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Baicalin and Baicalein, Constituents of an Important Medicinal Plant, Inhibit Intracellular Ca^{2+} Elevation by Reducing Phospholipase C Activity in C6 Rat Glioma Cells

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Abstract

Glial cells have a role in maintaining the function of neural cells. This study was undertaken to clarify the effects of baicalin and baicalein, flavonoids isolated from an important medicinal plant Scutellariae Radix (the root of *Scutellaria baicalensis* Georgi), on glial cell function using C6 rat glioma cells.

Baicalin and baicalein caused concentration-dependent inhibition of a histamine-induced increase in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). The potency of baicalein was significantly greater than that of baicalin. The noradrenaline- and carbachol-induced increase in $[Ca^{2+}]_i$ was also inhibited by baicalein and both drugs inhibited histamine-induced accumulation of total [^{3}H]inositol phosphates, consistent with their inhibition of the increase in $[Ca^{2+}]_i$.

These results suggest that baicalin and baicalein inhibit $[Ca^{2+}]_i$ elevation by reducing phospholipase C activity. The inhibitory effects of baicalin and baicalein on $[Ca^{2+}]_i$ elevation might be important in the interpretation of their pharmacological action on glial cells, such as inhibition of Ca^{2+}-required enzyme phospholipase A_2.

Glial cells, which outnumber neurons by approximately ten to one in the brain, provide mechanical and metabolic support for neurons (Somjen 1988) and so have an essential role in maintaining the function of neural cells. It has been shown that glial cells express neurotransmitter receptors, such as H_1-histamine (Nakahata et al 1986), muscarinic cholinergic- (Masters et al 1985) and α_1-adrenergic receptors (Agullo & Garcia 1992), which are coupled to phosphatidylinositol 4,5-bisphosphate-specific phospholipase C mediated via pertussis toxin-insensitive G protein, Gq (Taylor et al 1991). Stimulation of these receptors results in accumulation of inositol 1,4,5-trisphosphate and intracellular Ca^{2+} mobilization. Glial cells are assumed to be an important source of prostaglandins in the central nervous system (Seregi et al 1987). Prostaglandin E_2 (PGE_2), a major prostaglandin in glial cells (Ishimoto et al 1996), is produced by catalysis of phospholipase A_2 (PLA_2) and cyclooxygenase. There are two classes of PLA_2 (Dennis 1997), cytosolic PLA_2 ($cPLA_2$) and secretory PLA_2 ($sPLA_2$). $cPLA_2$ is generally believed to exert activity by means of micromolar concentrations of Ca^{2+} ions (Nakashima et al 1989), which could be supplied by receptor-mediated phospholipase C activation (Bass et al 1994).

In Japan and China the crude drug Ogon, Scutellariae Radix (the root of *Scutellaria baicalensis* Georgi), has been employed for centuries as an important medicine. We have previously demonstrated that baicalein, a flavonoid contained in Ogon, inhibited PGE_2 synthesis in C6 rat glioma cells (Nakahata et al 1998). In this study we examined whether baicalin and baicalein affected intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and phosphoinositide hydrolysis in C6 rat glioma cells. The results obtained suggest that these drugs inhibit receptor-mediated $[Ca^{2+}]_i$ elevation and phosphoinositide hydrolysis.

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Materials and Methods

Materials

Foetal bovine serum was obtained from Cell Culture Laboratory (Cleveland, OH), horse serum from Dainippon (Tokyo, Japan), F-10 (Nutrient Mixture: Ham) from Gibco BRL (Tokyo, Japan), fura 2-AM from Dojindo (Kumamoto, Japan), Triton X-100 from Wako (Tokyo, Japan), EGTA from Nakarai (Kyoto, Japan) and collagenase from Warthington (Freehold, NJ). Baicalin and baicalein, purified from Scutellariae Radix (root of *Scutellaria baicalensis* Georgi, 3·0 g), were dissolved in dimethylsulphoxide and used after dilution. Other chemicals and drugs were of reagent grade or of the highest quality available.

