\mu\text{mol/L}$ . Because the concentration 1  $\mu\text{mol/L}$ , is a supermaximal concentration for salivary fluid secretion, the moderate concentration was suitable to examine if the fluid secretion was promoted or not. In addition, they normalized the values for fluid secretion and oxygen consumption to avoid variations among individual glands. This measure was implemented because the responses to Chinese herb could vary among the individual rats and the surgical procedures employed were not fully developed yet by the young investigators (Murakami et al., 2009a). The collaborative study showed that DS promotes salivary fluid secretion, compared with CCh, and could be a promising drug in treatment for the relief of dry mouth caused by hypofunction of salivary gland. However, the characteristics and possible mechanisms associated with the sole use of DS have not been studied.

***IV-2. DS-induced salivary fluid secretion.*** Sole DS stimulation induced a fluid secretion by the isolated and perfused SMG of the rat. However, the time course of the secretion by DS was different from the CCh-induced fluid secretion. DS started fluid secretion with a time period latency and the secretion gradually increased to reach the highest value, which was 2.5 times higher than the fluid secretion at 5 min due to CCh. This type of high fluid secretion due to DS has not been reported previously. However, the fluid secretion slowly declined from the highest value to zero-level around 60 min from the start of the DS administration. These characteristics suggested the mechanism for DS-induced salivary fluid secretion was different from that induced by CCh or ACh.

CCh and ACh activate the  $M_3$  receptor to release  $IP_3$  and quickly release  $Ca^{2+}$  from  $IP_3$ -regulated calcium-stores (Menniti et al., 1991). The following process including channel activation and quick osmosis, results in a quick onset of fluid secretion. Fluid secretion can be quickly started by activation of the  $\alpha_1$  adrenergic receptors (Martinez et al., 1975; Bockman et al., 2004) and neurokinin A receptors (Qi et al., 2010). Therefore DS induced secretion could use a different signalling process, compared with  $IP_3$ -store  $Ca^{2+}$  release signalling.

The clinical dosage of DS ranges from 10 to 50 g/person because the treatment recipe is usually a mixture of several herbs, and the proportion of DS varies depending on the individual symptoms. For experimental convenience, we adopted an average dose of 25 g/person for the experiment. Assuming that all the DS will move to the blood circulation (5 L for 60 kg body weight), the concentration of DS in the blood will be 5 g/L. We took 5 g/L as a standard concentration of DS in the perfusion fluid. On the other hands, the relationship between DS dose and fluid secretion was examined using a series of doses at 1, 3, 5, 25, and 50 g/L. The highest fluid secretion increased with the higher dose of DS, while the latency was shorter at the higher doses of DS. The results of the dose of 5 g/L were slightly higher than the  $ED_{50}$ . These results suggested that we can control clinically the level of fluid secretion between 5 g/L and 25 g/L, which was also within the safe therapeutic dose. At doses higher than 25 g/L, the effect of the DS would not improve and side effects may appear, such as bleeding. Therefore, the administration of DS requires rigorous guidance and clinical observation. These results may be of some help for studies on the clinical applicability of DS.

The latency decreased as the dose of the DS was increased. This feature was apparently different from the instant reaction shown when the salivary fluid secretion

was stimulated by CCh through muscarinic receptors. Our previous study (Murakami et al., 2009a) showed that CCh rapidly stimulated salivary fluid secretion by the SMGs through the activation of muscarinic receptors. When perfused with DS, it took a long time to induce salivary fluid secretion, and there was no initial peak effect. However, when CCh was added to the DS perfusion at an early time, a marked superimposed peak in the salivary fluid secretion was shown. The salivary fluid secretion by the SMGs induced by DS decreased gradually after reaching the highest secretion ( $64.9 \pm 10.7 \mu\text{L/g-min}$ ,  $250.1 \pm 40.3\%$  of the CCh control, at 21.5 min), until the secretion stopped. Although continually perfused with DS, the gland did not secrete further after the secretion stopped. However, after washing with buffer solution, salivary fluid secretion was induced again when stimulated by DS. These phenomena indicated that DS may have other mechanisms to promote salivary fluid secretion, which were also different from that of the muscarinic and  $\alpha_1$  receptors.

In summary, at doses over 25 g/L, the effect of the DS would not improve and there is a risk of side effects, such as bleeding. Therefore, the administration of DS requires rigorous guidance and clinical observation.

***IV-3. Inhibition of  $\text{Na}^+/\text{K}^+$  ATPase during the DS-stimulation.*** The increase in oxygen consumption reflects the increased energy metabolism during fluid secretion (Murakami 1979, 1981). Because the increment of the oxygen consumption becomes the same as the increase in heat production, this suggests that the energy metabolism is mostly from oxidative phosphorylation in mitochondria. In addition, the increment of the oxygen consumption and the  $\text{K}^+$  uptake during the post-stimulatory activation of  $\text{Na}^+/\text{K}^+$  ATPase were compared, and the results showed that the increase in oxygen

consumption during fluid secretion is mostly from the activation of  $\text{Na}^+/\text{K}^+$  ATPase (Murakami et al., 1990). However, the protein synthesis and its secretion contributed less to the increase in the oxygen consumption during the combined stimulation of CCh and isoproterenol ( $\beta$ -adrenergic stimulant, Murakami et al., 2000). Finally, we managed to estimate the activation of  $\text{Na}^+/\text{K}^+$  ATPase from the decrement of oxygen consumption during ouabain application.

