Effects of Sho-saiko-to, San'o-shashin-to and Scutellariae Radix on Intracellular Ca^{2+} Mobilization in C6 Rat Glioma Cells

Reiki Kyo,^a Norimichi NAKAHATA,^{*a} Iwao SAKAKIBARA,^b Masayoshi KUBO,^b and Yasushi OHIZUMI^a

Department of Pharmaceutical Molecular Biology, Faculty of Pharmaceutical Sciences, Tohoku University,^a Aoba, Aramaki, Aoba-ku, Sendai 980–8578, Japan, and Tsumura Central Research Laboratories, Tsumura and Co.,^b 3586 Yoshiwara, Amimachi, Inashiki, Ibaraki 300–1192, Japan. Received March 9, 1998; accepted June 22, 1998

Glial cells are able to support neurons physically and functionally. The present study was undertaken to determine the effects of Kampo medicines on glial cell function, especially Ca^{2+} mobilization. C6 rat glioma cells expressed H_1 -histamine, muscarinic cholinergic and adrenergic α_1 -receptors, stimulation of which resulted in phosphoinositide hydrolysis and increase in intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$). The water extracts of Sho-saiko-to and San'o-shashin-to, Kampo medicines which contain Scutellariae Radix (Ogon, the root of *Scutellaria baicalensis* GEORGI), inhibited histamine (100 μM)-induced increase in $[\text{Ca}^{2+}]_i$ in a concentration-dependent manner. The water extract of Scutellariae Radix potently decreased $[\text{Ca}^{2+}]_i$ in a concentration-dependent manner. Sho-saiko-to, San'o-shashin-to and Scutellariae Radix significantly inhibited histamine-induced accumulation of total [^3H]inositol phosphates, consistent with their inhibition of the increase in $[\text{Ca}^{2+}]_i$. These results suggest that Sho-saiko-to, San'o-shashin-to and Scutellariae Radix inhibit Ca^{2+} mobilization mediated via an inhibition of phospholipase C. The inhibitory effect may be important in interpreting the pharmacological actions of above Kampo medicines.

Key words Sho-saiko-to; San'o-shashin-to; Scutellariae Radix; Ca^{2+} ; phosphoinositide hydrolysis; glial cell

Glial cells, which exist at about ten times the rate of neural cells in the brain, are known to control the liberation of neurotrophic factors¹⁾ or to retain the moisture of the brain. They therefore have a role in maintaining the function of neural cells.²⁾ It has been shown that glial cells express neurotransmitter receptors, such as H_1 -histamine-,³⁾ muscarinic cholinergic⁴⁾ and α_1 -adrenergic receptors,⁵⁾ which are coupled to phosphatidylinositol 4,5-bisphosphate (PIP_2)-specific phospholipase C mediated via pertussis toxin-insensitive G protein, Gq.⁶⁾ Stimulation of these receptors results in accumulation of inositol 1,4,5-trisphosphate (IP_3) and intracellular Ca^{2+} mobilization. Glial cells are assumed to exert prostaglandin (PG) synthesis in the central nervous system.⁷⁾ PGE_2 , a main product of PGs in glial cells,⁸⁾ is produced by catalysis of phospholipase A_2 (PLA_2) and cyclooxygenase (COX). There are two classes of PLA_2 ,⁹⁾ cytosolic PLA_2 (c PLA_2) and secretory PLA_2 (s PLA_2). c PLA_2 is generally believed to exert its activity with a micromolar concentration of Ca^{2+} ,¹⁰⁾ which is supplied by receptor-mediated phospholipase C activation.¹¹⁾ We previously demonstrated that several Kampo medicines affected PGE_2 synthesis in glial cells *in vitro*.¹²⁾ San'o-shashin-to caused the most potent inhibition of PGE_2 synthesis among Kampo medicines tested in the study.¹³⁾ Further analysis revealed that Scutellariae Radix, a crude drug composed of San'o-shashin-to caused a potent inhibition of PGE_2 synthesis in C6 rat glioma cells.¹⁴⁾ Thus, Scutellariae Radix is assumed to be a key crude drug inhibiting PGE_2 synthesis. It is also a crude drug composed of Sho-saiko-to, one of the most popular Kampo medicines.

