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Nicotinic receptor agonist-induced salivation and its cellular mechanism in parotid acini of rats

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ABSTRACT

Cigarette smoking and nicotine enhance parotid saliva secretion, however, the underlying mechanism is unclear. To address the mechanism of nicotine-induced salivation and to explore the possibility that nicotinic receptor agonists act as sialogogues, we investigated the effects of nicotinic receptor agonists on salivary secretion *in vivo* and on intracellular Ca^{2+} concentration in digested parotid acini *in vitro* in rats. In urethane-anesthetized rats, intravenous administration of nicotinic receptor agonists, nicotine and cytisine, at 3 μmol/kg increased whole saliva output accompanied by a pressor response with nicotine, but not with cytisine. Using Ca^{2+}-imaging system on digested parotid acini in which autonomic nerve terminals were kept intact, nicotine and cytisine dose-dependently increased intracellular Ca^{2+} concentration at μM level. This was not observed in single acinar cells containing no nerve terminal. The nicotine-induced Ca^{2+} response was largely blocked by a muscarinic receptor antagonist and partly blocked by an adrenergic receptor antagonist. Furthermore, the same nicotine-induced Ca^{2+} response was blocked by mecamylamine, a relatively selective nicotinic antagonist for α3β4 subtype receptor, but not by other selective antagonists, dihydro-β-erythroidine for α4-containing receptor and methyllycaconitine for α7 nicotinic receptors. These results suggest that nicotinic agonists-induced salivation is due to a release of acetylcholine and noradrenaline from autonomic nerve terminals through activation of α3β4 nicotinic receptor subtype. In addition, considering the blood pressure response and development of addiction with nicotine, cytisine may be a better therapeutic candidate to serve as a sialogogue for xerostomia patients.

1. Introduction

In the salivary gland, activation of muscarinic receptors leads to fluid secretion, elicited by enhancement of water permeability following increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in salivary acini (Melvin et al., 2005). Pilocarpine, a muscarinic acetylcholine receptor agonist, has been used as a sialogogue for treatment of xerostomia in patients with Sjögren's syndrome and after radiotherapy to head and neck cancer patients (Fox et al., 1991; Johnson et al., 1993; Jorkjend et al., 2008). However, such patients account for only a minute percentage of total xerostomia patients (Nagler and Hershkovich, 2005). Many cases of xerostomia are thought to be associated with aging, gender difference, systemic medications and small salivary gland size (Ben-Aryeh et al., 1984; Heft and Baum, 1984; Percival et al., 1994; Närhi, 1994; Ghezzi and Ship, 2003; Naglar and Hershkovich, 2005; Ono et al., 2009). Because pilocarpine can have severe adverse effects (e.g., hypertension), usage of the muscarinic sialogogue is cautiously prescribed to improve dry mouth complaints in xerostomia patients. Therefore, it is necessary to develop new sialogogues that can be used for many xerostomia patients without adverse effects.

The nicotinic receptor is another acetylcholine receptor subtype. Interestingly, four decades ago, it was reported that cigarette smoking increases parotid salivary secretion in humans (Barylko-Pikielna et al., 1968; Pangborn and Sharon, 1971), and intravenous injection of nicotine increases parotid salivary flow rate under parasympathetic nerve stimulation in rabbits (Inoki et al., 1971). The latter study suggests that the enhancement of parotid gland secretion following nicotine injection is partly caused by neurotransmitter release to the parotid gland without involving the sympathetic ganglion and the adrenal gland. However, the underlying mechanism of nicotine action has not been examined thus far because cellular responses in salivary acinar cells by cholinergic stimulation are largely blocked by muscarinic antagonists. There is only one study reported investigating salivary mechanisms at a cellular level during nicotinic receptor stimulation in the rat sublingual gland (Zhang and Melvin, 1994). This study demonstrated that nicotine increases $[Ca^{2+}]_i$ in rat sublingual mucous acini by acetylcholine release from parasympathetic nerve terminals, indicating that salivary acinar cells can be indirectly

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activated by nicotinic receptor on nerve terminals. If such nicotine-induced cellular mechanism exists in the other major salivary glands, which account for most of the saliva volume (Ferguson, 1975), nicotinic receptor agonists would be a new type of sialogogue, which may have a different mechanism from those of muscarinic sialogogues.

To learn more about the mechanism of nicotine-induced salivation and to explore the possibility that nicotinic receptor agonists act as sialogogues, we investigated the effects of nicotine on salivary secretion in anesthetized rats *in vivo*, and changes in $[Ca^{2+}]_i$ in parotid acini *in vitro*, using a Ca^{2+}-imaging system. Since nicotine has a well-known addiction response, we also investigated the effects of another nicotinic receptor agonist, cytisine, because the drug has been used to suppress nicotine addiction in smokers in Central and Eastern Europe since 40 years ago (Tutka and Zatoński, 2006).

2. Materials and methods

The study was approved by the institutional review board of Kyushu Dental College.

