Tumor and Stem Cell Biology

Cancer Research

Caffeine-Mediated Inhibition of Calcium Release Channel Inositol 1,4,5-Trisphosphate Receptor Subtype 3 Blocks Glioblastoma Invasion and Extends Survival

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Abstract

Calcium signaling is important in many signaling processes in cancer cell proliferation and motility including in deadly glioblastomas of the brain that aggressively invade neighboring tissue. We hypothesized that disturbing Ca^{2+} signaling pathways might decrease the invasive behavior of giloblastoma, extending survival. Evaluating a panel of small-molecule modulators of Ca^{2+} signaling, we identified caffeine as an inhibitor of glioblastoma cell motility. Caffeine, which is known to activate ryanodine receptors, paradoxically inhibits Ca^{2+} increase by inositol 1,4,5-trisphospate receptor subtype 3 (IP_3R3), the expression of which is increased in glioblastoma cells. Consequently, by inhibiting IP_3R3-mediated Ca^{2+} release, caffeine inhibited migration of glioblastoma cells in various *in vitro* assays. Consistent with these effects, caffeine greatly increased mean survival in a mouse xenograft model of glioblastoma. These findings suggest IP_3R3 as a novel therapeutic target and identify caffeine as a possible adjunct therapy to slow invasive growth of glioblastoma. *Cancer Res; 70(3); 1173–83. ©2010 AACR.*

Introduction

Glioblastoma, the most frequent and malignant tumor in the central nervous system, has a very poor prognosis, with a median survival of only 1 year after diagnosis (1, 2). Total surgical removal of glioblastoma is rarely possible because of the widespread infiltration of brain by neoplastic cells, and nearly all tumors will ultimately fail adjuvant therapy and recur. Thus, the fundamental source of treatment failure is the insidious propensity of tumor cells to invade normal brain structures (2, 3). A host of extracellular signaling molecules activates glioblastoma cells to affect proliferation, motility, and invasiveness. These signaling molecules include various growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor, and G protein–coupled receptor (GPCR) agonists, such as ATP, bradykinin, lysophosphatidic acid (LPA), S1P, thrombin, and plasmin (2). They in turn activate cell surface receptors, such as EGF receptor (EGFR), PAR1, B2, P2Y, LPA, and S1P receptors (4–6), and modulate downstream effectors of the intracellular signaling pathway. An important consequence of the intracellular signaling is an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which is well known to be a critical signal for gene expression, motility, differentiation, and survival. Furthermore, many GPCRs are known to transactivate and converge onto EGFRs in various cancer cells (4), aberrantly exacerbating the Ca^{2+} signaling and other signaling cascades.

Cancer cell migration depends mainly on actin polymerization and intracellular organization of various cytoskeletal proteins, which are influenced by a variety of actin binding proteins (7, 8). Regulation of actin binding protein activity is mediated by second messengers such as phosphoinositides and calcium (7, 8). Therefore, the precise mechanism of receptor-mediated Ca^{2+} increase in glioblastoma cells is an important factor for controlling proliferation, motility, and invasiveness of these cells (9, 10). However, to date, only a limited number of studies have been conducted with regard to Ca^{2+} signaling in glioblastoma cells.

Caffeine, a well-known activator of ryanodine receptor (RyR), has been reported to display anticancer effects (11, 12). Caffeine and its analogues have diverse effects on pain, Alzheimer's disease, asthma, cancer, diabetes, and Parkinson's

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disease (13). Recent studies have shown that caffeine inhibits metastasis in a mouse mammary tumor model and UV-induced skin cancer in nude mouse (11, 12). Therefore, we investigated the detailed Ca^{2+} signaling pathway of the glioblastoma cells in response to various receptor tyrosine kinase (RTK) and GPCR agonists and examined the possible target of caffeine.

Materials and Methods

Human surgical tissue samples. All the fresh, surgically removed tissue samples examined in this study were histologically diagnosed as glioblastoma according to WHO classification. The primary human glioblastoma cells and astrocytes were obtained from brain tissue of the Brain Bank of Seoul National University Hospital. This study was approved by the Institutional Review Board (IRB) of Seoul National University Hospital (IRB approval H-0B05-036-243).

Cell culture. The primary human glioblastoma cells and astrocytes from the Brain Bank of Seoul National University Hospital were enzymatically dissociated to single cell from mechanically dissected glioblastoma and temporal lobe tissues. The cells were then suspended in DMEM (Life Technologies) supplemented with 20% fetal bovine serum (FBS; Life Technologies). Cultured human glioblastoma cell lines (U178MG, U87MG, T98G, U373MG, and M059K) were maintained in DMEM supplemented with 10% FBS, penicillin (50 units/mL), and streptomycin (50 units/mL). wtEGFR and ΔEGFR are the U87MG cell line that has a wild-type EGFR or EGFRvIII (ΔEGFR) mutation, which causes a constitutively active tyrosine kinase activity. These cells were maintained in the same medium containing 200 μg/mL G418 as described previously (14–16).

Calcium imaging. For imaging, all cell lines were cultured as monolayers on 12-mm glass coverslips coated with poly-D-lysine (Sigma). Glioblastoma cells were incubated with 5 μmol/L Fura-2 AM plus 1 μmol/L pluronic acid (Molecular Probes) for 30 min at room temperature. External solution contained 150 mmol/L NaCl, 10 mmol/L HEPES, 3 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 22 mmol/L sucrose, and 10 mmol/L glucose (pH adjusted to 7.4 and osmolarity to 325 mOsm). INDEC Imaging Workbench version 5.2.10 was used for acquisition of intensity images and conversion to ratios. Thrombin (Sigma), S1P (Sigma), LPA (Sigma), TFLLR (Peptron), 2-methylthio-ATP (2-MT-ATP; Sigma), bradykinin (Sigma), and EGF (Sigma) were used as GPCR and RTK agonists.