Cell culture

C6 rat glioma cells were grown in F-10 medium containing 15% horse serum and 2·5% foetal bovine serum in a 37°C humidified incubator in an atmosphere of 5% CO_2 in air.

Measurement of intracellular free Ca^{2+} concentration with fura 2

Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured as described previously (Nakahata et al 1994). C6 rat glioma cells cultured on a 150-mm dish were washed three times with modified Tyrode solution (composition, mM: NaCl 137, KCl 2·7, $MgCl_2$ 1·0, $CaCl_2$ 0·18, glucose 5·6, HEPES 10, pH 7·4). The cells were separated from the dish by treatment with 0·1% collagenase and 1·0% bovine serum albumin in modified Tyrode solution (10 mL) for 15 min at 37°C, and were collected into a 50-mL tube and centrifuged at 250 *g* for 1 min. After washing once with modified Tyrode solution (10 mL), the cells ($1-5 \times 10^6$ mL^{-1}) were treated with 1 μM fura 2-AM for 15 min and washed twice with modified Tyrode solution. The cells were suspended ($1-5 \times 10^6$ mL^{-1}) and the cell suspension (1–2 mL) was used for fura 2 assay. Fluorescence of fura 2 at 510 nm after excitation at 340 and 380 nm was monitored simultaneously by spectrofluorimetry (Hitachi, F-2000). The ratio of fluorescence at 510 nm after excitation at 340 nm to that after excitation at 380 nm was calculated as relative $[Ca^{2+}]_i$. Occasionally $[Ca^{2+}]_i$ was calculated by using the K_d of fura 2 to Ca^{2+} as 224 nM.

Measurement of [^{3}H]inositol phosphates

Phosphoinositide breakdown was monitored by measuring [^{3}H]inositol phosphates as described previously (Nakahata et al 1996). Cells were seeded on 12-well plates at a density of 10^5 cells mL^{-1}. After two days the cells were labelled with F-10 containing [^{3}H]inositol (2 μCi mL^{-1}) for 24 h. Just before the assay the cells were washed with HEPES-buffered Eagle's medium (pH 7·35, 20 mM; 2 × 0·8 mL). The reaction was initiated by addition of the drugs in HEPES-buffered Eagle's medium (pH 7·35; 20 mM) containing 10 mM LiCl at 37°C. The reaction was terminated by the addition of 5% trichloroacetic acid (1 mL) after aspiration of the medium. Trichloroacetic acid in the supernatant was removed by washing three times with diethyl ether and the samples were then applied to anion-exchange columns (AG 1X-8). The columns were washed with water (6 mL) and ammonium formate (50 mM, 6 mL) to elute [^{3}H]inositol and [^{3}H]glycerophosphoinositol, respectively. Ammonium formate (1·0 M) in formic acid (0·1 M, 4 mL) was then added to the column to elute all [^{3}H]inositol phosphates. The effluent was counted by means of a liquid-scintillation spectrophotometer and a toluene-based scintillation fluid (8 mL).

Data analysis

The results obtained are expressed as means ± s.e.m. (standard error of the mean) and differences between results were evaluated by Student's *t*-test, with $P < 0.05$ being regarded as indicative of significance.

Results

The resting level of $[Ca^{2+}]_i$ in fura 2-loaded C6 rat glioma cells was 50–100 nM in the presence of extracellular Ca^{2+}. Although a low concentration (3 μM) of baicalin, an ingredient of Scutellariae Radix, slightly augmented the histamine-induced $[Ca^{2+}]_i$ elevation, high concentrations (> 10 μM) significantly reduced the $[Ca^{2+}]_i$ level in a concentration-dependent manner (Figure 1). Baicalein, another active ingredient of Scutellariae Radix, inhibited histamine-induced $[Ca^{2+}]_i$ elevation in a concentration-dependent manner (Figure 1). The potency of baicalein was greater than that of baicalin. The carbachol- or noradrenaline-induced increase in $[Ca^{2+}]_i$ was inhibited by baicalein (Figure 2), suggesting that it has a different underlying mechanism from the inhibition of the H_1 receptor. Baicalin also had a weak inhibitory effect on the carbachol- or noradrenaline-induced increase in $[Ca^{2+}]_i$ (data not shown).

