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European Journal of Pharmacology 461 (2003) 1–7
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Baicalein inhibits Raf-1-mediated phosphorylation of MEK-1 in C6 rat glioma cells

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Received 29 August 2002; received in revised form 18 December 2002; accepted 20 December 2002

Abstract

Baicalein is a flavonoid derived from the *Scutellaria* root. In investigations of the inhibitors of prostaglandin synthesis in C6 rat glioma cells, we found that baicalein had a potent inhibitory activity on prostaglandin synthesis induced by either histamine or A23187, a Ca^{2+} ionophore. Baicalein inhibited histamine- or A23187-induced phosphorylation of p42/p44 extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), which causes the phosphorylation of cytosolic phospholipase A_2 (PLA_2). Baicalein also inhibited the phosphorylation of MAPK kinase-1 (MEK-1) induced by histamine or A23187 in the cells. To examine the site of action of baicalein, MEK-1 and Raf-1 were prepared by immunoprecipitation with anti-MEK-1 and anti-Raf-1 antibodies, respectively. Baicalein inhibited the phosphorylation of exogenous MEK-1 by Raf-1 under cell-free conditions, while it did not change the phosphorylation of exogenous p42 MAPK by MEK-1. These results imply that baicalein inhibits the ERK/MAPK cascade, acting on the phosphorylation of MEK-1 by Raf-1.

Keywords: Baicalein; p42/p44 MAPK (ERK); MEK-1; Raf-1; Prostaglandin E_2

1. Introduction

Arachidonic acid, a precursor of eicosanoids, is liberated from membrane phospholipids by phospholipase A_2 (PLA_2). PLA_2 is subdivided into several groups based on its structure and enzymatic characteristics (Dennis, 1997; Leslie, 1997; Tischfield, 1997). $sPLA_2$ is a family of low-molecular mass (~ 14 kDa) enzymes, which require millimolar concentrations of Ca^{2+} for enzymatic activity. $cPLA_2$, or group IV PLA_2, is a ubiquitously distributed 85-kDa enzyme, the activation of which is tightly regulated by postreceptor transmembrane signaling (Clark et al., 1991; Sharp et al., 1991; Kim et al., 1991). $cPLA_2$ is activated by p42/p44 extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) when cytosolic Ca^{2+} concentrations are in the submicromolar or micromolar range (Lin et al., 1993; Pumiglia and Decker, 1997).

MAPK is a family of serine/threonine kinases and appears to be a common component of the signaling pathway initiated by a wide range of factors including hormones, differentiation factors and mitogens. MAPKs are now classified into three subgroups: p42/p44 ERK/MAPK, JNK protein kinase and p38 MAPK (Davis, 1994). p42/p44 ERK/MAPK is activated by phosphorylation on both tyrosine and threonine (Nishida and Gotoh, 1993). This reaction is catalyzed by a specific threonine/tyrosine-directed kinase, MAPK kinase-1 (MEK-1). In turn, MEK-1 is phosphorylated and activated by the serine/threonine kinase, Raf-1 (Nishida and Gotoh, 1993; Marshall, 1995). Thus, the signaling pathway of ERK/MAPK activation is called the MAPK cascade.

Glial cells, which outnumber neurons by about 10 to 1 in the brain, provide neurons with both mechanical and metabolic supports. Glial cells are assumed to be an important source of prostaglandins in the central nervous system (Minghetti et al., 1998). Prostaglandin E_2 (PGE_2), a main product of prostanoids in glial cells (Ishimoto et al., 1996), is

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doi:10.1016/S0014-2999(02)02950-3

