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Cholinergic and Neurokinergic Agonist-induced Ca^{2+} Responses in Rat von Ebner's Gland Acinar Cells

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Von Ebner's glands (vEG) are minor salivary glands associated with circumvallate and foliate papilla. The secretions of vEG consist of microenvironment of the taste buds in the circumvallate and foliate papillae, and thus saliva from vEG plays a role in the perception of taste. The Ca^{2+} signaling system in rat vEG acinar cell was examined using the Ca^{2+}-sensitive fluorescent indicator Fura-2. Agonist-induced increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) was stimulated by carbachol (CCh) and substance P (SP), but not by norepinephrine (NE), and recovered to control levels by their receptor antagonists dose-dependently. The effects were also observed in Ca^{2+}-free medium, suggesting mobilization from intracellular Ca^{2+} store. These results in the vEG acinar cell indicate that 1) $[Ca^{2+}]_i$ is at least regulated by muscarinic and neurokininergic (NK1) receptors; 2) the increases in $[Ca^{2+}]_i$ activated by CCh and SP are mainly mediated by discharge of cytosolic calcium pool.

Keywords: Carbachol, Norepinephrine, Substance P, von Ebner's glands, Calcium, Fura-2.

Introduction

Von Ebner's glands (vEG) are minor exocrine salivary glands that are embedded in muscle tissues beneath and anterior to the circumvallate papilla and medial to the foliate furrows in the posterior and pharyngeal portion of the tongue (Hand, 1970; Riva *et al*., 1985). The secretions of vEG bathe the taste buds that are found in the circumvallate and foliate papillae, and thus saliva from vEG plays a role in the perception of taste (Spielman, 1990). They contain serous acinar cells that are morphologically similar to those of the parotid gland and exocrine pancreas (Hand, 1970; Dixon, 1979; Leslie and Putney, 1983; Riva *et al*., 1985). These serous glands of the tongue are the sole source of an important digestive enzyme, lingual lipase (Hamosh and Scow, 1973) as well as amylase (Hamosh and Hand, 1978; Field *et al*., 1986; Field and Hand, 1987; Field *et al*., 1989).

The adrenergic and cholinergic regulation of protein secretion from vEG is particularly interesting because it is much more like that of the pancreas than those of the major salivary glands. Protein secretion from the parotid gland is mediated mainly by the β-adrenergic receptor and fluid secretion is mediated by the cholinergic receptor (Baum, 1987). However, lingual lipase and amylase are secreted from vEG mainly in response to cholinergic stimulation (Field and Hand, 1987). To a lesser extent protein secretion from vEG is evoked by β-adrenergic stimulation but not by α-adrenergic stimulation (Field and Hand, 1987). Histamine also stimulates protein secretion from vEG (Field and Chirtel, 1992). Substance P (SP), an undecapeptide, stimulates salivary flow from the rat parotid (Liang and Casccieri, 1979; Yu *et al*., 1983) and submandibular glands (SMG, Yu *et al*., 1983). SP also evokes amylase release from isolated parotid cells (Liang and Casccieri, 1979), parotid slices (Poat *et al*., 1987; Rudich and Butcher, 1976), guinea pig pancreas (Sjodin *et al*., 1980), and *in vivo* from rat parotid

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gland (Yu *et al.*, 1983). In addition, SP stimulates phosphoinositol turnover in the parotid (Hanley *et al.*, 1980; Rollandy *et al.*, 1989), submandibular (Hanley *et al.*, 1980; Martinez and Martinez, 1981), and sublingual glands (Hanley *et al.*, 1980). Thus there are considerable data on the effects of SP on the pancreas and major salivary glands.

