

ピロカルピン継続投与による唾液分泌量増加における細胞内シグナルの検討

坂詰 博仁

Intracellular Signaling Pathways Involved in the Regulation of Gene Expression
by Pilocarpine

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この論文を坂詰博仁の学位論文として提出すること、また、学術リポジトリとして
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Intracellular Signaling Pathways Involved in the Regulation of Gene Expression by Pilocarpine

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Abstract

Objectives: Pilocarpine which is commonly used in the clinic to treat dry mouth, is generally thought to stimulate Ca²⁺-responsive saliva secretion via activation of muscarinic receptors, yet the Ca²⁺-mobilizing effect of pilocarpine in salivary gland cells is extremely small. We hypothesized that pilocarpine alters gene expression in the salivary gland through muscarinic receptor stimulation. This study aims to investigate the effect of pilocarpine on gene expression changes mediated via mitogen-activated protein kinase (MAPK) activity.

Methods: The effect of pilocarpine on gene expression was investigated in rats and human salivary gland cells (HSY cells) using several inhibitors of intracellular signaling pathways. Gene expression in rat submandibular gland and HSY cells was determined by reverse transcription-quantitative polymerase chain reaction analysis of total RNA.

Results: In vivo, pilocarpine administration increased the expression of *Sgk1* and *Ctgf* in submandibular gland. In vitro, pilocarpine increased *Ctgf* expression in HSY cells. The MEK inhibitor, trametinib and the Src inhibitor, PP2 suppressed the effect of pilocarpine on gene expression.

Conclusions: Pilocarpine enhances *Sgk1* and/or *Ctgf* expression by activating Src-mediated MAPK activity. Although further studies are required to fully understand the role of *Sgk1* and *Ctgf* in the salivary secretion system, gene expression changes may play important roles in improving salivary secretion.

Keywords: Pilocarpine, MAPK, Ctgf, Gene expression, Src

1. Introduction

Pilocarpine is a muscarinic acetylcholine receptor (mAChR) agonist that is commonly used in the clinic to treat dry mouth caused by Sjögren's syndrome, or to improve salivary secretion after radiotherapy to treat head and neck cancer [1–4]. Salivary gland secretion is regulated by increases in intracellular Ca²⁺ levels, which activate ion channels [5]. The mechanism of pilocarpine is generally thought to be Ca²⁺-responsive saliva secretion via activation of Gq/11 protein-coupled mAChRs [6–8]. However, the Ca²⁺-mobilizing effect of pilocarpine in

salivary gland cells is extremely small, even though the long-term administration of pilocarpine improves salivary secretion more effectively than a single administration [9–11]. This observation indicates that the effect of pilocarpine is not limited to Ca²⁺-responsive saliva secretion. Therefore, we hypothesized that pilocarpine-mediated activation of mAChRs induces functional changes in gene expression in the salivary gland. Previous studies reported that activation of G protein-coupled receptors (GPCRs) stimulates the MAPK pathway to regulate gene expression, and pilocarpine acts on mAChRs to induce phosphorylation of MAPK [12–15].

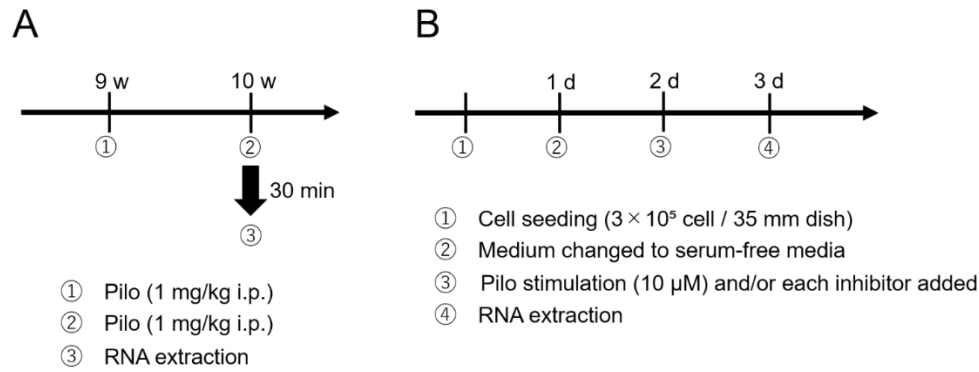


Fig. 1. The schedule of pilocarpine (Pilo) administration in vivo and in vitro. Pilocarpine administration to rats (A) and HSY cells (B).