***IV-3-1. Oxygen consumption during latency during DS stimulation.*** The oxygen consumption of the gland immediately increased after the administration of DS, even though there was no fluid secretion. This indicated that some energy consuming processes were activated by DS. Because the dilation of capillary bed and thus the promotion of microcirculation occurred simultaneously, the energy metabolism of the uncirculated region was possibly added due to the shunt closure. The fluid secretion started several minutes later, so these processes probably did not include fluid secretion. The promotion of microcirculation could probably to be one of these processes. Another possibility is the activation of the synthesis of secretory proteins. However, protein secretion was not measured in this study.

***IV-3-2. Oxygen consumption during DS-induced secretion.*** The time courses of the oxygen consumption and the salivary fluid secretion were similar during the DS stimulation, showing a slow increase and gradual decline. This suggests a close relationship between fluid secretion and the activation of  $\text{Na}^+/\text{K}^+$  ATPase. Ouabain (g-strophanthin) is a blocker of  $\text{Na}^+/\text{K}^+$  ATPase.  $\text{Na}^+/\text{K}^+$  ATPase is located on the basolateral membrane of the salivary acinar gland. According to the mostly accepted model for salivary fluid secretion mechanism (Catalan et al., 2009), cytosolic  $\text{K}^+$  is continuously released across the basolateral membrane through  $\text{Ca}^{2+}$ -activated  $\text{K}^+$

channels. The driving force for  $K^+$  release is the electrochemical potential of  $K^+$ , which is established by  $Na^+/K^+$  ATPase.  $Na^+/K^+$  ATPase pumps  $K^+$  in the cell and  $Na^+$  is pumped out. During the hydrolysis of one ATP,  $Na^+/K^+$  ATPase extrudes 3  $Na^+$  ions for the uptake of 2  $K^+$  ions, which produces a negative membrane potential. Therefore the enzyme also maintains a  $Na^+$  electrochemical potential for  $Na^+$  entry, which drives the  $Na^+/K^+/2Cl^-$  cotransporter for  $Cl^-$  uptake. The addition of ouabain blocked the  $Na^+/K^+$  ATPase, while the DS-induced fluid secretion significantly decreased to a plateau level. This decrease in salivary fluid secretion recovered with the removal of ouabain.

In the present experiment, the oxygen consumption due to the DS stimulation followed the time course of the fluid secretion. The highest level of the oxygen consumption due to the DS stimulation increased significantly, compared with that due to CCh. Importantly, ouabain suppressed the fluid secretion by 90%. These findings suggest that DS-induced fluid secretion is maintained by activation of  $Na^+/K^+$  ATPase and that the increased energy metabolism is mostly supplied for the DS-induced fluid secretion.

***IV-4. Inhibition of  $Na^+/K^+/2Cl^-$  cotransporter during the DS-stimulation.*** It has been widely accepted that the  $Na^+/K^+/2Cl^-$  cotransporter uptakes  $Cl^-$  from the basolateral side against the  $Cl^-$  electrochemical potential, making  $Cl^-$  the driving force for  $Cl^-$  release through the luminal  $Cl^-$  channel (TMEM16A). Bumetanide inhibits the activity of the  $Na^+/K^+/2Cl^-$  cotransporter. Bumetanide, at 100  $\mu\text{mol/L}$ , abolished the fluid secretion of the rat SMGs by ACh (1  $\mu\text{mol/L}$ ) during perfusion without bicarbonate (Murakami 1997, unpublished). However, bumetanide decreased the fluid secretion by 33% of the sustained fluid secretion during perfusion with bicarbonate (Murakami 1997,

unpublished). In both cases, the oxygen consumption remained at 70% of the control during stimulation (Murakami 1997, unpublished). These findings indicate that the  $\text{Na}^+/\text{H}^+$  antiporter was not inhibited by bumetanide, and that the  $\text{Cl}^-/\text{bicarbonate}$  antiporter can uptake  $\text{Cl}^-$  during perfusion with bicarbonate, but not without bicarbonate. In the present study, DS-induced salivary fluid secretion was decreased to 7% of the highest value by bumetanide (10  $\mu\text{mol/L}$ ) during bicarbonate-free perfusion. This indicated that the  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  cotransporter was almost fully activated during the DS-stimulation.

Ouabain inhibits the activation of  $\text{Na}^+/\text{K}^+$  ATPase. In rodents, including the rats, the susceptibility to ouabain is lower than that in other animals. Thus, a concentration of 1 mmol/L is not able to completely inhibit the fluid secretion, the oxygen consumption, or the  $\text{K}^+$  uptake during secretory stimulation (Murakami et al., 1990). Ouabain decreased the fluid secretion to 26% of control at 1 mmol/L of ouabain and 10% at 10 mmol/L during ACh stimulation at 1  $\mu\text{mol/L}$ . The difference in the inhibition of the fluid secretion by ouabain and bumetanide could be due to the incomplete inhibition by 1 mmol/L ouabain.