produced by catalysis of arachidonic acid by PLA_2 and cyclooxygenase. It has been shown that glial cells express neurotransmitter receptors, such as histamine-H_1 (Nakahata et al., 1986), muscarinic acetylcholine- (Masters et al., 1985) and α_1-adrenoceptors (Agullo and Garcia, 1992), which are coupled to phosphatidylinositol 4,5-bisphosphate (PIP_2)-specific phospholipase C mediated via pertussis toxin-insensitive G protein, $G_{q/11}$ (Taylor et al., 1991). Stimulation of these receptors results in accumulation of inositol 1,4,5-trisphosphate (IP_3) and intracellular Ca^{2+} mobilization. Cytosolic PLA_2 is generally believed to exert its activity at micromolar concentrations of Ca^{2+} (Lin et al., 1992; 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 *Scutellaria* root (the root of *Scutellaria baicalensis* Georgi) has been used for centuries as an important folk medicine. We previously demonstrated that baicalein, a flavonoid contained in *Scutellaria* root, inhibited prostaglandin E_2 synthesis and the phosphorylation of p42/44 ERK/MAPK in C6 rat glioma cells (Nakahata et al., 1998). In the present study, we examined the effect of baicalein on the ERK/MAPK cascade in C6 rat glioma cells. The results obtained suggest that baicalein inhibits the ERK/MAPK cascade by acting on the phosphorylation of MEK-1 by Raf-1.

2. Materials and methods

2.1. 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% CO_2 in air, as described previously (Nakatani et al., 2002).

2.2. Assay of prostaglandin E_2

C6 cells were seeded into 12-well plates at the density of 1.0×10^5 cells per well. The experiment was performed 2 days after cell seeding. The cells were washed twice with Eagle's minimum essential medium (EMEM) buffered with 20 mM HEPES, pH 7.35 (EMEM-HEPES) and were preincubated with or without baicalein for 10 min. The cells were further incubated with histamine or baicalein for an additional 10 min. The medium was acidified to pH 4.0 by addition of 1N HCl, and prostaglandin E_2 was extracted twice with ethyl acetate. After ethyl acetate was evaporated under a stream of N_2 gas, the sample was dissolved in 10 mM Tris–HCl (pH 7.6). Prostaglandin E_2 was determined by radioimmunoassay, as described previously (Nakahata et al., 1996).

2.3. Immunoblotting

C6 cells were seeded into 6-well plates at a density of 1.0×10^5 cells per well. Two days after seeding, the cells were washed twice with EMEM-HEPES and preincubated with or without baicalein for 10 min at 37 °C. After the cells were incubated with histamine or A23187 for an additional 2 min, the medium was aspirated. The cells were solubilized by the addition of Laemmli sample buffer (Laemmli, 1970), the composition of which was Tris–HCl 187.5 mM, sodium dodecyl sulfate (SDS) 6%, glycerol 30%, 2-mercaptoethanol 15%, pH 6.8. The sample was boiled at 95 °C for 5 min. Electrophoresis was performed on 11% acrylamide gels. Proteins were transferred electrically from the gel onto Immobilon polyvinylidene difluoride membranes (Millipore) by the semidry blotting method (Ohkubo et al., 1996). The immunoblots were blocked for 2 h with 2% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20 (TBST) at 25 °C, and incubated with anti-phospho-MAPK antibody (rabbit) or anti-phospho-MEK antibody (rabbit) at 1 μg/ml for 2 h at 25 °C. The immunoblots were washed several times and incubated overnight at 4 °C with a 1:2000 dilution of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) in TBST containing 2% bovine serum albumin. Blots were developed by using a chemiluminescence assay kit (Bio Rad) and visualized by exposing the membrane to Hyper-film ECL (Amersham).

2.4. Kinase assay in a cell-free condition

C6 rat glioma cells (2.0×10^5 cells/ml) cultured in 35-mm dishes were activated by 100 nM phorbol 12-myristate 13-acetate (PMA) and 1 μM A23187 for 10 min. The reaction was terminated by addition of 0.5 ml of lysate buffer, the composition of which was Tris–HCl 20 mM, NaCl 150 mM, EDTA 1 mM, EGTA 1 mM, Triton X-100 1%, β-glycerophosphate 1 mM, orthovanadate 1 mM, (*p*-amidinophenyl)methanesulfonyl fluoride 1 mM, leupeptin 1 μg/ml, aprotinin 10 μg/ml, pH 7.5. The cells were scraped off and homogenized by sonication, then the supernatant was obtained by centrifugation at $15{,}000 \times g$ for 5 min. The supernatant was incubated overnight at 4 °C with a 1:100 dilution of anti-phospho-MEK antibody (rabbit) or a 1:100 dilution of anti-Raf-1 antibody (rabbit) and then further incubated at 4 °C for 5 h after addition of 50 μl of protein A Sepharose. The immunoprecipitated materials were obtained by centrifugation at $5200 \times g$ for 3 min. After extensive washing with lysis buffer, the final pellet was resuspended in 1 ml of assay buffer, the composition of which was Tris–HCl 25 mM, β-glycerophosphate 5 mM, dithiothreitol 2 mM, orthovanadate 0.1 mM, $MgCl_2$ 10 mM, ATP 0.1 mM, pH 7.5. For analysis of MEK-1 activity, the sample was incubated at 37 °C for 30 min with a peptide of nonphosphorylated MAPK as substrate in 40 μl of assay buffer in a final volume of 50 μl. The reaction was terminated by addition of Laemmli sample buffer, followed by SDS-polyacrylamide electrophoresis (PAGE) and immunoblotting using anti-phospho-MAPK antibody as first antibody. For analysis of Raf-1 activity, the sample was