In addition, our comprehensive gene expression analysis revealed that pilocarpine increased the expression of *Sgk1* and *Ctgf* in rats (personal communication). In this study, we investigated changes in *Sgk1* and *Ctgf* expression induced by pilocarpine-mediated activation of the MAPK pathway in vivo and in vitro.

2. Materials and methods

2.1. Animals

Male Wistar/ST rats (9 weeks old) were purchased from Japan SLC (Shizuoka, Japan) and housed in conventional conditions in a temperature- and humidity-controlled room with a 12 h-12 h light-dark cycle. Rats were fed a normal diet (Clea Japan Inc., Tokyo, Japan) and provided with water ad libitum.

2.2. Materials

Pilocarpine hydrochloride (Wako Pure Chemical, Osaka, Japan) was used as a mAChR agonist. Trametinib (Selleckchem, Houston, TX, USA) was used as a MEK inhibitor [16], and PP2 (AOBIOUS, Gloucester, MA, USA) was used as a Src inhibitor.

2.3. Cell culture

The HSY-EA1 cell line (HSY) [17], which is derived from human salivary glands, was cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (Fujifilm Wako Pure Chemicals, Tokyo, Japan) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a humidified incubator with a 5% CO₂ atmosphere.

2.4. In-vivo experiments

A schematic of in-vivo experiments is shown in Fig. 1A. Rats were anaesthetized with ketamine (47 mg/kg body weight, i.p.) and xylazine (6.3 mg/kg body weight, i.p.). Then, they were placed on a heated pad and 1 mg/kg pilocarpine was administered intraperitoneally. Thirty minutes later, submandibular glands (SMGs) were isolated, frozen with liquid N₂ and stored at -80°C. Total RNA was extracted using Trizol (Thermo Fisher Scientific) according to the manufacturer's instructions.

2.5. In-vitro experiments

A schematic of in-vitro experiments is shown in Fig. 1B. Briefly, the cells were plated at a density of 3×10^5 cells in 35-mm culture dishes and allowed to grow for 24 h. Then, the medium was changed to serum-free medium. The next day, 10 μ M pilocarpine was added to the serum-free medium and the cells were exposed to pilocarpine for 24 h. For experiments that used inhibitors, each inhibitor was added 1h before stimulation with pilocarpine. The medium was removed, and cells were washed once with ice-cold D-PBS (-) (FUJIFILM Wako Pure Chemicals) to extract RNA. Total RNA was extracted using a miRNeasy extraction kit (QIAGEN, Venlo, the Netherlands) according to the manufacturer's instructions.

2.6. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

The primers used in RT-qPCR were designed by Primer 3 software. Reverse transcriptions were performed using ReverTra Ace (TOYOBO, Osaka, Japan), Random Hexamer primer (Thermo Fisher Scientific) and recombinant RNasin (Promega, Madison, WI, USA).

Real-time quantitative PCR (qPCR) amplification reactions were carried out using PowerTrack SYBR Green master mix (Thermo Fisher Scientific) and a

StepOnePlus real-time PCR system (Thermo Fisher Scientific) according to the manufacturer's instructions. The expression level of each gene was normalized to that of the housekeeping gene *GAPDH*, and the relative expression was shown as the cycle threshold ($2^{-\Delta\Delta Ct}$) value. The qPCR assay was performed in duplicate.

The nucleotide sequences of the primers used are shown below:

rat *Sgk1* forward: 5'-CTAAGCCGGTCTCACTGCTC-3',

rat *Sgk1* reverse: 5'-GCGATGAGGATTGCTACCAT-3',

rat *Ctgf* forward: 5'-TAGCAAGAGCTGGGTGTGTG-3',

rat *Ctgf* reverse: 5'-TTCAGTTGCCACAAGCTGTC-3',

rat *GAPDH* forward:

5'-GGCATTGCTCTCAATGACAA-3',

rat *GAPDH* reverse:

5'-TGTGAGGGAGATGCTCAGTG-3',

human *Sgk1* forward:

5'-GCAGAAGGACAGGACAAAGC-3',

human *Sgk1* reverse:

5'-CAGGCTCTTCGGTAAACTCG-3',

human *Ctgf* forward:

5'-CCGTACTCCCAAATCTCCA-3',

human *Ctgf* reverse:

5'-GTAATGGCAGGCACAGGTCT-3',

human *GAPDH* forward:

5'-CGACCACTTTGTCAAGCTCA-3',

human *GAPDH* reverse:

5'-AGGGGTCTACATGGCAACTG-3'.