2.1. Measurements of salivary secretion and cardiovascular responses

Male Wistar rats (8 weeks old) were used in the present study. Following anesthesia with urethane (1.0 g/kg, intraperitoneally), rat was placed in a cylindrical heating system that was set to 38 °C after the surgical implantation of an indwelling catheter into the right femoral vein. Mean blood pressure (BP) was continuously recorded every 1 min throughout the experiment with a tail-cuff system (BP-98A, Softron, Tokyo, Japan) and analyzed with computer software (BP98AW Ver. 3.10, Softron, Tokyo, Japan). After observing a stable recording of mean BP over a 10-min period, saline (vehicle, serving as a control), nicotine ((−)-nicotine ditartrate, Research Biochemicals International, MA, USA) and cytisine ((−)-cytisine, Chemical Diversity Labs, CA, USA) were administrated intravenously (0.2 ml). The concentrations of nicotine and cytisine used were 1 and 3 μmol/kg in saline. Because rats occasionally died after intravenous injections of the nicotinic receptor agonists at doses of 10 μmol/kg, doses did not exceed 3 μmol/kg for the rest of the present experiments. Whole saliva was collected from the oral cavity by using pre-weighted cottons for 30 min. One milligram of whole saliva was regarded as 1 μL. To confirm success of the injection procedure, pilocarpine hydrochloride (Kanto Chemical, Tokyo, Japan) at 4 μmol/kg was administrated intravenously at the end of the experiments. The concentration of pilocarpine has been reported to induce large amount of salivary secretion with pressor response (Moreira et al., 2003).

2.2. Parotid cell preparation and Ca^{2+}-imaging technique

The cell preparation and imaging technique were previously described in detail (Inagaki et al., 2010). Following deep anesthesia with intraperitoneal administration of pentobarbital (60 mg/kg), the parotid gland was immediately removed and placed in cold balanced salt solution (BSS) containing (mM): 120 NaCl, 5 KCl, 1 Na_2HPO_4, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 20 Hepes (adjusted to pH 7.4 by NaOH) as well as 0.5% bovine serum albumin. After mincing, the material was digested for 30 min at 37 °C with 50 U/mL collagenase (Wako, Osaka, Japan) in BSS, and the suspension was gently passed through a pipette 10 times every 10 min. After filtering through a 150-μm nylon mesh, the cell preparations were loaded with 2 μM fura-2 AM (Dojindo, Kumamoto, Japan), which was suspended in 10 mL of BSS, and incubated for 30 min at room temperature. After rinsing twice, the preparation was stored at 4 °C until the start of Ca^{2+}-imaging experiment.

After dispersing the cell preparation on poly-D-lysine-coated glass bottom dishes (P35GC-0-10-C, MatTek Corporation, MA, USA), the dishes were mounted on the stage of an inverted fluorescence microscope (IX71, Olympus, Tokyo, Japan) and perfused with BSS at a rate of 1 mL/min at room temperature. Excitation of fura-2 was made every 1 s by an alternate illumination of 340 and 380 nm light, and the resultant fluorescence (510–550 nm, F340 and F380) was collected using an objective lens (UPlanApo 20×/340, Olympus) and a silicon-intensified target camera (Hamamatsu Photonics, Hamamatsu, Japan). Acini were observed as spherical or ovoid structures that had a globular appearance, whereas the ducts showed obviously tubular structures (Inagaki et al., 2010).

2.3. Drugs

Drugs were purchased from the following companies: carbachol chloride (CCh), $CdCl_2$, atropine chloride (ATR), hexamethonium chloride (Hex), D-tubocurarine chloride, mecamylamine (MEC), dihydro-β-erythroidine (DHβE) and methyllycaconitine (MLA) from Sigma (MO, USA); tetrodotoxin (TTX) from Wako (Osaka, Japan) and phentolamine mesylate (Phent) from ICN Biochemical Inc (OH, USA). All drugs were applied to cells by superfusion from separate storage bottles already containing the medium to which they had been added. When a high K^+ solution was needed, 100 mM NaCl in BSS was exchanged with KCl at the same concentration, according to previous studies investigating sublingual acini-innervated nerve terminals (Zhang et al., 1996; Zhang and Melvin, 1994). Repetitive applications of stimulants (high K^+ solution, nicotine) were performed at 3–4 min intervals, and channel and receptor blockers were applied before 1 min of stimulant application. As an adrenergic blocker, we used the α1-adrenergic receptor antagonist Phent in the Ca^{2+}-imaging study because our previous study demonstrated that $[Ca^{2+}]_i$ in rat parotid acini was increased by α1-adrenergic receptor agonist only, but not α2- and β-adrenergic receptor agonists (Inagaki et al., 2010).

2.4. Statistical analysis

The numerical data were expressed as means ± SEM, and *n* represents the number of rats and parotid acini (or single-isolated cells) that were tested. The change in mean BP was indicated as the difference of values (Δ MBP) against the averaged value for 5 min before drug administration. All Ca^{2+} responses of drugs were expressed as Δ ratio, by subtracting base ratio values before drug applications from peak ratio values during drug applications. For the analysis of time-course changes of Δ MBP and dose-dependencies of nicotinic agonists, a two-way analysis of variance, followed by the Bonferroni *post-hoc* test, was used to compare saline (as control) with each nicotinic agonist, and between nicotinic agonists, respectively. For the analysis of total amounts of whole saliva produced for 30 min and drug effects on high K^+ solution- and nicotine-induced Ca^{2+} responses, Student's unpaired t-tests were used. *P* values less than 0.05 were considered to be statistically significant.

3. Results

To examine the sialogogic effects of nicotinic agonists, nicotine and cytisine were intravenously administrated in urethane-anesthetized rats and whole saliva secretions were evaluated for 30 min with measurements of BP. Averaged mean BPs for 5 min before drug administration did not differ in the five treatment groups (saline: 81 ± 4 mmHg, nicotine at 1 μmol/kg: 78 ± 4 mmHg, cytisine at 1 μmol/kg: 77 ± 4 mmHg, nicotine at 3 μmol/kg: 75 ± 5 mmHg, each $n = 4$, and cytisine at 3 μmol/kg: 82 ± 7 mmHg, $n = 5$). At 1 μmol/kg, both drugs did not increase whole saliva secretion and mean blood pressure (Fig. 1A and B, respectively). At 3 μmol/kg, they significantly increased total amounts of whole saliva to a similar level (nicotine: