Abstract

Effects of muscarinic receptor agonist pilocarpine on intracellular signaling in salivary gland cells and salivary secretion

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[Introduction]

The cholinergic signaling through muscarinic acetylcholine receptor (mAChRs) play an essential role in salivary fluid secretion. Pilocarpine hydrochloride, a cholinergic drug, has been used as a stimulant for salivary secretion in animal experiments and the treatment of dry mouth. The increase salivary secretion elicited by pilocarpine is thought to be mediated by Ca^{2+} response via activation of muscarinic receptor (mAChR) in salivary acinar cells. However, the induction of the Ca^{2+} responses by pilocarpine is extremely weak and limited, and thus the precise biological mechanism of pilocarpine to promotes salivary secretion is still unclear. The present study was aimed to investigate the effect of pilocarpine on Ca^{2+} responses and β-arrestin pathway using in vitro models with rat salivary gland cells and the HSY human salivary gland cell line. In addition, the mechanisms of salivary water and protein secretion by pilocarpine (direct effect and indirect effect) were investigated using in vivo mouse and rat models.

[Materials and Methods]

HSY salivary cell line were cultured in DMEM F-12 supplemented with 10% NCS and penicillin (100 U/mL)-streptomycin (100 μg/mL) at 37°C in a humidified 5% CO_2-atmosphere incubator. Rat SMG and parotid cells were prepared from male Wistar/ST rats (9-10 weeks) using collagenase P. The Ca^{2+} response was determined by spectrofluorometer and calcium imaging system using Ca^{2+} indicator Fura 2-AM. Gene expression of mAChRs subtypes were determined by PCR. Phosphorylation of ERK1/2 (p-ERK1/2) was analyzed with Western blot using primary antibody p44/p42^{MAPK} (ERK1/2) and phosphor-p44/p42^{MAPK} (p-ERK1/2). Salivary secretions were measured using male ddy mice (7-8 weeks old) and male Wistar/ST rats (9-10 weeks) under anesthesia with urethane (1 g/kg body weight, i.p). Whole saliva was collected by inserting cotton pellet into the mouth, and then separate the saliva by centrifugation. The saliva subsequently will use for protein measurement using Pierce™ *BCA Protein Assay* Kit and amylase activity measurement by the method of Bernfeld.

[Results and Discussion]

1. Role of M1R and M3R in Pilocarpine induces Ca^{2+} response in salivary acinar cells

Pilocarpine can induce a small Ca^{2+} response in rat salivary cells, with a minimum concentration of 1 μM and near-maximal response at 10 μM. On the other hand, higher concentrations (3-10 μM) were required for bethanechol to elicit Ca^{2+} responses, with even

greater Ca^{2+} responses compare to pilocarpine. Moreover, only ~50% cluster of acinar cells that responding against pilocarpine stimulation in the maximum concentration. This number was smaller compare to other muscarinic agonist carbachol and bethanechol, in which most of cluster acinar cells (~80% and ~95%, respectively) was response. By contrast, 100 μM pilocarpine failed to induce Ca^{2+} response in human salivary cell, HSY cells, while 100μM carbachol induce a strong Ca^{2+} response. Interestingly, HSY cells cultured in the absence of serum [HSY cell (-) serum] showed weak Ca^{2+} responses by stimulation with pilocarpine and a low concentration of carbachol (1 μM). PCR analysis revealed that HSY cells in normal condition express M3R, M4R, and M5R, whereas HSY cell (-) serum express M1R in addition to M3R, M4R, and M5R subtypes of mAChRs. It was also demonstrated that dispersed cells from rat submandibular and parotid glands showed small Ca^{2+} response by pilocarpine, and that these cells expressed M1R and M3R. These results suggest that not only M3R, the M1R also essential for pilocarpine to induce Ca^{2+} responses.