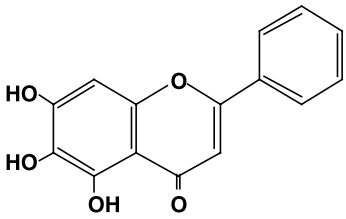

Fig. 1. Chemical structure of baicalein.

incubated at 30 °C for 30 min with a peptide of His-MEK-1 as substrate in the assay buffer containing 166 μCi/ml of [γ-^{32}P]ATP in a final volume of 30 μl. The reaction was terminated by addition of Laemmli sample buffer. After SDS-PAGE, the phosphorylated His-MEK was analyzed by using a molecular imager (GS363, Bio-Rad, Hercules, CA).

2.5. Data analysis

The statistical significance of differences between values ($P<0.05$ or $P<0.01$) was determined with analysis of variance (ANOVA).

2.6. Materials

Fetal bovine serum was obtained from Cell Culture Laboratory (Cleveland, OH). Horse serum was obtained from Dainippon Pharmaceutical (Tokyo, Japan). F-10 (Nutrient Mixture: Ham) was from GIBCO BRL (Tokyo, Japan). EMEM was purchased from Nissui Pharmaceutical (Tokyo, Japan). Prostaglandin E_2 and anti-prostaglandin E_2 antibody were generous gifts from Ono Pharmaceuticals (Osaka, Japan). [^{3}H] prostaglandin E_2 (200 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA, USA). Anti-phospho-ERK/MAPK antibody (rabbit), anti-phospho-MEK-1 antibody (rabbit), anti-ERK/MAPK antibody (rabbit), anti-MEK-1 antibody (rabbit), alkaline phosphatase-conjugated goat anti-rabbit IgG, inactive MAPK (ERK 2) and 2′-amino-3′-methoxy-flavone (PD98059) were obtained from New England Biolabs (Beverly, MA, USA). Anti-Raf-1 antibody (rabbit) and MEK-1 (full length) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Baicalein (Fig. 1) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in dimethylsufoxide to make a concentration of 50 mM for use. Other chemicals and drugs were of reagent grade or of the highest quality available.

3. Results

3.1. Effect of baicalein on histamine- or A23187-induced prostaglandin E_2 release from C6 rat glioma cells

Histamine stimulated prostaglandin E_2 release from C6 rat glioma cells mediated by histamine H_1 receptor. The histamine-induced release of prostaglandin E_2 was inhibited by baicalein in a concentration-dependent manner with an IC_{50} value of about 5 μM (Fig. 2A). A23187, a Ca^{2+} ionophore, also stimulated prostaglandin E_2 release from C6 cells. The A23187-induced release of prostaglandin E_2 was also inhibited by baicalein in a concentration-dependent manner with an IC_{50} value of about 8 μM (Fig. 2B). These results suggest that the site of action of baicalein may be downstream of receptor-G protein-phospholipase C, because it inhibited A23187-induced prostaglandin E_2 release.