Ca^{2+} acts as a universal intracellular messenger to modulate many processes such as neurotransmission, enzyme and hormone secretion as well as many biological processes (Berridge *et al.*, 1998). Accordingly, measurement of intracellular Ca^{2+} level would be useful to detect the condition of a cell. To study the dynamics of Ca^{2+} in the cytosol, optical techniques in combination with sensitive fluorescent dyes have become the most frequently employed method (Takahashi *et al.*, 1999). It is well known in major salivary acinar cells that stimulation of muscarinic, α-adrenergic, or SP receptors induces an increase the $[Ca^{2+}]_i$ in the intracellular free Ca^{2+} concentration (Merritt and Rink, 1987; Seo *et al.*, 1999). All of these receptors are coupled via G proteins to phospholipase C (PLC) and hence promote to production of D-*myo*-inositol 1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 in turn induces an increase in $[Ca^{2+}]_i$ by mobilization of Ca^{2+} from internal (or external) stores. However, it is doubt that a similar pathway may exist in vEG acinar cells.

The present study was undertaken to determine whether acetylcholine (ACh), norepinephrine (NE), and SP is a modulator of intracellular calcium levels in rat vEG acinar cells. Agonists and antagonists of muscarinic, adrenergic, and NK1 receptors were evaluated for their effects on intracellular calcium levels.

Materials and Methods

Tissue Preparation

All surgical and experimental procedures used in this study were approved by the animal use and care committee of College of Dentistry, Kangnung National University. VEG acini were isolated from male Spraque-Dawley rats by our previous method (Kim, 2000). In brief, rats were anesthetized with CO_2 gas, and vEG were isolated and dissected in an ice-chilled HEPES buffered tyrode solution (in mM, 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 5 glucose, 5 pyruvate, adjusted pH 7.3 with NaOH) equilibrated with 100% O_2 from the tongues with the aid of microtome and dissecting microscope. Care was taken not to include any mucous glands that are located posterior and lateral to vEG.. The glands were then minced and digested serially with trypsin (0.4 mg/ml) for 20 min, and then with collagenase (type IV from Sigma, USA; 60 U/ml) for 30 min in a HEPES-buffered physiological saline. During digestion, the cells were mechanically dissociated by gentle pipetting. The cell clusters and small acini containing 3-5 cells were obtained by this procedure.

Fura-2 loading and $[Ca^{2+}]_i$ mesurements

Acinar cells were loaded with fura-2 by incubation with fura-2 AM in the HEPES-buffered solution for 30 min at room temperature under 100% O_2. They were washed three times, further incubated for 20 min at room temperature in a shaker at 60 cycles / min, and then resuspended in a HEPES buffered tyrode solution. Small portions of this cell suspension were transferred to a chamber (volume ~ 0.3 ml) precoated with Cel-Tek (BD Bioscience, USA) on the stage of a Nikon inverted microscope for $[Ca^{2+}]_i$ measurements. Once the cells were adhered to the coverslip that formed the base of the experimental chamber, they were continuously superfused with HEPES buffered tyrode solution at a flow rate of 2 ml/min. In Ca^{2+}-free solutions, $CaCl_2$ was omitted and 1 mM EGTA was added. Optical measurements were carried out at room temperature throughout the experiments. Using Metafluor (version 5.0, Universal Imaging Corp., USA) and a computer-controlled DG-4 shutter system (Sutter Instruments, USA), images were taken at two wavelengths (340 and 380 nm), which were generated from a 75-W Xenon lamp. Emitted fluorescent beams were transmitted through the objective, the dichroic mirror and emission filters and collected by a cooled charge-coupled device (CCD) camera (HQ, Roper, Japan) mounted on an inverted Nikon microscope (Nikon TE-2000, Japan). The routine exposure time was 200 ms and the acquisition frequency was one image per 10 or 15 s to minimize dye photobleaching. Storage on a high capacity hard driver permitted further off-line quantitative analysis. The fluorescence ratio of 340 nm over 380 nm was used as an indicator of changes in intracellular calcium concentration.

Drug application

Drugs were applied in known concentrations in the superfusing solution by switching from the control solution to one which differed only in its content of the drug. All chemicals were purchased from Sigma (USA), except for fura 2-AM, which was obtained from Molecular Probes (USA) and was made up as a 1 mM stock solution in DMSO. Data are presented as means ± S.E.M. Statistical analysis was carried out using unpaired Student's *t*-test.