In the present study, we analyzed intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and phosphoinositide hydrolysis to examine whether Sho-saiko-to, San'o-shashin-to and Scutellariae Radix (Ogon) affected $[\text{Ca}^{2+}]_i$ and phosphoinositide hydrolysis in C6 rat glioma cells. The results suggest that these drugs inhibit the receptor-mediated phosphoinositide hydrolysis and the increase in $[\text{Ca}^{2+}]_i$.

MATERIALS AND METHODS

Materials Fetal bovine serum was obtained from the Cell Culture Laboratory (Cleveland, OH, U.S.A.). Horse serum was obtained from Dainippon Pharmaceutical Co., Ltd. (Tokyo, Japan). F-10 (Nutrient Mixture: Ham) was obtained from GIBCO BRL (Tokyo, Japan). Fura-2-AM was from Dojindo (Kumamoto, Japan). Triton X-100 was from Wako Pure Chemicals (Tokyo, Japan). EGTA was from Nakarai Chemicals (Kyoto, Japan). Collagenase was obtained from Warthington Biochemical Co. (Freehold, NJ, U.S.A.).

Kampo medicines were obtained from Tsumura and Co. (Ibaraki, Japan). San'o-shashin-to was prepared as follows: A mixture of crude drugs consisting of Scutellariae Radix (root of *Scutellaria baicalensis* GEORGI 3.0 g), Coptidis Rhizoma (rhizome of *Coptis japonica* MAKINO, 3.0 g) and Rhei Rhizoma (rhizome of *Rheum palmatum* L., 3.0 g), was extracted with water (285 ml) at 100 °C for 1 h. The extract solution was filtered and spray-dried to obtain the dry extract powder as San'o-shashin-to. Sho-saiko-to was prepared as follows: a mixture of crude drugs consisting of Bupleuri Radix (root of *Bupleurum falcatum* LINNÉ, 7.0 g), Pinelliae Tuber (tuber of *Pinellia ternata* BREITENBACH, 5.0 g) and Scutellariae Radix (root of *Scutellaria baicalensis* GEORGI, 3.0 g), Zizyphi Fructus (fruit of *Zizyphus jujuba* MILLER var. *inermis* REHDER, 3.0 g), Ginseng Radix (root of *Panax ginseng* C.A. MEYER, 3.0 g), Glycyrrhizae Radix (root of *Glycyrrhiza uralensis* FISCH *et.* DC., 2.0 g), Zingiberis Rhizoma (rhizome of *Zingiber officinale* ROSCOE, 1.0 g) was extracted with water (285 ml) at 100 °C for 1 h. The extract solution was filtered and spray-dried to obtain the dry extract powder as Sho-saiko-to. Scutellariae Radix (10 g) was extracted with water (100 ml) at 100 °C for 1 h. The dried extracts of Kampo medicines or a crude drug were dissolved in 0.9% NaCl to make a concentration of 10 mg/ml by sonication. The solution was diluted 10 times by the culture medium. Other

* To whom correspondence should be addressed.

chemicals and drugs were of reagent grade or highest quality available.

Cell Culture C6 rat glioma cells were grown in F-10 medium containing 15% horse serum and 2.5% fetal bovine serum in a 37°C humidified incubator in an atmosphere of 5% CO₂ in air.