In the present experiment, we used bicarbonate-free perfusion. The intracellular  $\text{HCO}_3^-$  could be less than 1 mmol/L ICF and the  $\text{Cl}^-$  uptake by the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger may have a very minor contribution to the total  $\text{Cl}^-$  uptake from ECF. Thus, NKCC1 plays a major role for  $\text{Cl}^-$  uptake from ECF. The salivary fluid secretion in NKCC1 knockout mouse dropped over 60% even during perfusion with  $\text{HCO}_3^-$  (Evans et al., 2000). The oxygen consumption remained at 63% of the control during ouabain inhibition, and at 56% of the control during bumetanide inhibition. This similarity in the inhibition suggests that the remaining oxygen consumption could include the lesser

activity of the compensation of the  $\text{Na}^+/\text{H}^+$  exchanger. Further studies will be required to clarify the issue, including perfusion with bicarbonate and measurements of the cytosolic pH.

***IV-5. Can Danshen stimulate muscarinic or  $\alpha_1$  adrenergic receptors?*** This is the most important question, related to whether DS is a stimulator of the muscarinic or  $\alpha_1$ -adrenergic receptors, similar to the present therapeutics for xerostomia. The superior salivatory nucleus at the medulla oblongata sends the neuron to the SMG of the stimulation of the fluid secretion. The  $\text{M}_3$  muscarinic receptors play a main role in the initiation and maintenance of fluid secretion at the secretory end-piece cells (i.e. acinar cells). The accepted present terminology allows for acinar cells in rat SMGs, but not in human SMGs (secretory end-piece cells are allowed in both). The  $\alpha_1$ -adrenergic receptors also plays a role in the production of salivary fluid. In addition, substance P and VIP are also physiologically active substances related to salivary fluid secretion. Acetylcholine, noradrenaline and substance P bind with the  $\text{M}_3$  muscarinic receptors, the  $\alpha_1$ -adrenoreceptors and the substance P receptors, respectively, and produce inositol trisphosphate ( $\text{IP}_3$ ).  $\text{IP}_3$  binds with the  $\text{IP}_3$  receptors of the  $\text{Ca}^{2+}$  stores, which releases  $\text{Ca}^{2+}$  and increases cytosolic  $\text{Ca}^{2+}$  level. Then, the  $\text{Ca}^{2+}$  activates the  $\text{K}^+$  and  $\text{Cl}^-$  release from the cell and the water movement starts through the aquaporins. The possible contribution of the muscarinic and  $\alpha_1$ -adrenergic receptors was denied by the results of the present experiments using atropine or phentolamine, which are potent inhibitors for both receptors. These results suggest that DS does not bind with either the  $\text{M}_3$  muscarinic or the  $\alpha_1$ -adrenergic receptors. Therefore we consider that DS is a promising secretagogue which could avoid the systemic side effects induced by the recent

muscarinic drugs such as cevimeline, pilocarpine and so on.

**IV-6. Does Danshen stimulation require extracellular Ca?** Extracellular  $\text{Ca}^{2+}$  entry plays a role in the maintenance of salivary fluid secretion in CCh-induced salivary fluid secretion (Ambudkar, 2000). Cytosolic  $\text{Ca}^{2+}$  is the most important signal that activates both the luminal  $\text{Cl}^-$  and basolateral  $\text{K}^+$  channels, which thus evokes a salivary fluid secretion. The  $\text{Ca}^{2+}$  release from stores increases the cytosol  $\text{Ca}^{2+}$  level quickly, while it also quickly empties the cytosolic store, which sends a signal for  $\text{Ca}^{2+}$  entry from outside of the cell (Capasitative  $\text{Ca}^{2+}$  entry theory, Putney 1986). Thus the cytosolic  $\text{Ca}^{2+}$  level is maintained to support continuous fluid secretion.

Under calcium-free perfusion, DS stimulation did not induce any salivary fluid secretion, either with or without  $\text{Ca}^{2+}$  chelating agent. This result suggested that  $\text{Ca}^{2+}$  entry is essential for DS to induce salivary fluid secretion.  $\text{Ca}^{2+}$  entry probably provided a slow but continuous increase in cytosol calcium level, which provides support for DS to induce fluid secretion. The secretory characteristics, the slow increase and then the slow decrease, suggested that there is probably a change in  $[\text{Ca}^{2+}]_i$  during the time course of the DS-induced salivary fluid secretion.

**IV-7. Intracellular Ca.** In the classic model of salivary fluid secretion, intracellular  $\text{Ca}^{2+}$  acts as second messenger in the signal transduction and regulates the ion movements.  $\text{Ca}^{2+}$  entry existed during the DS-induced salivary fluid secretion. Therefore, we are keen to measure  $[\text{Ca}^{2+}]_i$  on dispersed salivary acinar cells. However, DS and SAB could absorb light below 400 nm.

***IV-8. Paracellular fluid transport during DS perfusion.*** Imai (1976) demonstrated the relationship between the fluid secretion rate and the hydrostatic pressure using *in vivo* SMGs of the dog under electrical stimulation on chorda tympani (the parasympathetic nerve of SMG). These results indicated a linear relationship between the fluid secretion rate and the retrogradely applied hydrostatic pressure, giving a constant hydraulic permeability across the epithelia from the slope. This includes both transcellular and paracellular fluid secretion according to their different secretory route. Murakami and his group have been keen to investigate this issue using isolated perfused salivary glands of rat (Murakami et al., 2001; Segawa et al., 2003). Because the paracellular fluid secretion was estimated as 60% of the whole fluid secretion during sustained stimulation with CCh (Segawa et al., 2003), the DS-induced salivary fluid secretion was also examined in the present study.