3.2. Effect of baicalein or PD98059 on the phosphorylation of ERK/MAPK

p42/44 ERK/MAPK is known to stimulate cytosolic PLA_2 by phosphorylation (Pumiglia and Decker, 1997;

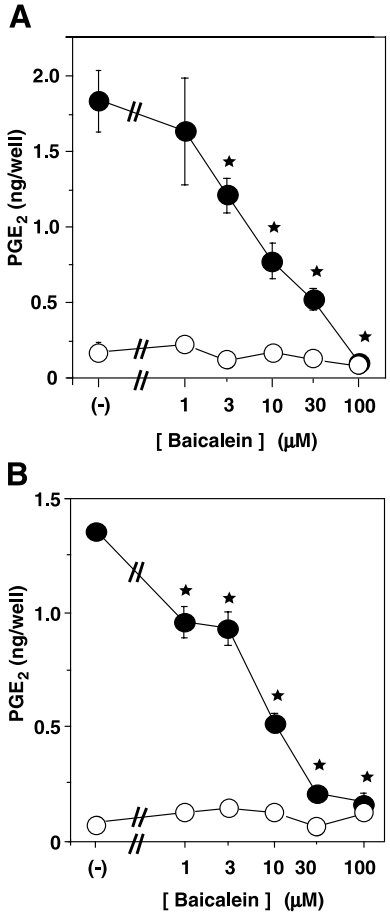

Fig. 2. Effect of baicalein on prostaglandin E_2 release from C6 cells. (A) The cells were preincubated with indicated concentrations of baicalein for 10 min, and then they were stimulated by 100 μM histamine (●) or vehicle (○) for 10 min. (B) The cells were preincubated with indicated concentrations of baicalein for 10 min, and then they were stimulated by 10 μM A23187 (●) or vehicle (○) for 10 min. The released prostaglandin E_2 (PGE_2) in the medium was determined by radioimmunoassay. Each point represents the mean with S.E. of three determinations. The data are representative of three independent experiments. Significant difference from without histamine or A23187 (*$P<0.05$).

Davis, 1994; Nishida and Gotoh, 1993). Histamine caused the potent phosphorylation of p42/44 ERK/MAPK, which was a concentration dependently inhibited by baicalein (Fig. 3A). Furthermore, baicalein was able to inhibit A23187-induced phosphorylation of p42/44 ERK/MAPK in a concentration-dependent manner (Fig. 3B). PD98057, a known inhibitor of MAPK kinase (MEK) (Pang et al., 1995), also inhibited A23187-induced phosphorylation of p42/44 ERK/MAPK in a concentration-dependent manner (Fig. 3C). Thus, it is suggested that the site of action of baicalein may be upstream of p42/44 ERK/MAPK.

3.3. Effect of baicalein or PD98059 on the phosphorylation of MEK

Since MEK-1 is known to cause the phosphorylation of p42/44 ERK/MAPK (Nishida and Gotoh, 1993; Marshall, 1995), we next examined whether baicalein affected the phosphorylation of MEK-1. Histamine caused the phosphorylation of MEK-1, which was inhibited by baicalein in a concentration-dependent manner (Fig. 4A). Furthermore, baicalein potently inhibited A23187-induced phosphorylation of MEK-1 in a concentration-dependent manner (Fig. 4B). PD98059 also inhibited A23187-induced phosphorylation of MEK-1 in a concentration-dependent manner (Fig. 4C).

3.4. Effect of baicalein or PD98059 on the activity of MEK-1 or Raf-1 under cell-free conditions

To clarify the target protein of baicalein in the ERK/MAPK cascade, an in vitro kinase assay was carried out by using the immunoprecipitate with anti-MEK-1 antibody or anti-Raf-1 antibody. The cells were incubated with A23187 and PMA for 10 min to activate the ERK/MAPK cascade. Then, the activated MEK-1 was immunoprecipitated with