Measurement of Intracellular Free Ca²⁺ Concentration with Fura-2 Intracellular free Ca²⁺ concentration ([Ca²⁺]_i) was measured by the fura-2 method as described previously.¹⁵⁾ C6 rat glioma cells cultured on a 150 mm dish were washed three times with a modified Tyrode solution (composition, mM: NaCl 137, KCl 2.7, MgCl₂ 1.0, CaCl₂ 0.18, glucose 5.6, HEPES 10, pH 7.4). The cells were freed from the dish by treatment with 0.1% collagenase and 1.0% bovine serum albumin (BSA) in 10 ml of the modified tyrode solution for 15 min at 37°C, and were collected in a 50 ml tube followed by centrifugation at 250 *g* for 1 min. After washing once with 10 ml of the modified tyrode solution, the cells (1–5 × 10⁶/ml) were treated with 1 μM fura-2-AM for 15 min, and washed twice with the same solution. Cells were then suspended in concentrations of 1–5 × 10⁶/ml, and 1–2 ml of the cell suspension was used for fura-2 assay. Fluorescence of fura-2 at 510 nm by excitation waves at 340 and 380 nm was monitored simultaneously by a spectrofluorometer (Hitachi, F-2000). The maximum ratio of fluorescence at 510 nm by the excitation wave of 340 nm to that by 380 nm was obtained in the presence of 0.1% Triton X-100, and the minimum fluorescence ratio was obtained in the presence of 2 mM EGTA. Free Ca²⁺ concentration was calculated using the *K*_d of fura-2 to Ca²⁺ as 224 nM.

Measurement of [³H]Inositol Phosphates Phosphoinositide breakdown was monitored by measuring [³H]inositol phosphates as described.¹⁶⁾ Cells were seeded on 12-well plates at a density of 10⁵ cells/ml; Two days later they were labeled with F-10 containing [³H]inositol (2 μCi/ml) for 24 h.

The cells were washed twice with 0.8 ml of 20 mM HEPES-buffered Eagle's medium (pH 7.35) just prior to the assay. The reaction was initiated by the addition of drugs in 20 mM HEPES-buffered Eagle's medium (pH 7.35) containing 10 mM LiCl at 37°C. The reaction was terminated by addition of 1 ml of 5% trichloroacetic acid (TCA) after aspiration of the medium. TCA in the supernatant was removed by three washings with diethyl ether, and then the samples were applied to an anion-exchange column (AG 1X-8). The columns were washed with 6 ml of water and 6 ml of 50 mM ammonium formate to elute [³H]inositol and [³H]glycerophosphoinositol, respectively. To elute all [³H]inositol phosphates, 4 ml of 1.0 M ammonium formate in 0.1 M formic acid was added to the column. The eluate was counted by a liquid scintillation spectrophotometer with 8 ml of toluene-based scintillation cocktail.

Data Analysis The results obtained in separate experiments were expressed as mean ± S.E., and a significant difference (*p* < 0.05) was determined with ANOVA.

RESULTS

A resting level of [Ca²⁺]_i in fura-2-loaded C6 rat glioma cells was 50–150 nM in the presence of extracellular Ca²⁺. Histamine, carbachol and norepinephrine at the concentration of 100 μM increased [Ca²⁺]_i in the presence of extracellular Ca²⁺, as shown in Fig. 1. Histamine-induced increase in [Ca²⁺]_i was mediated by H₁-receptor, because the increase was completely inhibited by chlorpheniramine, a H₁-receptor antagonist (Fig. 1). Carbachol- and norepinephrine-induced increases in [Ca²⁺]_i were inhibited by atropine and prazosin, respectively (Fig. 1); carbachol therefore stimulates muscarinic receptor and norepinephrine stimulates α₁-adrenergic receptor. These agonists increased [Ca²⁺]_i in a concentration-dependent manner, as shown in Fig. 2.

Fig. 1. Effects of Histamine, Carbachol and Norepinephrine on [Ca²⁺]_i.