***IV-8-1. Increase in the area of the capillary bed due to DS.*** It is well known that the capillary circulation increases in gland during secretory stimulation. This is the case also in the SMG (Murakami et al., 1980). This is caused by kallikrein, which is released by the ductal cell upon parasympathetic stimulation (Beilenson et al., 1968; Hojima et al., 1977). Kallikrein converts kininogen to bradykinin, a potent endothelium-dependent vasodilator that opens the precapillary sphincter, leading the blood to the capillary net. This is measured as an increase in circulation in the *in vivo* gland, and as a decrease in A-V pressure difference of the isolated and perfused gland at a constant rate. In the present study the A-V pressure decreased immediately upon DS stimulation. This quick response is similar to that seen with CCh in the perfused SMGs, suggesting a similar mechanism by kallikrein. In the perfused gland at a constant rate, the decrease in A-V pressure means an increase in the circulation surrounding the acinar cells, and the local

hydrostatic pressure surrounding the cell could be increased.

The decrement in the A-V pressure difference was sustained at a plateau level during the DS stimulation. This observation suggested that the promoted microcirculation probably did not contribute to the slow increase of the DS-induced fluid secretion. In general, promotion of the microcirculation might increase the nutrient supplies, oxygen supplies, and eliminate waste products of the salivary gland. These would help to provide a better environment for cell function. The increased blood volume in the nearby capillary bed will also provide more plasma and tissue fluid for production of saliva. These factors could probably be among the reasons DS promotes salivary fluid secretion.

It was reported that DS clinically relieved xerostomia and xerophthalmia (Jiang et al., 2005; Luo et al., 2011). Medication for hypertension caused xerostomia and xerophthalmia in 16.99 % patients suffering from dry mouth (Kumar et al. 2012). DS could decrease the risk of hypotension. DS could prevent atherosclerosis by inhibiting the proliferation and cell migration of vascular smooth muscle cells. DS also enhances the regression of atherosclerosis. DS could decrease the blood viscosity. In addition, another study (Dong, 2001) also showed the relationship between blood circulation disorders and Sjögren's syndrome, and many of the dry mouth suffers had high blood viscosity, which could be reduced by DS (Tang, 2004).

In traditional Chinese medicine, salivary fluid secretion plays an important role in the body fluid metabolism. The role of fluid secretion is a mirror image with that of blood. When the blood flow is disordered, the distribution of body fluid will also be changed. Traditional Chinese doctors consider that xerostomia is closely related to blood stasis (Zhong et al., 2009; Gu et al., 2002). Traditional Chinese medical theory

assumes that either the lack of body fluid, or blood stasis, which obstructs the body fluid transmission channel, leads to the dry mouth. When traditional Chinese doctors treated xerostomia, they always used blood-activating agents and *yin*-nourishing agents at the same time (Wang, 2008; Cao, 2009). Also in TCM, DS is a representative blood-activating agent and it is commonly used for the gynecological disorders in old women, because in the TCM clinics, those disorders are usually thought to be related to blood stasis. Hypofunction of salivary gland is also recognized now in relation to blood stasis. Despite different explanation between TCM and western medicine, to have a common pathogenesis, and DS is probably one of the best drugs to treat it.

***IV-8-2. Increase in the paracellular transport was measured by LY secretion.*** In paracellular fluid secretion, water moves from the interstitial space to the lumen through the tight junction (TJ). The TJ of the salivary acinus is not closed, even without stimulation (Murakami, unpublished). There are at least two paracellular routes, a smaller route which allows for the passage of the molecules with a radius less than 5 Å, and a larger route that allows for the passage of macromolecules. Thus, a water molecule can move freely through the TJ (Murakami et al., 2001). LY is a fluorescent dye with a large molecular weight of 521, so LY can not enter the cell. LY was carried by water transported through the larger route. Therefore, the LY secretion could reflect the fluid secretion through the larger paracellular route.

CCh stimulation allows for a paracellular fluid component that composes 65% of the whole fluid secretion (Segawa et al., 2003). Assuming that we could estimate the paracellular fluid secretion from LY secretion, because the highest LY secretion was 60% of the control LY secretion induced by CCh, the paracellular fluid secretion could be estimated as 40% of the fluid secretion induced by CCh. However, DS increased the

fluid secretion to 250% of the fluid secretion induced by CCh, and as a result, the paracellular component due to DS should decrease to 16% of the DS-induced fluid secretion. This estimation suggests that the transcellular fluid secretion was increased to 84% of the whole secretion. In comparison, DS could activate the mechanism for transcellular secretion more dominantly than that for the paracellular secretion.

