Increase of Cl⁻ Secretion Induced by Kampo Medicine (Japanese Herbal Medicine), Sai-rei-to, in Mongolian Gerbil Middle Ear Epithelium

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ABSTRACT—Sai-rei-to, a type of Kampo medicine (Japanese herbal medicine), has been shown to be clinically effective in treating patients with otitis media with effusion. The effect of Sai-rei-to on the ion transport of the middle ear surface epithelium cultured from the Mongolian gerbil was investigated by using an Ussing chamber. Application of Sai-rei-to to the mucosal bath but not the serosal bath induced an increase in the short-circuit current (Isc) in the basal state. The increase in Isc was almost completely inhibited by addition of diphenylamine-2-carboxylic acid but not by amiloride, indicating enhancement of Cl⁻ secretion. On the basis of the lack of changes in the intracellular Ca²⁺ concentration and a sidesiness of action, the effect of Sai-rei-to on Isc is thought to be a direct and selective activation on the apical Cl⁻ channel.

Keywords: Short-circuit current, Cl⁻ secretion, Basal secretion, Otitis media with effusion, Mucociliary transport

Electrolyte transport coupled with water movement by airway epithelial cells controls the quantity and composition of respiratory tract fluid, thereby contributing to normal mucociliary clearance. Recent development and establishment of a middle ear epithelial (MEE) culture system has enabled electrolyte transport studies to be performed with an Ussing chamber (1-3). Cultured MEE have been shown to be capable of actively absorbing Na⁺ from the mucosal to the serosal surface through amiloride-sensitive Na⁺ channels and for active Cl⁻ secretion to occur from the serosal to the mucosal surface (1-3). Although Herman et al. (2) reported a lack of the basal Cl⁻ secretion in their cultured cells from Mongolian gerbils, our cultured cells from the same species showed the presence of basal Cl⁻ secretions sensitive to diphenylamine-2-carboxylic acid (DPC) (4).

Disturbance of periciliary fluid regulation in the otitis media with effusion (OME) has been reported in several studies. Inagaki et al. (5) found that the rows of cilia were buried so deeply in the mucosal layer, indicating a reduction in the depth of the periciliary fluid layer. One of the authors (T.T.) also demonstrated the same finding in OME induced in animals experimentally (6). The reduced depth of the periciliary layer characterized in OME may explain mucociliary dysfunction as evidenced by radio-labeled molecular studies (7-9).

We previously reported the clinical efficacy of a traditional herbal medicine, Sai-rei-to, in patients with OME (10). Oral administration of Sai-rei-to resulted in objective improvement of 78% as judged by pure-tone and impedance audiology. One of the underlying mechanisms for the efficacy of Sai-rei-to included improvement in mucociliary clearance, which was demonstrated in the animal OME model (11). This study was designed to elucidate the effect of Sai-rei-to on the electrolyte transport of the cultured middle ear epithelium in order to enhance our understanding of the basic mechanism for its clinical efficacy.

MATERIALS AND METHODS

Cell culture

Gerbils ranging from 2 to 3 months of age were anesthetized with diethyl ether and decapitated. The tympanic bulla was removed and rinsed to wash out mucus with ice-cold Dulbecco's phosphate-buffered saline (PBS) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin sulfate, 50 μg/ml gentamicin sulfate and 2.5 μg/ml amphotericin B. The tympanic bullae were then exposed overnight at 4°C to a 0.1% protease XIV and 0.01% deoxyribonuclease solution in PBS. Enzymatically dissociated cells were harvested, centrifuged at 1000 x g
for 5 min and then resuspended in Dulbecco's modified Eagle medium (DMEM). Cell viability was assessed according to trypan blue exclusion (95%). Plating was performed in a 1:1 mixture of DMEM and Ham's F-12 (F-12) containing 5% fetal calf serum, 10 ng/ml epidermal growth factor, 5 mg/ml transferrin, 2 nM triiodothyronine, 10 μg/ml insulin, 10⁻⁶ M hydrocortisone, 7.5 μg/ml endothelial cell growth supplement, 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 2 mM L-glutamine. For measuring the short-circuit current (Isc), cells were plated at 10⁶ cells /cm² onto Coster Transwell membranes (tissue culture treated polycarbonate filters with 0.4-μm pore diameter; Coster, Cambridge, MA, USA). Cells were grown with an air interface; i.e., no media were added to mucosal surface. All culture vessels were coated with rat tail collagen (20 μg/cm²).