Cells loaded with fura-2 were suspended in modified tyrode solution. 100 μM of each of histamine (HA), carbachol (CARB) and norepinephrine (NE) (a–c) increased [Ca²⁺]_i. Chlorpheniramine (CP, 10 μM), atropine (ATR, 10 μM) and prazosin (PRA, 10 μM) was added 120 s before addition of each stimulant (d–f).

The water extract of Sho-saiko-to, a traditional Kampo medicine, inhibited histamine-induced increase in $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 3-a). San'o-shashin-to also potently inhibited histamine-induced increase in $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 3-b). The inhibitory effect of San'o-shashin-to on $[Ca^{2+}]_i$ was much more potent than that of Sho-saiko-to. The water extract of Scutellariae Radix in a concentration range of 1–30 $\mu\text{g/ml}$ inhibited histamine-induced increase in $[Ca^{2+}]_i$ (Fig. 4). The water extract of Scutellariae Radix at 30 $\mu\text{g/ml}$ also inhibited carbachol- and norepinephrine-induced increases in $[Ca^{2+}]_i$ (Fig. 5).

It is known that histamine-induced Ca^{2+} mobilization is due to IP_3 produced by activation of phospholipase C mediated via a G-protein.¹⁷⁾ Figure 6 shows the effects of Sho-saiko-to, San'o-shashin-to and Scutellariae Radix on histamine-induced accumulation of $[^3H]$ inositol phosphates. Sho-saiko-to (100 $\mu\text{g/ml}$), San'o-shashin-to (100 $\mu\text{g/ml}$) and Scutellariae Radix (50 $\mu\text{g/ml}$) significantly inhibited histamine (100 μM)-induced accumulation of $[^3H]$ inositol phosphates.

Fig. 2. Concentration-Dependent Increase in $[Ca^{2+}]_i$ by Histamine, Carbachol or Norepinephrine

Each point represents the mean value $\pm$ S.E. of three separate experiments. Abscissa: concentration of drugs (μM). Ordinate: drug-induced peak increase in $[Ca^{2+}]_i$ (nM). ●: Histamine, ▲: Carbachol, ■: Norepinephrine.

Fig. 3. Effects of Sho-saiko-to (a) and San'o-shashin-to (b) on Histamine Induced Increase in $[Ca^{2+}]_i$

Abscissa: concentration of Sho-saiko-to or San'o-shashin-to ($\mu\text{g/ml}$). Ordinate: histamine (100 μM)-induced increase in $[Ca^{2+}]_i$ (% of control). Each point represents the mean value $\pm$ S.E. of three separate experiments. ★, significant difference from histamine alone ($p < 0.05$).

DISCUSSION

C6 rat glioma cells are known to express various receptors such as endothelin_A,¹⁸⁾ angiotensin₁,¹⁹⁾ 5-HT₂,²⁰⁾ and adrenergic β -receptors.²¹⁾ In this paper, we demonstrated that C6 rat glioma cells have H₁-histamine, muscarinic cholinergic and adrenergic α_1 -receptors, which are coupled to PIP_2 -specific phospholipase C leading to $[Ca^{2+}]_i$ elevation. These receptors are assumed to have a role in the neuron-glia interaction.

Sho-saiko-to and San'o-shashin-to, traditional Chinese medicines (Kampo medicines), inhibited receptor-mediated $[Ca^{2+}]_i$ elevation, concomitant with the inhibition of phosphoinositide hydrolysis. San'o-shashin-to had a greater potency than Sho-saiko-to. Scutellariae Radix, composed of both these herbal medicines, also potently inhibited receptor-mediated $[Ca^{2+}]_i$ elevation. One interpretation of the different inhibitory potencies of Sho-saiko-to and San'o-shashin-to is the difference in content of Scutellariae Radix in these medicines. However, more detailed analysis is necessary to establish the contribution of crude drug to the inhibition of $[Ca^{2+}]_i$ elevation.