## **V. Further studies.**

There are many possible mechanisms that may induce salivary fluid secretion, such as aquaporin (AQP5 in the salivary gland, Murakami et al., 2006). However, no report has been published on the relationship between fluid secretion and the incidence of AQP5. Ma reported that the salivary fluid secretion decreased in the AQP5 KO mice (Ma et al., 1999). Other studies have confirmed that salivary secretion is also affected by the regulation of other neurotransmitter receptors, such as the vasoactive intestinal polypeptide receptor (VIPR) (Ulrich et al., 1998), the tachykinin receptor (Sugiyama et al., 1998), the purine receptor (Gallacher, 1982), the vanilloid receptor (VR) (Dunér-Engström et al., 1986), and the cannabinoid receptor (CB) (Busch et al., 2004). The issue of whether or not these newly discovered receptors are involved in the role of DS-induced salivary fluid secretion requires further experiments. Further studies are also necessary to elucidate the mechanisms involved, including screening of the membrane calcium channels such as a TRPC1 and Orail for store-operated  $Ca^{2+}$  entry (SOCE, Putney, 1990; Beech et al., 2003; Liu et al., 2000 and 2007). TRPC4 and TRPV6 are also considered to be candidates for the molecular components of SOCE channels (Freichel et al., 2001; Schindl et al., 2002).

In addition, we can use the experimental plan employed in this study in order to

assess the capability for fluid secretion of newly discovered drugs and classical Chinese herbs, in the future.

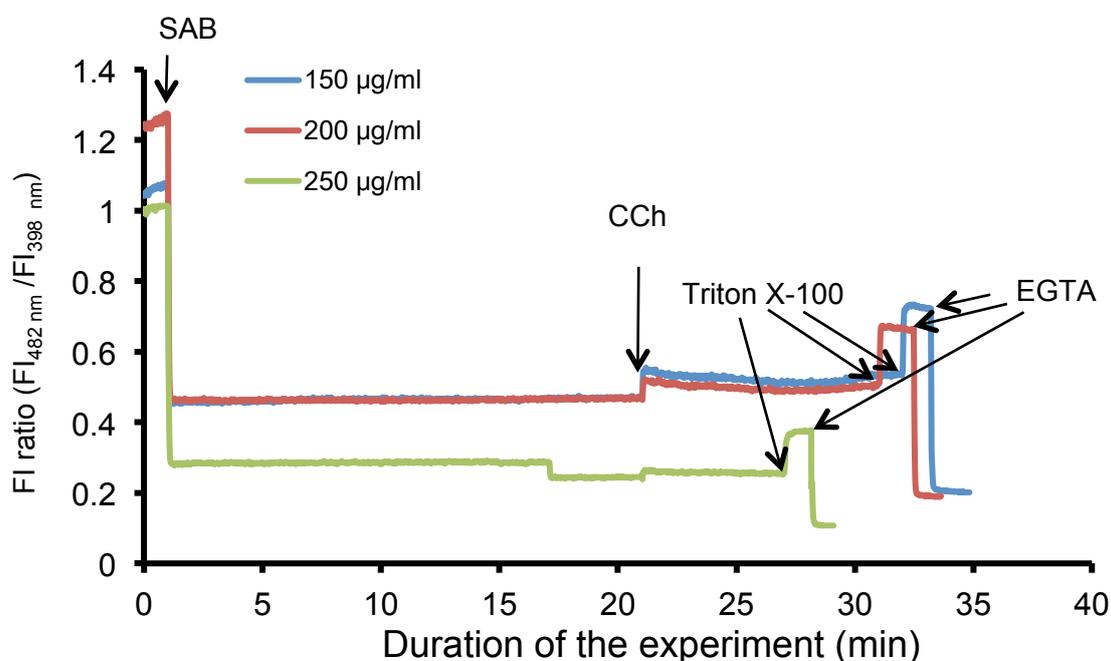
## **VI. Conclusions.**

- 1) DS induced salivary fluid secretion on isolated and perfused SMG of rat.
- 2) DS does not activate  $M_3$  or  $\alpha_1$  receptors.
- 3) DS uses  $Na^+/K^+$  ATPase and NKCC1 activation to maintain  $Cl^-$  release and  $K^+$  release for fluid secretion.
- 4)  $Ca^{2+}$  entry and the cytosolic  $Ca^{2+}$  metabolism are essential to activate those channels for DS-induced salivary fluid secretion.
- 5) The slow increase and decline of fluid secretion and oxygen consumption suggest that DS uses a  $Ca^{2+}$  signaling pathway distinct from  $IP_3$  signaling.
- 6) DS increases the microcirculation of the salivary gland.
- 7) The transcellular fluid secretion was estimated as dominant in the whole fluid secretion.
- 8) The present findings support the fundamentals of DS application to provide relief for salivary gland hypofunction (SGH), including xerostomia.

***Appendix I. Unsuccessful Measurement of intracellular  $Ca^{2+}$  by double wavelength fluorometry.*** The dark color of DS granules masked the fluorescent excitation and the emission signals for  $Ca^{2+}$  measurement. Thus, we used a main component of DS, Salvianolic acid B (SAB), which was purchased from Shaanxi Sciphar Biotechnology Co., Ltd., China (DS111217).

***A1-1. Indo-1 AM.*** A double wavelength fluorospectrophotometer (CAF-110, JASCO,

Tokyo, Japan) was used to measure the  $\text{Ca}^{2+}$  of the isolated SMG cells. SAB was added to the SMG cell suspension separately at concentrations of 150, 200, and 250  $\mu\text{g}/\text{mL}$ . Indo-1-AM was used in order to reduce the autofluorescence from the SAB. The intracellular  $\text{Ca}^{2+}$  level was measured from the ratio of the emitted fluorescent intensities (FI ratio, 405 nm (bound) and 500 nm (free) against 330 nm excitation). At different doses, the SAB was added at 1 min, and CCh (0.2  $\mu\text{mol}/\text{L}$ ) was added at 21 min. Then triton X-100 (Sigma, MO, USA) and ethylene glycol bis (2-aminoethyl) tetra acetic acid (EGTA, Sigma, MO, USA) were added, respectively, as shown in Fig. A-1.