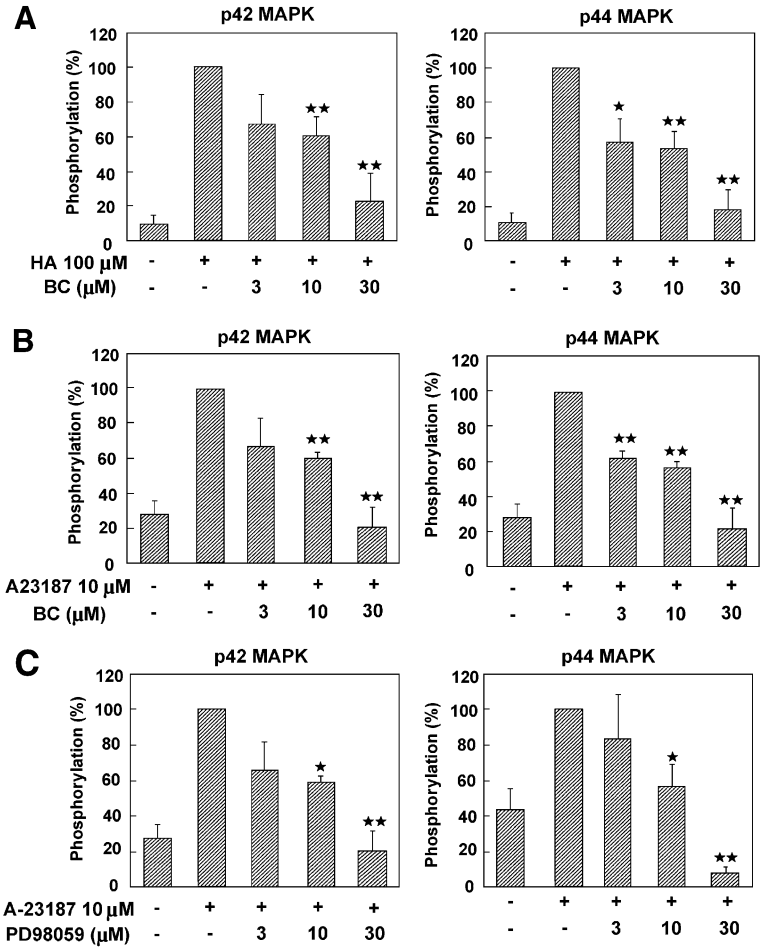

Fig. 3. Effect of baicalein or PD98059 on histamine- or A23187-induced phosphorylation of ERK/MAPK in intact C6 cells. The cells were preincubated with indicated concentrations of baicalein (BC) or PD98059 for 10 min, and then they were stimulated by 100 μM histamine or 10 μM A23187 for 2 min. The samples were separated by SDS-PAGE followed by immunoblotting using anti-phospho-ERK/MAPK antibody and anti-ERK/MAPK antibody. The density of phospho-ERK/MAPK was normalized to the density of ERK/MAPK in each sample. Phosphorylation is expressed as percentage of that in response to histamine or A23187 alone. The results of p42 MAPK and p44 MAPK are shown on the left and right, respectively. (A) Effect of baicalein on histamine-induced phosphorylation. (B) Effect of baicalein on A23187-induced phosphorylation. (C) Effect of PD98059 on A23187-induced phosphorylation. Each column represents the mean with S.E. of three determinations. Significant difference from histamine or A23187 alone (*$P<0.05$; **$P<0.01$).

anti-phospho-MEK-1 antibody. The immunoprecipitate was used as enzyme in the kinase assay with exogenous p42 ERK/MAPK (ERK 2) as substrate. p42 ERK/MAPK was phosphorylated during incubation for 30 min at 30 °C with the immunoprecipitated MEK-1 in vitro, determined by anti-phospho-ERK/MAPK antibody (Fig. 5A). Baicalein did not inhibit the phosphorylation of p42 ERK/MAPK by MEK-1 in vitro. PD98059 also failed to inhibit the in vitro phosphorylation of p42 ERK/MAPK (data not shown). Next, the activity of Raf-1 was investigated in vitro by determining the phosphorylation of exogenous MEK-1 as substrate for Raf-1, using the immunoprecipitate with anti-Raf-1 antibody and [γ-^{32}P]ATP. The in vitro phosphorylation of exogenous MEK-1 was clearly inhibited by baicalein in a concentration-dependent manner (Fig. 5B). The in vitro phosphorylation of MEK-1 was also inhibited by PD98059 (data not shown). Thus, baicalein is assumed to be an inhibitor of the phosphorylation of MEK-1 by Raf-1.