Fig. 4. Concentration-Dependent Inhibition by Scutellariae Radix of Histamine-Induced Increase in $[Ca^{2+}]_i$

Abscissa: concentration of Scutellariae Radix ($\mu\text{g/ml}$). Ordinate: histamine (100 μM)-induced increase in $[Ca^{2+}]_i$ (% of control). Each point represents the mean value $\pm$ S.E. of three separate experiments. ★, significant difference from histamine alone ($p < 0.05$).

Fig. 5. Inhibitory Effects of Scutellariae Radix on Carbachol and Norepinephrine-Induced Increase in $[Ca^{2+}]_i$

Cells loaded with fura-2 were suspended in modified tyrode solution, and drugs were added to the cell suspensions. Results were expressed as ratio of the fluorescence activated at 340 nm to that at 380 nm. (a) Carbachol (CARB, 100 μM), (b) Scutellariae Radix (30 $\mu g/ml$) was added 120 s before addition of CARB (100 μM), (c) Norepinephrine (NE, 100 μM), (d) Scutellariae Radix (30 $\mu g/ml$) was added 120 s before addition of NE (100 μM).

Fig. 6. Effects of Sho-saiko-to, San'o-shashin-to and Scutellariae Radix on Histamine-Induced Accumulation of $[^3H]$ Inositol Phosphates

The cells were incubated for 10 min with Sho-saiko-to, San'o-shashin-to or Scutellariae Radix, and were further incubated with (right) or without (left) histamine (100 μM) for an additional 10 min. Ordinate: $[^3H]$ inositol phosphates (dpm/well). (a) Sho-saiko-to (100 $\mu g/ml$), (b) San'o-shashin-to (100 $\mu g/ml$), (c) Scutellariae Radix (50 $\mu g/ml$). Each point represents the mean value $\pm$ S.E. of three separate experiments. *, significant difference from histamine alone ($p < 0.05$).

Sho-saiko-to is one of the most popular herbal medicines in Japan, and has been used for the treatment of various chronic diseases,²²⁾ because of its anti-allergy and anti-inflammatory activities.²³⁾ There are also several reports about the effectiveness of Sho-saiko-to on several disorders: an anti-human immunodeficiency virus (HIV),²⁴⁾ inhibition of the proliferation of a human hepatocellular carcinoma cell line (KIM-1) by inducing apoptosis and arresting at the G_0/G_1

phase *in vitro*,²⁵⁾ and an antimutagenic activity against AF-2 (2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide)-induced mutagenesis in *Salmonella typhimurium* TA-100.²⁶⁾

The water extract of Scutellariae Radix had a potent inhibitory effect on receptor-mediated $[Ca^{2+}]_i$ elevation. However, the extract directly decreased fura-2 fluorescence (Fig. 4), showing that the compound(s) in Scutellariae Radix interfered with the fura-2 fluorescence. This idea was supported

by the results that *Scutellariae Radix* did not directly affect phosphoinositide hydrolysis (Fig. 6). Furthermore, the inhibition of receptor-mediated $[Ca^{2+}]_i$ elevation by *Scutellariae Radix* was concomitant with the inhibition of receptor-mediated phosphoinositide hydrolysis. Since $[Ca^{2+}]_i$ elevation by stimulation of three kinds of receptors was inhibited by *Scutellariae Radix*, it is assumed that the site of action of this root is not the receptors but G-protein or phospholipase C. To our knowledge, this is the first demonstration that the water extract of *Scutellariae Radix* inhibits receptor-mediated phosphoinositide hydrolysis and $[Ca^{2+}]_i$ elevation.

In conclusion, the present study was the first to demonstrate that Sho-saiko-to, San'o-shashin-to and *Scutellariae Radix* inhibit Ca^{2+} mobilization through an inhibition of receptor-mediated activation of PIP_2 -specific phospholipase C. The inhibiting effects of Sho-saiko-to and San'o-shashin-to on PGE_2 synthesis may be explained, in part, by the inhibition of Ca^{2+} supply.

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