**Figure A-1. The ratio of fluorescent intensities of Indo-1 in the isolated cells of the rat SMG during application of SAB, CCh, Triton-X and EGTA.** The intracellular  $\text{Ca}^{2+}$  level was measured as the ratio of the emitted fluorescent intensities for 20 min after SAB administration. Different doses of SAB were added at 1 min, and CCh (0.15  $\mu\text{mol}/\text{L}$ ) was added at 21 min. Then triton X-100 and EGTA were added, respectively.

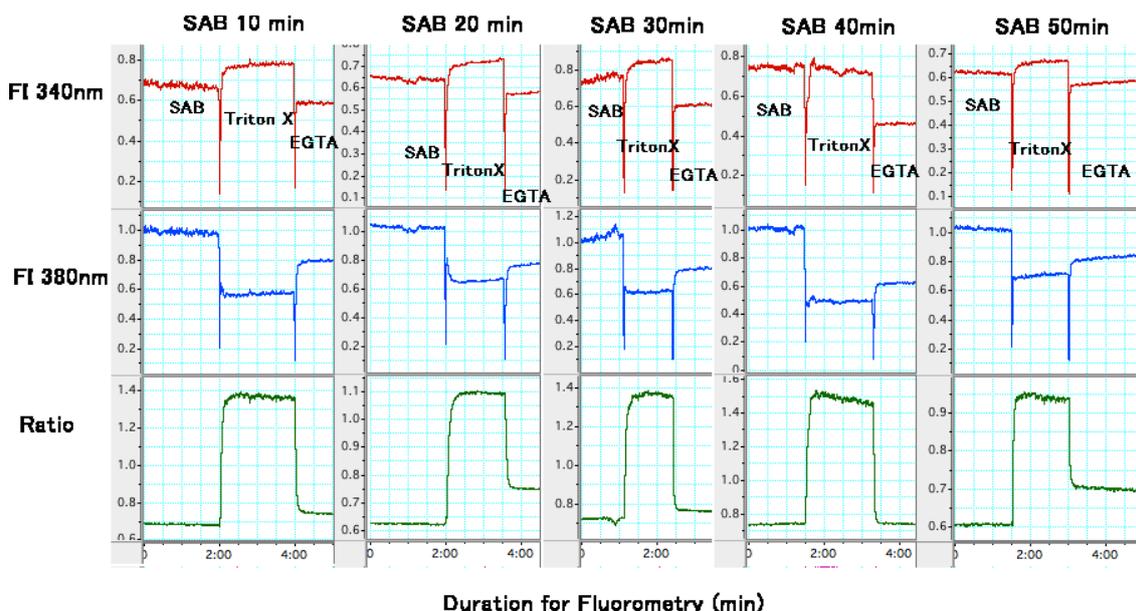
Upon the addition of SAB at 1 min from the start of the  $\text{Ca}^{2+}$  measurements, the FI

ratio decreased quickly and remained at that low level for 20 min, suggesting that the FI was decreased by the dark color of the SAB itself. The FI ratios were 0.46, 0.49, and 0.27 for 150, 200, and 250  $\mu\text{g/mL}$ , respectively. At 21 min, 0.15  $\mu\text{mol/L}$  of CCh was added, and the FI ratios showed transient small increases as 0.5, 0.52, and 0.26 (from 0.25) for 150, 200, and 250  $\mu\text{g/ml}$ , respectively. The administration of triton X- 100 broke the cell membrane and allowed for massive  $\text{Ca}^{2+}$  entry to bind with Indo-1. Then the FI ratio must have immediately reach to the highest value. However, in the present experiment, triton X- 100 lead to a small increase in the FI ratio, but the ratio was still much lower than the level at the start of the experiment. This finding suggested that the reduction of the FI ratio was due to SAB administration. Administration of EGTA was conducted to chelate all of the  $\text{Ca}^{2+}$  in the system, and following that, the fluorescence intensity ratio decreased to the minimum. The FI ratio did not changed during the addition of the SAB, thus there was no sign of  $[\text{Ca}^{2+}]_i$  changes.

**A1-2. CaTM-2 AM.** The newly developed  $\text{Ca}^{2+}$  indicator, CaTM-2<sup>TM</sup> AM (Goryo Chemicals, Tokyo, Japan), was used. Because the maximum fluorescent emission is at 609 nm with excitation at 597 nm and the range for measurement was shifted to “red”, called as a red fluorescent  $\text{Ca}^{2+}$  indicator), we thought that this indicator minimize the masking effect by the SAB. The CaTM-2-AM was applied to the cell suspension and intracellular accumulation of the dye was observed by the confocal microscope. However, even the administration of CCh to the cells did not change the cell size and their color. This observation suggests that the cells were damaged already by the administration of the CaTM-2.