2.7. Statistical analysis

Statistical analysis was performed using the Mann-Whitney U test. Results are shown as the mean \pm SE. P-values of <0.05 and <0.01 represent statistically significant differences.

3. Results

3.1. Pilocarpine-induced gene expression changes in rats

Expression of *Sgk1* and *Ctgf* in SMGs 7 days after pilocarpine stimulation was significantly higher than in control (non-stimulated) SMGs (Fig. 2).

3.2. Pilocarpine-induced *Ctgf* expression in HSY cells

One day after HSY cells were stimulated with pilocarpine, *Sgk1* and *Ctgf* expression was examined. The expression of *Ctgf* was significantly higher in pilocarpine-stimulated cells than in control (non-stimulated) cells (Fig. 3). Pilocarpine did not alter the expression of *Sgk1* (data not shown).

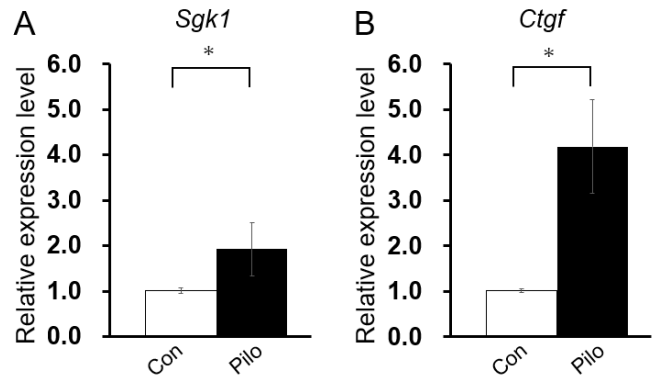


Fig. 2. RT-qPCR analysis of the relative expression level (fold-change) of *Sgk1* and *Ctgf* expression after pilocarpine (Pilo) stimulation in vivo. (A) *Sgk1* expression in SMGs 7 days after pilocarpine stimulation or control (Con) without stimulation. (B) *Ctgf* expression in SMGs 7 days after pilocarpine stimulation or control without stimulation. Data are presented as the mean \pm SE (control n=6, pilocarpine n=7). * $p<0.05$.

3.3 The effects of inhibitors on pilocarpine-stimulated *Ctgf* expression in HSY cells

We used several inhibitors of intracellular signaling pathways to determine the pathways involved in pilocarpine-induced changes in *Ctgf* expression. The MEK inhibitor, trametinib (Fig. 4A) and the Src inhibitor, PP2 (Fig. 4B) suppressed pilocarpine-induced changes in *Ctgf* expression.

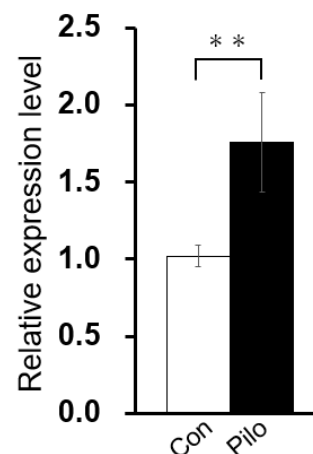


Fig. 3. The change in *Ctgf* expression in pilocarpine-stimulated HSY cells. RNA was extracted from HSY cells 1 day after pilocarpine stimulation (10 μ M) or control to determine the changes in *Ctgf* expression using RT-qPCR. Data are presented as the mean \pm SE (control n=9, pilocarpine n=14). ** $p<0.01$.