It is well known that histamine-induced Ca^{2+} mobilization is a result of inositol 1,4,5-trisphosphate produced by activation of phospholipase C (Nakahata & Harden 1987). Histamine caused an accumulation of [^{3}H]inositol phosphates in C6 rat glioma cells labelled with [^{3}H]inositol. Baicalin and baicalein (30 μM) significantly

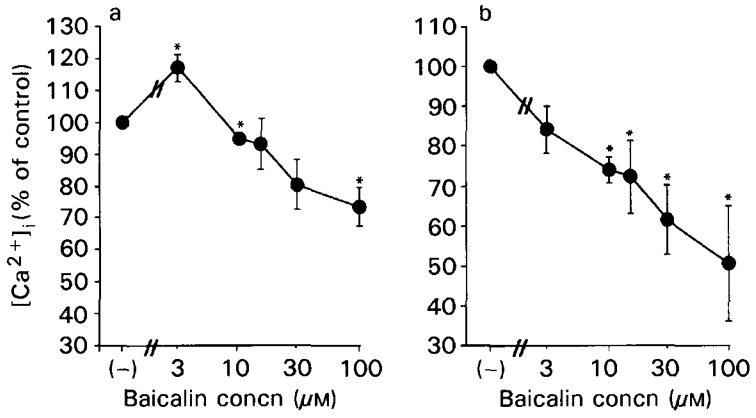

Figure 1. Effects of baicalin (a) and baicalein (b) on the histamine-induced increase in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). Abscissa—concentration of baicalin or baicalein (mM). Ordinate—histamine (100 mM)-induced increase in $[Ca^{2+}]_i$ (% of control). Each point represents the mean ± s.e.m. of results from three separate experiments. * $P < 0.05$, significantly different from result for histamine alone.

inhibited the histamine-induced accumulation of [^{3}H]inositol phosphates (Table 1). Baicalein was a much more potent inhibitor than baicalin, a result consistent with their effects on $[Ca^{2+}]_i$.

Discussion

Baicalin and baicalein, major flavonoids from Scutellariae Radix, clearly inhibited receptor-mediated Ca^{2+} mobilization and phosphoinositide hydrolysis in C6 rat glioma cells. Baicalein was much more effective than baicalin. Kimura et al (1987, 1997) reported that baicalein inhibited Ca^{2+} ionophore A23187-induced $[Ca^{2+}]_i$ elevation in polymorphonuclear leukocytes and thrombin receptor-mediated $[Ca^{2+}]_i$ elevation in umbilical-vein endothelial cells in man. This study provides evidence that inhibition of $[Ca^{2+}]_i$ elevation by flavonoids is because they inhibit phosphoinositide hydrolysis in C6 rat glioma cells. To the best of our knowledge the inhibition of phosphoinositide hydrolysis by baicalein and baicalin has not been reported. Because baicalin and baicalein reduce $[Ca^{2+}]_i$ elevation in C6 rat glioma cells, they will modify the receptor-mediated, Ca^{2+}-required function of glial cells. Glial cells might contribute to a pyrogenic reaction in the brain by production of PGE_2. $cPLA_2$, a Ca^{2+}-sensitive enzyme, induces liberation of arachidonic acid, a precursor of prostaglandins. The inhibitory effects of baicalin and