**A1-3. Fura-2 AM.** In order to reduce the masking effects induced by the SAB, before the measurement we removed as much of the SAB as possible. We used Fura-2 AM for

the  $\text{Ca}^{2+}$  measurements. The fluorescence of Fura-2-loaded cells was measured with a CAF-110 spectrofluorometer (Nihon Bunko, Japan) with excitation at 340 nm and 380 nm and emission at 500 nm. The data were recorded in computer with LabChart software (AD Instrument, NSW, Australia).

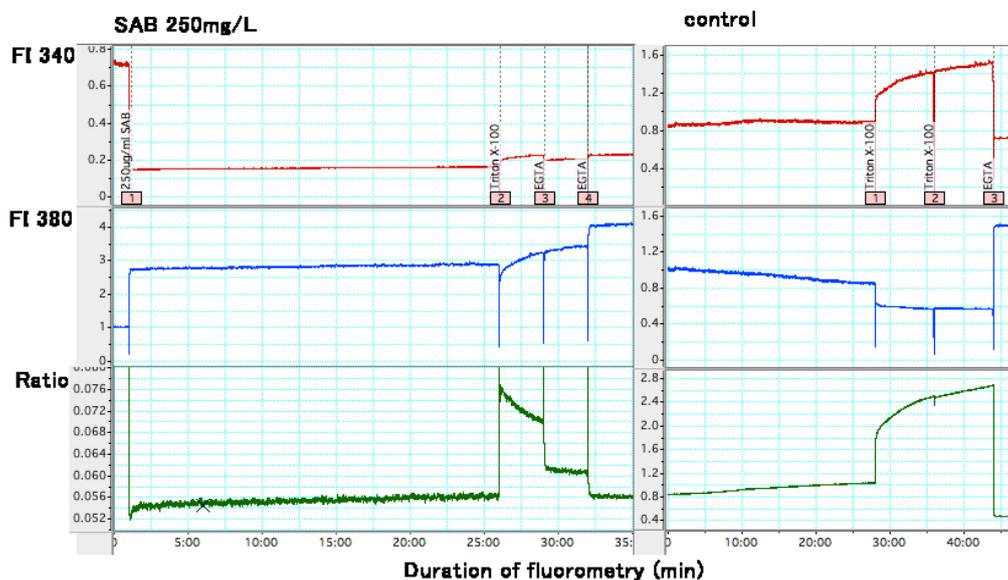


**Figure A1-2.** The ratio of fluorescent intensities of Fura-2 in the isolated cells of the rat SMG during the application of SAB. The sample was removed from the suspension pool containing the SAB, the incubation medium was replaced with the control medium, and its  $\text{Ca}^{2+}$  signal (initial trace) was measured by a fluorometer. Thereafter Triton-X 100 was added to increase  $[\text{Ca}^{2+}]_i$  and the EGTA was overloaded for removal of  $\text{Ca}^{2+}$ .

The addition of 100  $\mu\text{mol/L}$  CCh increased the fluorescence excited at 340 nm ( $\text{FI}_{340}$ ) and decreased the  $\text{FI}_{380}$ , thus the ratio ( $\text{FI}_{340} / \text{FI}_{380}$ ) increased, to show an increase in  $[\text{Ca}^{2+}]$  (not shown). At the end of the fluorometry, Triton-X 100 was added to induce  $\text{Ca}^{2+}$  entry from the medium and the  $\text{FI}_{340}$  was elevated highly. Thereafter, the addition of EGTA removed the free  $\text{Ca}^{2+}$  and lead to a decrease in the  $\text{FI}_{340}$  and an increase in the  $\text{FI}_{380}$ . Finally, the ratio decreased to show an increase in  $[\text{Ca}^{2+}]_i$ . During

the stimulation with SAB, the medium was removed and replaced. As shown in Fig A1-2, the ratio was slightly increased by SAB. However, the FI<sub>380</sub> did not increase to a higher level than that during SAB stimulation by EGTA, suggesting that the SAB still remained in the measurement system. It was considered that this was due to the entry of the SAB in the cell.

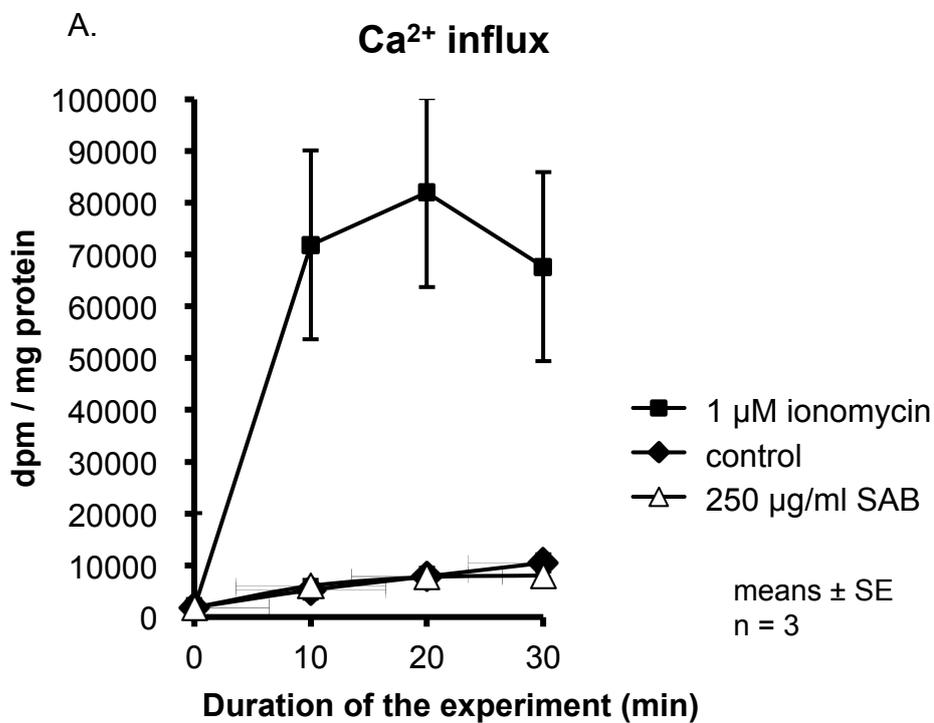
The continuous measurements of the dispersed cells are performed by Fura-2 AM, as shown in Fig. A1-3. The ratio increased gradually for 30 min from the start of the SAB stimulation. However, the ratio also gradually increased for 30 min even without stimulation. This finding suggests the gradual release of Fura-2 during the long incubation. As a result, we gave up on the usage of fluorometry of the cell suspension to measure  $[Ca^{2+}]_i$ .