Results

To elucidate which type of receptors mediate $[Ca^{2+}]_i$ mobilization in the vEG acinar cells, muscarinic, α-adrenergic and neurokininergic (NK) receptors, the major stimulatory receptor systems in exocrine system, were tested. Carbachol (CCh), NE and SP were used for agonists of muscarinic, α-adrenergic and NK receptors, respectively. CCh and SP increased $[Ca^{2+}]i$ (Fig. 1). However, the NE-induced increase was not detected (Fig.1 C). These results suggest that the intracellular Ca^{2+} response is likely mediated by cholinergic and NK receptors.

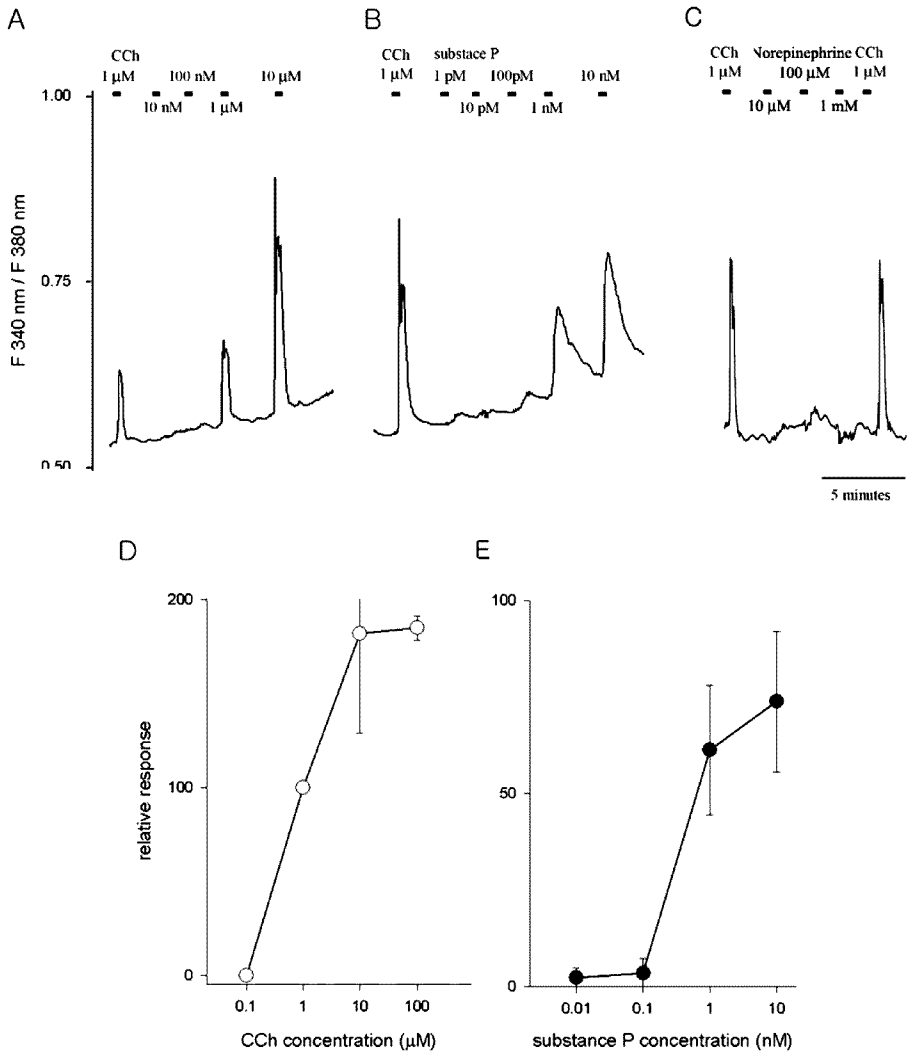

Fig. 1. The concentration-response relationship of the $[Ca^{2+}]_i$ increase activated by carbachol (CCh), substance P (SP) or norepinephrine(NA). Von Ebner's acinar cells were loaded with Fura-2 AM in normal tyrode solution at room temperature. Cells were exposed to CCh at doses of 0.01-100 μM (A and D), SP at doses of 0.001-10 nM (B and E) or NA at doses of 10 to 1000 μM(C) for 1 min. The increase in $[Ca^{2+}]_i$ was plotted. Values are means±SEM of at least five separate experiments. Relative response in D and E means per cent of response elicited by 1 μM CCh.