2. Indirect effect of pilocarpine to promotes salivary secretion

A possible involvement of indirect effects of pilocarpine through the central nervous system (CNS) and autonomic ganglia in salivary secretion was examined using pharmacological approach. Pilocarpine (1 mg/kg BW i.p.)-induced salivary secretions in mice were not much affected by pretreatment of cholinesterase inhibitor, physostigmine (0.1 mg/kg BW) and post-ganglionic nicotinic receptor antagonist, hexamethonium (20 mg/kg BW). However, pretreatment with β-adrenergic receptor (β-ARs) antagonist, propranolol (1mg/kg BW) decreased salivary protein and amylase secretions by pilocarpine. Consistent results also obtained when using rat models. Moreover, pilocarpine failed to induce amylase release from rat parotid gland cells in vitro, excluding the possibility that pilocarpine may act directly on the β-ARs of salivary acinar cells to release amylase. These results indicate the possibility of pilocarpine to activate sympathetic autonomic ganglia and cause releasing a neurotransmitter that lead to activate β-adrenergic receptor to promotes protein and amylase release.

3. Distinct mechanism of pilocarpine and carbachol to induce ERK1/2 activation

Carbachol and pilocarpine induced phosphorylation of ERK1/2 (p-ERK1/2) that act maximum at concentration 100 μM and peak at 10 min. Pretreatment with atropine (1 μM) a mAChRs antagonist and epidermal growth factor receptor (EGFR) antagonist, gefitinib (1 μM) completely block carbachol and pilocarpine to induce p-ERK1/2, indicating that carbachol and

pilocarpine induced p-ERK1/2 by mAChR via transactivation of EGFR. Moreover, pretreatment with nonselective PKC inhibitor, GF109203X (1 μM) showed partial inhibition of carbachol-induced p-ERK1/2, but no effect on pilocarpine-induced p-ERK1/2. While pilocarpine-and carbachol-induced p-ERK1/2 was reduce by the presence of barbadin (50 μM), a novel β-arrestin2 inhibitor. Indicating that, unlike carbachol that activate ERK1/2 through PKC-dependent and β-arrestin-dependent pathways, pilocarpine activates ERK1/2 only by β-arrestin-dependent pathways. Pretreatment with GPCR kinase 2 (GRK2) inhibitor reduced carbachol-induced p-ERK1/2 but not for pilocarpine, suggesting that GRKs other than GRK2 isoforms are involved in the effects of pilocarpine. Examinations using clathrin inhibitor, pitstop-2 (15 μM) and dynamin inhibitor, dynole 34-2 (15 μM) showed the inhibition of carbachol-induced p-ERK1/2. While pilocarpine-induced p-ERK1/2 was completely block by the addition of src inhibitor PP2 (1 μM). These finding indicating that pilocarpine and carbachol activate β-arrestin signaling pathways by different mechanisms. Carbachol-induced p-ERK1/2 was β-arrestin-clathrin dependent and pilocarpine-induced p-ERK1/2 was β-arrestin-Src dependent. Since ERK1/2 inhibitor does not alter the pilocarpine-induced salivary secretion in mice, involvement of p-ERK1/2 in the salivary fluid secretions are less likely. Several studies implicated roles of ERK1/2 activation in various of cellular aspects in salivary system such as regeneration, cell proliferation, morphogenesis, and expressions of transporter and ion channels. The roles of ERK1/2 activation in the functions of salivary gland is still poorly understood and further studies are clearly required.

[Conclusion]

Pilocarpine can induce a small but clear Ca^{2+} response in salivary acinar cell through mAChRs. In addition to M3R, the expression of M1R is also important for pilocarpine to induce Ca^{2+} response. Moreover, pilocarpine acts as β-arrestin bias agonist to activate ERK1/2 signaling through src-dependent pathways. However, the role of ERK1/2 activation in salivary gland was still unknown. Pilocarpine was also found to stimulate salivary secretion by two mechanism; direct activations of mAChRs in salivary gland and indirect action through the release of neurotransmitter via sympathetic autonomic ganglia.