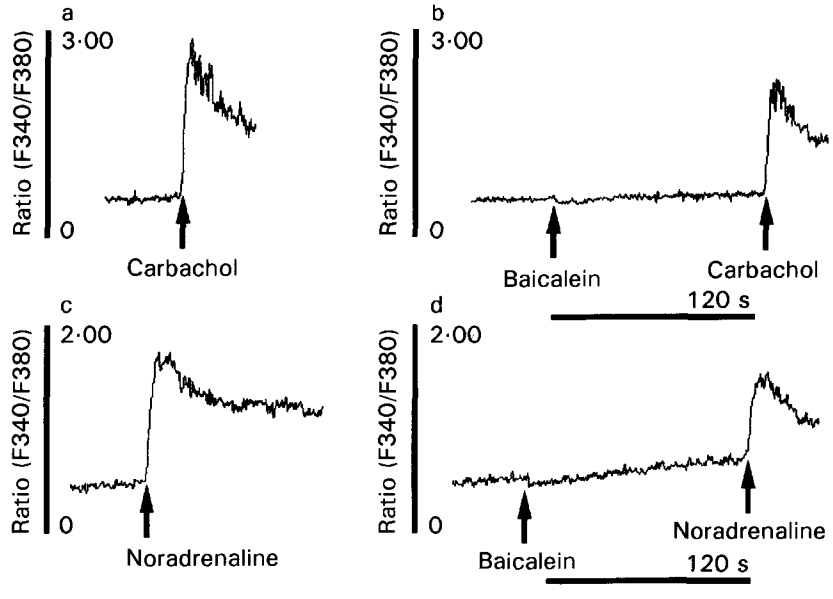

Figure 2. Inhibitory effects of baicalein on carbachol- or noradrenaline-induced increases in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). Cells loaded with fura 2 were suspended in modified Tyrode solution and the drugs were added. a. Carbachol (100 μM); b. baicalein (100 μM) added 120 s before addition of carbachol (100 μM); c. noradrenaline (100 μM); d. baicalein (100 μM) added 120 s before addition of noradrenaline (100 μM).

Table 1. Effects of baicalin and baicalein on histamine-induced accumulation of [^{3}H]inositol phosphates.

	Amount of [^{3}H]inositol phosphates (disintegrations min^{-1}/well)
Control	2258 ± 209
Control + 30 μM baicalin	2119 ± 355
Control + 30 μM baicalein	2267 ± 110
Control + 100 μM histamine	7918 ± 512
Control + 100 μM histamine + 30 μM baicalin	5572 ± 695*
Control + 100 μM histamine + 30 μM baicalein	3764 ± 79*

The cells were incubated for 10 min with or without the drug, and were further incubated with or without histamine (100 μM) for a further 10 min. Data are means ± s.e.m. of results from three separate experiments. * $P < 0.05$, significantly different from result for histamine alone.

baicalein on $[Ca^{2+}]_i$ elevation might be related to their inhibition of $cPLA_2$ by reducing the supply of Ca^{2+}. It is well known that baicalin and baicalein have important pharmacological activity, including functioning as anti-allergens (Koda et al 1970). Baicalin has been reported to inhibit the activity of reverse transcriptases from murine leukaemia and human immunodeficiency virus (HIV) (Zhang et al 1991), and lipid peroxidation by scavenging hydroxyl and superoxide radicals (Honglian et al 1995). Baicalein is also a selective inhibitor of platelet 12-lipoxygenase (Sekiya & Okuda 1982). The inhibition of phosphoinositide hydrolysis and Ca^{2+}mobilization by baicalin and baicalein should be regarded as another important pharmacological activity of flavonoids. In conclusion, this research demonstrates for the first time that baicalin and baicalein act as potent inhibitors of $[Ca^{2+}]_i$ elevation by reducing agonist-induced activation of phosphatidylinositol 4,5-bisphosphate-specific phospholipase C.

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References

Agullo, L., Garcia, A. (1992) Characterization of noradrenaline-stimulated cyclic GMP formation in brain astrocytes in culture. Biochem. J. 288: 619–624

Bass, D. A., Seeds, M. C., Jones, D. F., Chilton, F. H., Bauldry, S. A. (1994) Priming of phospholipase A_2 of human neutrophils by tumor necrosis factor. Chest 105: 99–100

Dennis, E. A. (1997) The growing phospholipase A_2 superfamily of signal transduction enzymes. Trends Biochem. Sci. 22: 1–2

Honglian, S., Baolu, Z., Wenjuan, X. (1995) Scavenging effects of baicalin on free radicals and its protection of erythrocyte membranes from free radical injury. Biochem. Mol. Biol. Int. 35: 981–994