To determine the optimal dose of CCh, experiments were conducted in which dissociated vEG acinar cells were incubated with 0.01 (n=5), 0.1 (n=5), 1.0 (n=5) and 10 (n=5) μM CCh. Fig. 1C shows the dose-response curve. Very little or no $[Ca^{2+}]_i$ elevation was evoked by 0.1 μM, somewhat more by 1.0 μM, and the maximal elevation by 10 μM CCh. The percentage elevation of $[Ca^{2+}]_i$ evoked by 100 μM was almost the same as by 10 μM (Fig. 1C).

Using concentrations of 0.001 (n=5), 0.01 (n=5), 0.1 (n=5) and 1 (n=5), 10 (n=5) nM SP, the optimal dose for SP was also determined to be 1.0 nM (Fig. 1D). Statistical analyses were not performed on these data.

The effect of a muscarinic receptor antagonist, atropine, was used to determine if stimulatory effect of CCh return to control levels. Fig. 2 shows the Ca^{2+} mobilizations elicited by CCh during incubation of vEG acinar cells with atropine. Statistical analyses demonstrated a significant inhibitory effect of atropine on the CCh-evoked Ca^{2+} elevations. Atropine had no significant effect on basal Ca^{2+} activities, when incubated alone (data not shown).

L-732,138 is an inhibitor of NK1 receptors that is not a peptide or an analog of SP. Fig. 3 shows the results of five experiments in which 100 nM L-732,138 was preincubated for (2 min) with dissociated acinar cell and was then incubated with 1 nM SP and L-732,138 for 30 sec. When combined with SP, a trend was seen towards inhibition of SP-evoked elevation of $[Ca^{2+}]_i$. L-732,138 had no significant effect on $[Ca^{2+}]_i$ when incubated with the acinar cell alone at this concentration. The percentage reductions in SP-induced elevation of $[Ca^{2+}]_i$ were 27.5±8.0, 65.9±9.4, and 92.0±2.3 at doses of 10, 100, and 1,000 nM, respectively.

To rule out the possibility that the extracellular Ca^{2+}

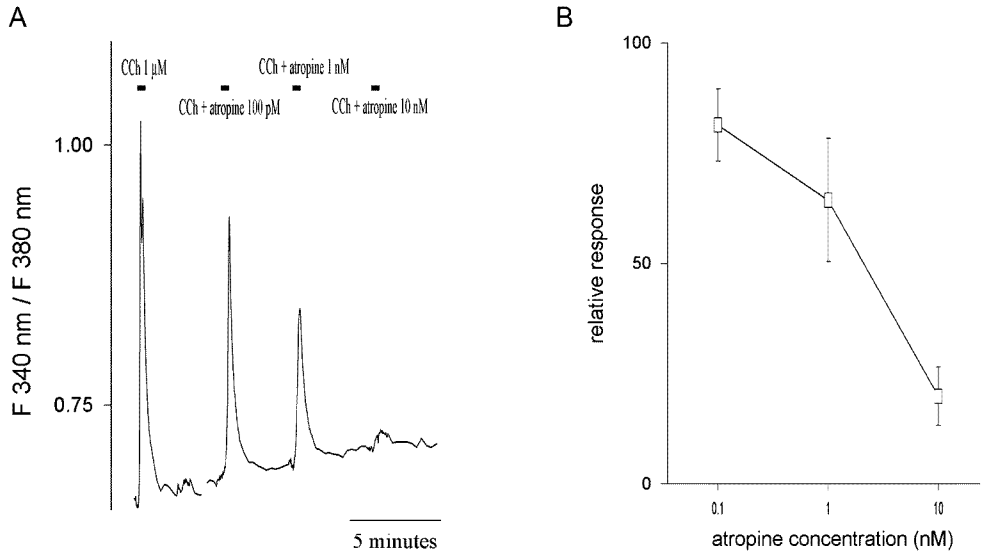

Fig. 2. The effect of a cholinergic receptor antagonist, atropine, on CCh-induced Ca^{2+} elevation. Dissociated von Ebner's acinar cells were superfused with CCh (1.0 μM) alone or preincubated with atropine (0.1-10 nM) for 5 min and then superfused with CCh combined with atropine for 2 min. Data are the mean±S.E.M. of of at least five separate experiments. . Relative response in C and D means per cent of response elicited by 1 μM CCh.