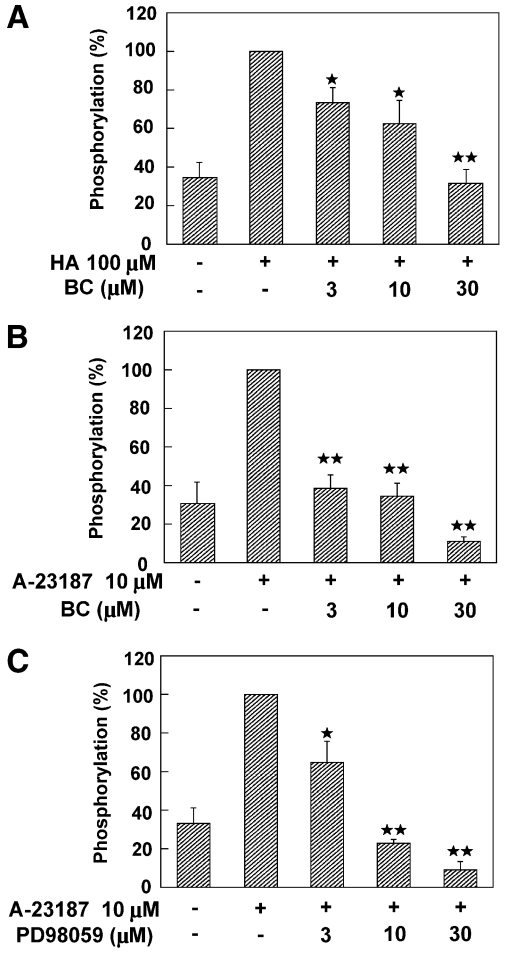

Fig. 4. Effect of baicalein or PD98059 on histamine- or A23187-induced phosphorylation of MEK in intact C6 cells. The cells were preincubated with indicated concentrations of baicalein (BC) or PD98059 for 10 min, then they were stimulated by 100 μM histamine or 10 μM A23187 for 2 min. The samples were separated by SDS-PAGE followed by immunoblotting using anti-phospho-MEK antibody and anti-MEK antibody. The density of phospho-MEK was normalized to the density of MEK in each sample. Phosphorylation is expressed as percentage of that in response to histamine or A23187 alone. (A) Effect of baicalein on histamine-induced phosphorylation. (B) Effect of baicalein on A23187-induced phosphorylation. (C) Effect of PD98059 on A23187-induced phosphorylation. Each column represents the mean with S.E. of three determinations. Significant difference from histamine alone (*$P<0.05$; **$P<0.01$).

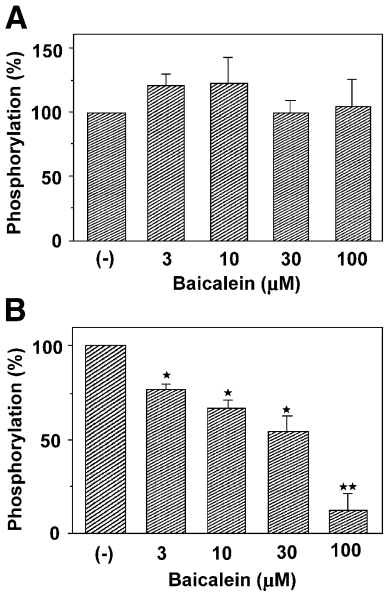

Fig. 5. Effect of baicalein on the activity of MEK-1 or Raf-1 in a cell-free system. (A) Effect of baicalein on the activity of MEK-1. In vitro kinase assay was carried out as described in Materials and methods. The samples were preincubated with indicated concentrations of baicalein (BC) for 10 min, and then they were incubated with nonphosphorylated p42 MAPK for 30 min. The phosphorylated p42 MAPK was detected by using anti-phospho-MAPK antibody and immunoblotting. Phosphorylation was calculated from the density as the phosphorylation in the absence of baicalein of 100%. Each column represents the mean with S.E. of three determinations. (B) Effect of baicalein on the activity of Raf-1. In vitro kinase assay was carried out as described in Materials and methods. The samples were preincubated with indicated concentrations of baicalein (BC) for 10 min, and then they were further incubated with [γ-^{32}P]ATP and His-MEK-1 for an additional 30 min. The samples were separated by SDS-PAGE, and the ^{32}P-phosphorylation of MEK-1 was determined by using a molecular imager. Phosphorylation was calculated from the density as the phosphorylation in the absence of baicalein of 100%. Each column represents the mean with S.E. of three determinations. Significant difference from without baicalein (*$P<0.05$; **$P<0.01$).