Ishimoto, H., Matsuoka, I., Nakanishi, H., Nakahata, N. (1996) A comparative study of arachidonic acid metabolism in rabbit cultured astrocytes and human astrocytoma cells (1321N1). Gen. Pharmacol. 27: 313–317

Kimura, Y., Okuda, H., Arichi, S. (1987) Effects of baicalein on leukotriene biosynthesis and degranulation in human polymorphonuclear leukocytes. Biochim. Biophys. Acta 922: 278–286

Kimura, Y., Yokoi, K., Matsushita, N., Okuda, H. (1997) Effects of flavonoids isolated from Scutellariae Radix on the production of tissue-type plasminogen activator and plasminogen activator inhibitor-1 induced by thrombin and thrombin receptor agonist peptide in cultured human umbilical vein endothelial cells. J. Pharm. Pharmacol. 49: 816–822

Koda, A., Nagai, H., Toshida, Y., Lauw, H. K. (1970) Pharmacological actions of baicalin and baicalein. 3. Action on experimental asthma. Folia Pharmacol. Japon. 66: 471–486

Masters, S. B., Martin, M. W., Harden, T. K., Brown, J. H. (1985) Pertussis toxin does not inhibit muscarinic receptor-mediated phosphoinositide hydrolysis or calcium mobilization. Biochem. J. 227: 933–937

Nakahata, N., Harden, T. K. (1987) Regulation of inositol trisphosphate accumulation by muscarinic cholinergic and H_1-histamine receptors on human astrocytoma cells. Biochem. J. 241: 337–344

Nakahata, N., Martin, M. W., Hughes, A. R., Hepler, J. R., Harden, T. K. (1986) H_1-histamine receptors on human astrocytoma cells. Mol. Pharmacol. 29: 188–195

Nakahata, N., Ishimoto, H., Mizuno, K., Ohizumi, Y., Nakanishi, H. (1994) Dual effects of mastoparan on intracellular free Ca^{2+} concentrations in human astrocytoma cells. Br. J. Pharmacol. 112: 299–303

Nakahata, N., Imata, K., Ohkawa, T., Watanabe, Y., Ishimoto, H., Ono, T., Ohizumi, Y., Nakanishi, H. (1996) Mastoparan elicits prostaglandin E_2 generation and inhibits inositol phosphate accumulation via different mechanisms in rabbit astrocytes. Biochim. Biophys. Acta 1310: 60–66

Nakahata, N., Kutsuwa, M., Kyo, R., Kubo, M., Hayashi, K., Ohizumi, Y. (1998) Analysis of inhibitory effects of Scutellariae Radix and baicalein on prostaglandin E_2 production in rat C6 glioma cells. Am. J. Chin. Med. In press

Nakashima, S., Sugamura, A., Sato, M., Tohmatsu, T., Nozawa, Y. (1989) Mechanism of arachidonic acid liberation in platelet-activating factor-stimulated human polymorphonuclear neurophils. J. Immunol. 143: 1295–1302

Sekiya, K., Okuda, H. (1982) Selective inhibition of platelet lipoxygenase by baicalein. Biochem. Biophys. Res. Commun. 105: 1090–1095

Seregi, A., Keller, M., Hertting, G. (1987) Are cerebral prostanoids of astroglial origin? Studies on the prostanoid forming system in developing rat brain and primary cultures of rat astrocytes. Brain Res. 404: 113–120

Somjen, G. G. (1988) Nervenkitt: notes on history of the concept of neuroglia. Glia 1: 2–9

Taylor, S. J., Chae, H. Z., Rhee, S. G., Exton, J. H. (1991) Activation of the beta 1 isozyme of phospholipase C by alpha subunits of the Gq class of G proteins. Nature 350: 516–518

Zhang, X., Tang, X., Chen, H. C. (1991) Inhibition of HIV replication by baicalin and *S. baicalensis* extracts in H9 cell culture. Chin. Med. Sci. J. 6: 230–232