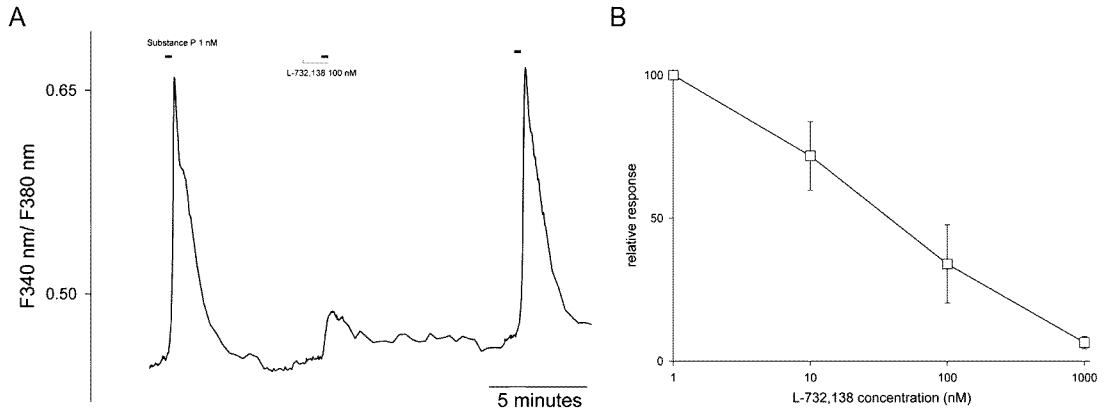

Fig. 3. The effect of a neurokinergic receptor antagonist, L-732,138, on SP-induced Ca^{2+} elevation. Dissociated von Ebner's acinar cells were stimulated with SP (1.0 nM) alone or preincubated with L-732,138 (1-1000 nM) for 5 min and then incubated with SP combined with L-732,138 for given times. Data are the mean±S.E.M. of of at least five separate experiments. . Relative response in B means per cent of response elicited by 1 nM SP.

(1 mM) in this medium may partially contribute to the $[Ca^{2+}]_i$ increases in response to CCh and SP, the same measurements were conducted in Ca^{2+}-free medium containing 1 mM EGTA. As shown in Fig. 4, EGTA-containing medium had little effects for the CCh or SP-induced $[Ca^{2+}]_i$ increase. These results suggest that the $[Ca^{2+}]_i$ increases in response to CCh and SP are due to Ca^{2+} release from intracellular Ca^{2+} stores, and are not derived from Ca^{2+} influx.

Finally, to test whether Ca^{2+} elevation activated by CCh and SP is through the same entry pathway, we measured Ca^{2+} elevation in the cells stimulated with CCh, SP or combinations of CCh and SP. As shown in Fig. 5, combining these stimuli did not alter the Ca^{2+} changes.

Discussion

The Ca^{2+} imaging results of the present study clearly show that regulation of Ca^{2+} mobilization occurs primarily through muscarinic and NK (but not adrenergic) receptors in vEG acinar cells. Stimulation of muscarinic and NK receptors elicited a rapid intracellular Ca^{2+} increase which did not need an influx of extracellular calcium.