Bioelectric measurements

For measurement of ion transport, cells grown on the Transwell filter were mounted in an Ussing chamber. Experiments were performed in bicarbonate-buffered Krebs-Henseleit solution (pH 7.4) bubbled with 95% O₂ - 5% CO₂ at 37°C. Isc was measured continuously on a pen recorder, and resistance (R) was determined from the size of current pulses of 200-msec duration passed across short-circuited tissues every 20 sec so as to displace the transepithelial potential difference (PD) by +0.2 - +1 mV from zero. After cell sheets were mounted, we waited for 15 min before adding drugs to allow the baseline electrical properties to stabilize; after this initial period, R and Isc showed negligible change with time. Only tissues with R>100 Q • cm² were used. All drugs were added to the tissues as 100-fold dilutions.

[Ca²⁺]i measurements

For the measurement of intracellular Ca²⁺ concentration ([Ca²⁺]i), 3 μM fura-2 acetoxymethyl ester (fura-2 AM) was loaded in the tissue. Fura-2 AM was dissolved in dimethyl sulfoxide and mixed with an equal volume of 10% w/v Pluronic F-127 at a concentration of 5 mM. After dye loading for 15 min, the tissue was rinsed with the standard solution. A glass cover slip was attached on the bottom of the perfusion chamber with a bath capacity of 0.2 ml and sealed with silicone grease. During the experiments, the specimen was continuously superfused with the desired solutions, oxygenated and warmed to 37°C at a rate of 1.6 ml/min, ensuring exchange of the bathing solution within a few seconds. Instrumentation used for the digital imaging has been reported previously (12). All images were obtained for paired excitation radiations by averaging 16 frames per 0.53 sec for each excitation wavelength, usually at 10-sec intervals. The excitation radiation was only transmitted during the data collection cycle. After background subtraction, the images of the fluorescence ratio were obtained by dividing, pixel by pixel, at 340 and 380 nm excitation (F₃₄₀ and F₃₈₀, respectively). The fluorescence intensity ratio R = F₃₄₀/F₃₈₀ was converted to [Ca²⁺]i, by the following equation (13):

\[
[Ca^{2+}]_i = K \cdot (R - R_{\min}) / (R_{\max} - R)
\]

To estimate the fluorescence ratio of fura-2 when saturated with Ca²⁺ (Rmax), the tissue preparation was washed with the standard solution containing 10 μM ionomycin and 20 mM CaCl₂ for 5 -10 min. The fluorescence ratio of the Ca²⁺-free dye (Rmin) was obtained after perfusing and incubating the same preparation with Ca²⁺-free solution containing the ionophore and 10 mM EGTA. The parameter K is the product of K₀(F₀/F₃), where K₀ is a dissociation constant of 224 nM and F₀/F₃ is the ratio of the excitation efficiencies of free indicator to Ca²⁺-bound indicator at 380 nm. The overall [Ca²⁺]i of the cell was determined by averaging the [Ca²⁺]i for each pixel.

Chemicals and solutions

Amiloride and rat tail collagen were purchased from Sigma Chemical (St. Louis, MO, USA). Cell culture media and growth factors were from Gibco/BRL Life Technologies Inc. (Palo Alto, CA, USA). Other chemicals were purchased from Wako Jun-yaku (Osaka). Bioelectric measurements were performed in a Krebs-Henseleit solution composed of 118 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM NaH₂PO₄, 25.5 mM NaHCO₃ and 5.6 mM glucose. Sai-rei-to was a gift from Tsumura & Co. (Tokyo) and was made from the crude extract of eight medicinal plants: Bupleurum falcatum, Alisma orientale, Pinellia ternata, Scutellaria baicalensis, Zizyphus jujuba, Panax ginseng, Cinnamomum cassia and Zingiber officinale. The decoction was made from Sai-rei-to after gently boiling for 60 min and was used for the experiments.

Statistics

Results are expressed as the mean ± S.E. Statistical analyses was performed by the paired or unpaired Student's t-test. P<0.05 was considered statistically significant.

RESULTS

Baseline Isc and transepithelial resistance in the cultured MEE used in the present study were 15.3 ± 1.0 μA /cm² and 191 ± 14 Ω • cm, respectively (n = 13). Application of Sai-rei-to (10 mg/ml) to the serosal side caused little
change in $I_{SC}$ ($0.2 \pm 0.1$, $n=4$, paired test) whereas a significant increase in $I_{SC}$ ($1.0 \pm 0.2 \mu A/cm^2$, $n=4$, paired test) was observed in the mucosal application (Fig. 1A).