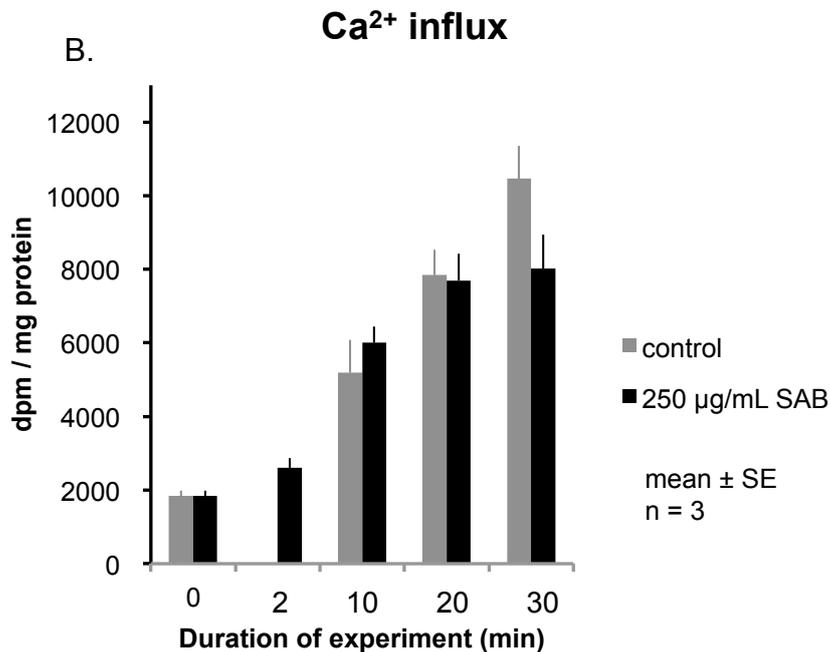


**Figure A1-3. The ratio of fluorescent intensities of Fura-2 in the isolated cells of the rat SMG during application of SAB.**

*Appendix II. Measurement of  $^{45}Ca^{2+}$  uptake.* The  $^{45}Ca$  uptake by the SMG cells was

measured by following the protocol by Yang et al (2006). Along with the addition of SAB, the  $\text{Ca}^{2+}$  influx slowly increased along the time course of the experiment, but not significant change from the control. In comparison with ionomycin, the SAB induced a slower  $\text{Ca}^{2+}$  influx and the maximum influx was lower ( $P < 0.05$ ). The time course of the  $\text{Ca}^{2+}$  influx (Figure A2-B) was similar to that of fluid secretion induced by the DS stimulation as shown in Figure 1-1.





**Figure 5.** <sup>45</sup>Ca influx of isolated rat SMG cells. A. With SAB (250 µg/mL, white triangle), ionomycin (1 µmol/L, filled square) and control (filled orthogonal). B. Comparison in detail between control (gray column) and SAB (filled column). At 30 min the <sup>45</sup>Ca uptake decreased significantly by SAB.

The details in difference between the SAB-induced <sup>45</sup>Ca uptake and the control were shown in Figure A2-B. SAB tended to increase more than the control at 2-10 min. Subsequently the SAB decreased <sup>45</sup>Ca uptake in another 10 min, thereafter the uptake remained at the similar rate. Although the number of experiments required more, the Ca<sup>2+</sup> uptake increased and its level was maintained at the significantly lower level than ionomycin-induced Ca<sup>2+</sup> uptake. Concerning the relation to the latency, the shorter interval of sampling is required.

The present finding suggests that the Ca<sup>2+</sup> influx slowly increased upon SAB stimulation and reached the maximum around 20 min from the start of the SAB stimulation, thereafter the uptake rate remained at 30 min. These results support the

assumption that the increase in the  $\text{Ca}^{2+}$  influx elevates the  $[\text{Ca}^{2+}]_i$ , which appears to cause activations of  $\text{Ca}^{2+}$  activated channels for  $\text{K}^+$  and  $\text{Cl}^-$ , enabling fluid secretion due to the DS stimulation.

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