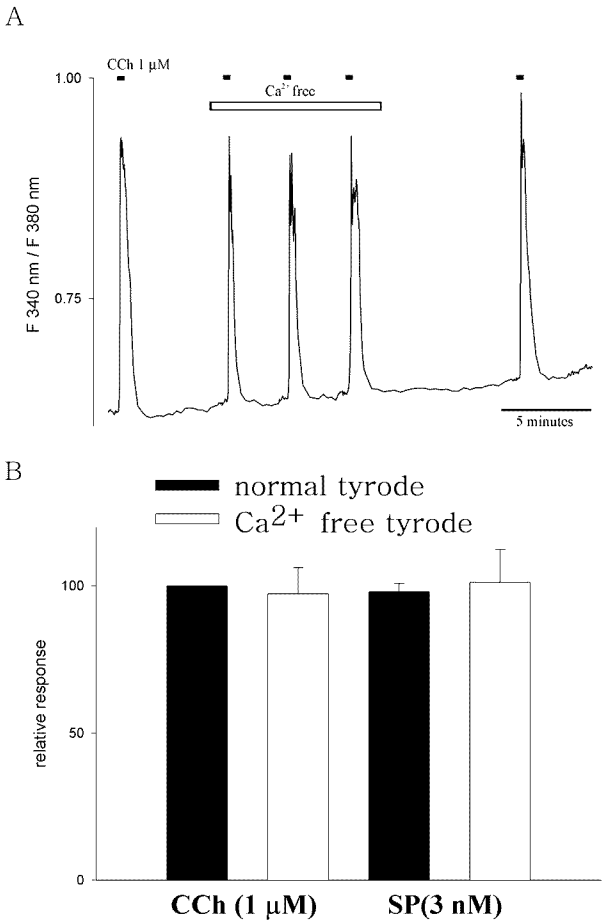

Fig. 4. CCh or SP-induced Ca^{2+} elevation is not influenced by removal of extracellular Ca^{2+} in the von Ebner's acinar cells. **A.** No change in CCh-induced Ca^{2+} elevation in 0 $[Ca^{2+}]_o$. **B.** Summary of relative response produced by 0 $[Ca^{2+}]_o$. Relative response in B means per cent of response elicited by 1 μM CCh.

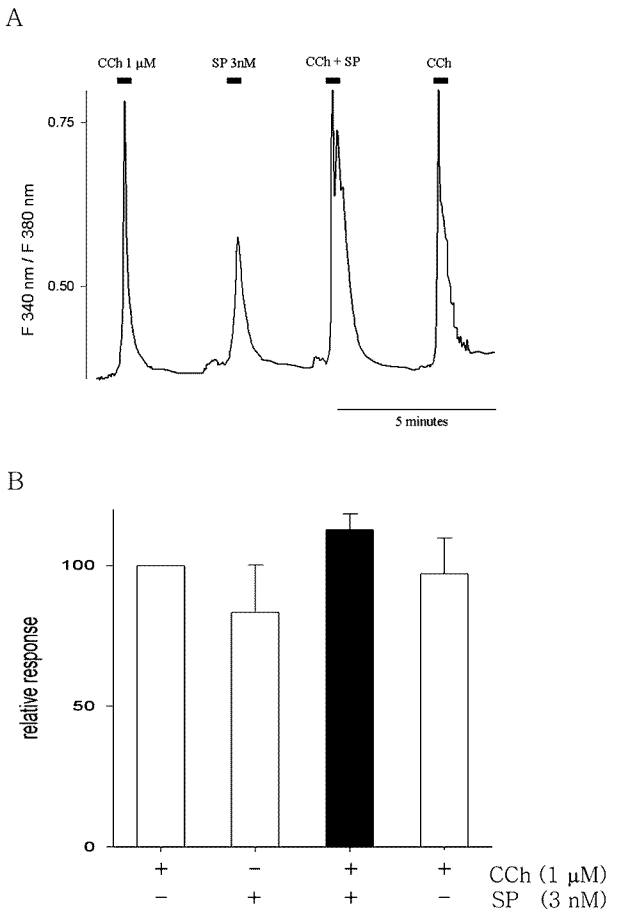

Fig. 5. Effect of CCh (1 μM), SP (3 nM) and CCh plus SP on Ca^{2+} mobilization in the von Ebner's acinar cells. **A.** Representative traces. **B.** Summary. Combining these two stimuli did not alter the Ca^{2+} changes, suggesting that the discrete pathway mediates the Ca^{2+} elevations. Relative response in B means per cent of response elicited by 1 μM CCh.