The previous study demonstrated the presence of both amiloride-sensitive Na$^+$ channels and DPC-sensitive Cl$^-$ channels in the apical membrane (4). The next series of experiments were conducted to reveal the components of Sai-rei-to-induced increase in $I_{SC}$. Application of amiloride (10 $\mu$M) to the mucosal side promptly and markedly decreased the $I_{SC}$ by $12.5 \pm 0.9 \mu A/cm^2$ ($n=5$, paired test). In the presence of amiloride (10 $\mu$M), mucosal application of Sai-rei-to (10 mg/ml) still maintained the increase in $I_{SC}$ ($2.2 \pm 0.3 \mu A/cm^2$, $n=5$, Fig. 1B), which was not significantly different from when amiloride was absent (unpaired test). In comparison, in the presence of DPC (1 mM) which induced a small but significant drop in $I_{SC}$ by $1.1 \pm 0.6 \mu A/cm^2$ ($n=4$, paired test), Sai-rei-to applied apically changed the basal $I_{SC}$ by $0.2 \pm 0.2 \mu A/cm^2$ ($n=4$, Fig. 1C), which was significantly lower than that in the absence of DPC (unpaired test). The effect of Sai-rei-to in each condition is summarized in Table 1.

Thus, the increase of $I_{SC}$ induced by Sai-rei-to is brought about by activation of the apical Cl conductance.

In the previous study, addition of extracellular ATP induced an increase in opening K$^+$ channels at the basolateral membrane, resulting in an increase in the driving force for Cl$^-$ exit at the apical membrane (4). To determine the underlying mechanism of the activation of Cl$^-$ channels induced by Sai-rei-to, we examined the effect of Sai-rei-to on $[Ca^{2+}]_i$. The application of Sai-rei-to (10 mg/ml) to both sides of the cultured MEE produced no change in $[Ca^{2+}]_i$ (data not shown). Thus, it is unlikely that the Cl$^-$ secretion by Sai-rei-to results from the increase in $[Ca^{2+}]_i$.

**DISCUSSION**

The present study demonstrated that the Kampo medicine Sai-rei-to induced Cl$^-$ secretion in the cultured MEE from the Mongolian gerbil. The cultured MEE from the same species showed no evidence of Cl$^-$ secretion in the basal state, which could be explained by the electrochemical equilibrium of Cl$^-$ across the apical membrane (2). However, the apical Cl$^-$ secretion in the basal state was actually proven in our cultured cells since addition of DPC to the mucosal bath reduced the basal $I_{SC}$. In addition, our preliminary study demonstrated the presence of the Cl$^-$ channel in the apical membrane, which was activated by the increase in the intracellular Ca$^{2+}$ concentration and the membrane hyperpolarization resulting from the activation of the basolateral K$^+$ channel. The mode of activation seems to be a direct and selective action on the Cl$^-$ channel since i) the apical but not the basolateral application acted on the cell, ii) the increased $I_{SC}$ induced by Sai-rei-to was inhibited by the Cl$^-$ channel blocker, DPC, and iii) the increase in $[Ca^{2+}]_i$, which activates the basolateral K$^+$ channel, thereby inducing a favorable electrochemical gradient for Cl$^-$ exit, was not recognized.

**Table 1. Effects of Sai-rei-to on the short-circuit current ($I_{SC}$, $\mu A/cm^2$) in the cultured middle ear epithelial cells**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>$I_{SC}$ (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$1.0 \pm 0.2$ (4)</td>
</tr>
<tr>
<td>Amiloride</td>
<td>$2.2 \pm 0.3$ (5)</td>
</tr>
<tr>
<td>DPC</td>
<td>$0.2 \pm 0.2$ (4)</td>
</tr>
</tbody>
</table>

* N.S.: not significant.

In all the experiments, serosal application of Sai-rei-to was performed. Both amiloride and diphenylamine-2-carboxylic acid (DPC) were serosally applied to the cells. Parentheses: the number of experiments. *: P<0.05.
Although another possibility is activation of apical Cl$^-$ channels via cAMP-protein kinase A-dependent phosphorylation induced by Sai-rei-to as seen in the respiratory epithelia, this would be unlikely based on the ineffectiveness of the basolateral application of Sai-rei-to. Thus, it is likely that Sai-rei-to directly and selectively activates the apical Cl$^-$ channel, although there remains a possibility that an unknown second messenger system may be involved in the activation. More direct evidence should be obtained by a single channel analysis using the patch-clamp method.

An insufficient volume of periciliary fluid has been suggested as a possible etiopathology of OME (5, 6). Altered composition and volume of airway mucus would lead to a decrease of clearance and bacterial infection (7–9). Net epithelial secretion of Cl$^-$, along with Na$^+$, by Sai-rei-to, controls the volume and hydration of middle ear mucus, resulting in increased mucociliary clearance and a beneficial effect in the treatment of OME.

Sai-rei-to is a compound drug made from the crude extract of various medicinal plants. These plants and extracts have been reported to have an anti-inflammatory action (10). Further studies are required to determine the chemical component inducing Cl$^-$ secretion, which would substantially contribute to the treatment of OME.

In conclusion, the present study revealed that Sai-rei-to showed a direct and selective activation of the apical Cl$^-$ channel in the cultured MEE. The underlying mechanism shown here can well explain the clinical efficacy of Sai-rei-to in the treatment of OME.

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REFERENCES