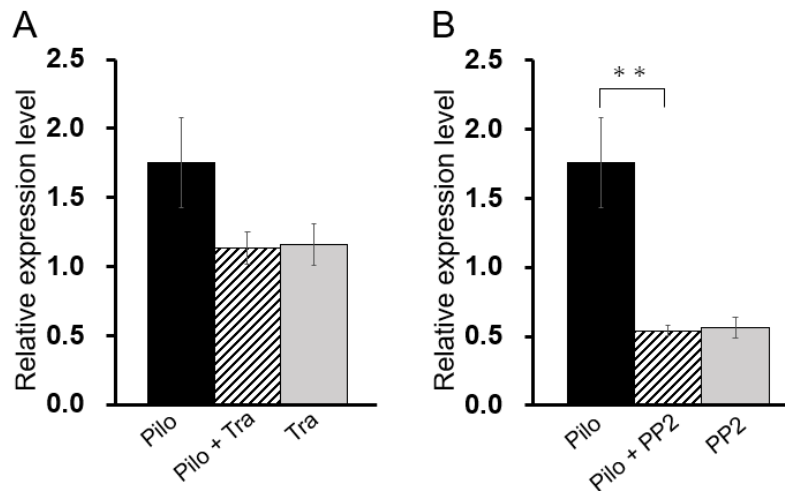


Fig. 4. The effects of inhibitors on pilocarpine-mediated *Ctgf* expression in HSY cells.

Specific inhibitors were used to investigate the intracellular signaling pathways involved in pilocarpine (Pilo)-induced changes in *Ctgf* expression in HSY cells. (A) The effect of 10 nM trametinib (Tra) on pilocarpine-induced changes in *Ctgf* expression. (B) The effect of 1 μ M PP2, a Src inhibitor, on pilocarpine-induced changes in *Ctgf* expression. Data are presented as the mean \pm SE (pilocarpine n=14, trametinib n=10, pilocarpine plus trametinib n=10, PP2 n=6, pilocarpine plus PP2 n=7). ** $p < 0.01$.

4. Discussion

In this study, pilocarpine altered the expression of *Sgk1* and *Ctgf* in vivo and the expression of *Ctgf* in vitro. To the best of our knowledge, this is the first report to show that pilocarpine can change gene expression via MAPK.

Our results showed that pilocarpine-induced changes in *Ctgf* gene expression were mediated by MAPK. Previous studies have already reported that gene expression is regulated by activation of GPCRs through the MAPK pathway, and that pilocarpine phosphorylates the MAPK system via muscarinic receptors [12–15,18,19]. However, there are few reports of the effect of pilocarpine on gene expression [10].

We found that Src is involved in the signaling pathway following pilocarpine stimulation; this finding is consistent with the report of Dikic et al. showing that Src activation leads to MAPK activation [20]. Our results indicate that pilocarpine may increase *Ctgf* expression by activating Src-mediated MAPK activity.

Our in-vivo study showed that pilocarpine increased the expression of *Sgk1* and *Ctgf* in SMGs. *Sgk1* is a serine/threonine protein kinase that regulates processes such as cell survival, neuronal excitability and renal sodium excretion [21–23]. *Sgk1* has been also reported to activate ion transporters such as K^+ and Cl^- channels and the $Na^+-K^+-2Cl^-$ cotransporter NKCC, which have important roles in the salivary secretion system [21,23,24]. Therefore, pilocarpine might promote saliva secretion by stimulating *Sgk1* activity.

In contrast to our in-vivo findings, pilocarpine did not increase *Sgk1* in HSY cells. Presumably, this is because HSY cells were not the most appropriate cell line for the

experiment. *Sgk1* is highly expressed in nervous system cells and therefore neural-derived cultured cells such as SH-SY5Y cells may be more suitable for investigating the expression of *Sgk1* [26].

Ctgf is a matricellular protein of the CCN family that mediates the interaction between cell-surface and matrix proteoglycans [25, 26]. *Ctgf* is involved in angiogenesis, which increases the amount of water supplied during salivary secretion [26, 27]. *Ctgf* may play a role in the promotion of salivary secretion by pilocarpine.

Although further studies are required to investigate the full functions of *Sgk1* and *Ctgf* in the salivary secretion system, our results suggest that pilocarpine improves salivary secretion by activating Src-mediated MAPK activity to enhance *Sgk1* and/or *Ctgf* expression.

5. Conclusion

In this study, our results showed the possibility that pilocarpine can change gene expression via MAPK. Our findings would help clarify the molecular mechanism of pilocarpine and may provide future strategies to improve saliva secretion in patients.

Ethical approval

Experimental procedures performed on animals were conducted with the approval of the ethical review board for experiments using animals of the Nippon Dental University School of Life Dentistry at Niigata (approval number: 247).

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Conflicts of Interest

The authors declare no conflict of interest.

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