4. Discussion

The present study demonstrated that baicalein, a flavonoid of natural origin, potently and selectively inhibits the ERK/MAPK cascade in C6 rat glioma cells, resulting in the inhibition of prostaglandin E_2 release from the cells. The site of action of baicalein is assumed to be the phosphorylation of MEK-1 by Raf-1, since the activity of Raf-1, but not MEK-1, was clearly inhibited by baicalein (Fig. 6).

Baicalein is a flavonoid derived from *Scutellaria* root, which has been used as a crude drug in the treatment of inflammation for many centuries in Japan and China.

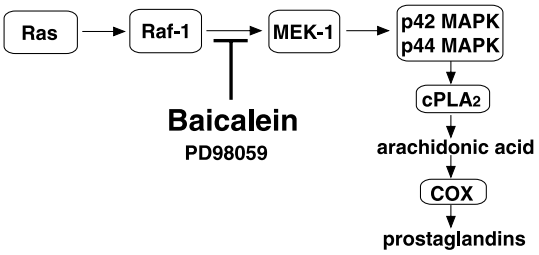

Fig. 6. Possible mechanism of baicalein in causing inhibition of the ERK/MAPK cascade.

Baicalein has been reported to possess several pharmacological activities, such as anti-inflammatory actions (Lin and Shieh, 1996), inhibition of leukotriene production (Kimura et al., 1987), inhibition of prostaglandin synthesis (Nakahata et al., 1998), inhibition of platelet lipoxygenase (Sekiya and Okuda, 1982), inhibition of sialidase (Nagai et al., 1989), antitumor effects in human cancer cell lines (Motoo and Sawabu, 1994), induction of apoptosis (Chang et al., 2002; Liu et al., 2002), antiproliferative effects (Huang et al., 1994; Nagao et al., 2002), inhibition of xanthine oxidase (Chang et al., 1993) and potentiation of smooth muscle contraction (Berger et al., 1992; Miyamoto et al., 1997). The present finding that baicalein inhibits the ERK/MAPK cascade can explain several pharmacological effects of baicalein, such as its anti-inflammatory effect, inhibition of prostaglandin synthesis, inhibition of leukotriene production, antiproliferative effect and antitumor effect. We previously reported that baicalein inhibited the receptor-mediated activation of phospholipase C, resulting in the inhibition of Ca^{2+} mobilization in cells (Kyo et al., 1998). Thus, it is assumed that baicalein inhibits arachidonic acid liberation through its inhibition of the ERK/MAPK cascade and receptor-mediated Ca^{2+} supply in intact cells.

It has been shown that two inhibitors of the ERK/MAPK cascade, PD98059 (Pang et al., 1995) and 1,4-diamino-2,3-dicyano-1, 4-bis[2-aminophenylthio]butadiene (U0126) (Favata et al., 1998), are commonly used for analysis or evaluation of the ERK/MAPK cascade in cells. PD98059 was discovered through the investigation of its inhibitory activity on NGF-induced neurite outgrowth (Pang et al., 1995). U0126 was identified as a functional inhibitor of transcriptional factor AP-1 (Favata et al., 1998). In the present study, we showed that baicalein is a functional inhibitor of prostanoid biosynthesis. Thus, three different drugs having inhibitory effects on the activity of MEK-1 have been found from the investigation of different biological responses. Therefore, the ERK/MAPK cascade is assumed to be an important signaling pathway leading to multiple cellular responses.

Baicalein is a flavonoid of natural origin. It is known that there are many kinds of flavonoids in several plants, including vegetables and fruits. It is assumed that there may be flavonoids other than baicalein with the ability to inhibit the ERK/MAPK cascade. If we take a meal containing flavonoids, they may influence the ERK/MAPK cascade in the cells of our body. Thus, the present results prompt investigation of the effects of flavonoids other than baicalein on the ERK/MAPK cascade in the future. Although its specificity for the inhibition of the ERK/MAPK remains to be evaluated, baicalein may be a useful drug with inhibitory activity on the ERK/MAPK cascade.

In conclusion, we show for the first time that baicalein, a flavonoid of natural origin, inhibits the ERK/MAPK cascade and prostaglandin synthesis, acting on the phosphorylation of MEK-1 by Raf-1.

Acknowledgements

This work was partly supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan (No. 14370737 to NN).

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