The muscarinic receptor is one of the major receptor types in the regulation of fluid and electrolyte secretion in salivary acinar cells. The CCh-induced increase in $[Ca^{2+}]_i$ was observed dose-dependent manner and was blocked by its antagonist atropine dose-dependently. This result is consistent with several reports that determined the Ca^{2+} responses of acinar cells by muscarinic receptor stimulation in murine submandibular glands (Dinudom *et al.*, 1993; Xu *et al.*, 1996).

Receptors for SP have also been reported to implicate in the secretory responses of the submandibular (Fleming *et al.*, 1984; Hanley *et al.*, 1980; Yu *et al.*, 1983), parotid (Gallacher, 1983; Hanley *et al.*, 1980; Merritt and Rink, 1987; Yu *et al.*, 1983), and sublingual glands (Hanley *et al.*, 1980), and of the exocrine pancreas (Jensen *et al.*, 1984; Song *et al.*, 1988). In vEG, recent histochemical studies revealed degranulation of acinar cells in response to SP (Ueba and Uchihashi, 1991) and the presence of SP immunoreactivity in selective cells (Roberts *et al.*, 1991) and neuronal elements (Oomori *et al.*, 1995). Our experimental results showed clearly that SP strongly stimulated Ca^{2+} mobilization in acinar cells dose-dependent manner which was reversibly inhibited by 100 nM L-732,138, an NK1 receptor antagonist. Taken together, it is highly speculative to conclude that NK1 receptors are implicated in the secretory responses of rat vEG.

The present study also showed that stimulation of α-receptors with NA did not induce a substantial Ca^{2+} mobilization in vEG acinar cells, consistent in part with the report by Quissell *et al.*(1997), who found that Ca^{2+} increase in response to the α_1-agonists epinephrine and phenylephrine were minimal in the submandibular gland acinar cells. This may be due to the lack or less expression of the receptor than other receptors in these cells. One possible explanation for these differences can be drawn from reports that the adrenergic receptor has species- and organ-specificity. For example, in freshly isolated submandibular gland (SMG) acinar cells, α_1-receptors may not play a critical role in regulating the Ca^{2+} signal. It has been reported that parotid gland (PG) acinar cells respond strongly to α_1-

agonists, manifesting large increases in IP_3 formation and Ca^{2+} mobilization (Ambudkar *et al.*, 1988; Melvin and Zhang, 1993), although Soltoff *et al.*(1989) observed that stimulation of rat PG acinar cells with 10 μM phenylephrine only slightly (35%) increased $[Ca^{2+}]_i$. In freshly isolated SMG acinar cells, IP_3 formation and Ca^{2+} release from intracellular stores in response to α_1-receptor stimulation are relatively small (Wells *et al.*, 1997). The receptor expression or pharmacological analyses with α- and β-adrenoceptor antagonists in vEG acinar cells would be great interest.

NK1 and muscarinic receptors are all members of the same family of G-protein-coupled receptors (Berridge, 1993; Bonner *et al.*, 1987; Hershey and Krause, 1990) and are all thought to activate the Ca^{2+} intracellular messenger pathway. More specifically, salivary gland muscarinic receptor is thought to be the M3 subtype (Dai et al., 1991; Merritt and Rink, 1987; Sawaki *et al.*, 1995), and this and NK1 receptors all couple to G proteins that stimulate PLC and then increases $[Ca^{2+}]_i$ via a common intracellular signalling pathway in salivary acinar cells (Merritt and Rink, 1987; Sawaki *et al.*, 1995). However, it was unexpected results that a synergic effect for Ca^{2+} increase between NK1 and muscarinic receptors was not observed in vEG acinar cells. It may be due to a species- and organ-specificity, but further studies are necessary for explanation the result.

In summary, these results indicate that 1) $[Ca^{2+}]_i$ signaling in vEG acinar cells is regulated by muscarinic and NK1 receptors; 2) the increases in $[Ca^{2+}]_i$ activated by CCh and SP are mediated not by Ca^{2+} entry pathway but by discharge of cytosolic calcium pool. vEG are closely associated with taste bud and supply the microenvironment of these taste buds. Thus, the secretion mechanism of these glands may also be very important in the mechanism of taste transduction.

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