Scrape motility assay. All cell lines were grown as monolayers in 12-well culture plates in serum-containing medium. Scrapes were made with a 10 μL pipette tip. After caffeine (Sigma), thapsigargin (Tocris), and ryanodine (Tocris) were added, plates were returned to the incubator. To prevent proliferation, 10 μmol/L fluorodeoxyuridine/uridine (Sigma) was added. After incubation for 24 h, the cells were fixed in 4% paraformaldehyde. The areas of repopulation of three 100× fields within the scrape areas were determined, and the mean percentage of scrape area wound closure was determined.

Matrigel invasion assay. Cell invasion was assayed using Transwell inserts containing 8-μm pore size (Corning) in 24-well culture plates. For invasion assay, inserts were coated with 2 mg/mL basement membrane Matrigel (BD Biosciences). Cells (1×10^5) in serum-free medium were plated onto the upper side of insert, and complete medium was placed in the lower chamber to act as a chemoattractant. After 24 h of incubation at 37°C, the cells on the upper side of insert were removed by wiping with a cotton swab. The cells that migrated to the lower side of membrane were stained with 4′,6-diamidino-2-phenylindole (DAPI) and randomly photographed under microscope at ×200 magnification. The mean number of untreated cells was considered as 100% invasion. Each condition was duplicated, and five fields were randomly selected and counted for each assay. Caffeine, DPCPX (Tocris), bicuculline (Tocris), and IBMX (Sigma) were added at the time of cell plating.

Soft agar colony formation assay. Cells (1×10^4) were seeded into six-well plates in a soft agar (0.3%) overlaying a 0.6% base agar. The solidified cell layer was covered with medium containing caffeine, which was replaced every 4 d. Cells were incubated at 37°C for 14 to 17 d to allow colonies to develop. Afterward, colonies were stained with 0.05% cresyl violet and photographed. Each experiment was done in triplicate.

Transfection. HEK 293T cells were transfected with plasmids carrying various inositol 1,4,5-trisphosphate receptor (IP_3R) subunits using Effectene Transfection Reagent (Qiagen) according to the manufacturer's instructions. U178MG cells were transfected with plasmids carrying green fluorescent protein (GFP) or IP_3R subtype 3 (IP_3R3)–small hairpin RNA (shRNA)–GFP by using electroporation. A single voltage (1,100 V) was given for 30 ms by Microporator (Seltagen).

IP_3 uncaging. U178MG cells were colabeled with 200 μmol/L NPE-caged IP_3R (Invitrogen) and 100 μmol/L Oregon Green 488 BAPTA-2 (Invitrogen) by using electroporation. Colabeled U178MG cells were visualized using Olympus FluoView FV1000 confocal microscope. Caged IP_3 was uncaged by 50% of 405-nm laser for 100 ms. Increases in fluorescence intensity over baseline within region of interest were analyzed using FluoView version 1.7a software.

Organotypic brain slice invasion model. Organotypic hippocampal slice cultures were prepared and maintained as described previously (17). Organotypic glioma invasion model was followed by some modification of the procedure as previously described (18). Briefly, DiI-stained U178MG cells (~5,000 cells) were gently placed on the slices in the presence or absence of caffeine 6 d after slice preparation. After 1 and 120 h, movement of the glioma cell in the slices was detected with an inverted confocal laser scanning microscope (Zeiss LSM5, Carl Zeiss). ImageJ software (NIH) was used to calculate the invasion area of DiI-stained cells. Invasion area (%) = (area of DiI-stained cells at 120 h/area of DiI-stained cells at 1 h) × 100.

Tumor xenografts in nude mice. We used U87MG cell line in the tumor xenograft models because this cell line has been widely used due to their high tumorigenicity in nude mouse. The U178MG cells did not show any tumorigenicity in nude mouse in the initial attempts to establish xenograft model. Five-week-old athymic mice (BALB/c *nu/nu*) were obtained from Central Laboratory Animal, Inc. For the xenograft tumor

growth assay, U87MG cells (3×10^6/150 μL PBS) were injected s.c. into the right flanks of the mice (n = 5–10 mice per group). From the day 7 after injection and on, caffeine was given as drinking water (1 mg/mL). The control animals were given distilled water. BALB/c nude mice bearing U87MG were randomized into two groups (control and caffeine; n = 13 per group) on day 7 after tumor inoculation. Caffeine (1 mg/mL) was delivered orally on the day of randomization. Tumor size was measured weekly for 28 d with the use of caliper. Tumor size was measured twice per week for 4 wk, and tumor volumes were calculated by the following formula: volume = length × width^2/2. The effect of the caffeine was determined by the growth delay. To investigate the effects of caffeine on the survival, an orthotopic implantation model was established with U87MG. U87MG cells (2.5×10^5/5 μL PBS) were implanted by intracranial injections in the left frontal lobe at coordinates 2 mm lateral from the bregma, 0.5 mm anterior, and 3.5 mm intraparenchymal. Caffeine (1 mg/mL) was given to mice from 1 wk before inoculation. Mice were monitored daily for general appearance, behavioral changes, and neurologic deficits. Mice were sacrificed when moribund. All protocols were approved by the Gyeongsang National University Institutional Animal Care and Use Committee. The survival data were analyzed by log-rank Kaplan-Meier method using SigmaStat (version 3.0).

Caffeine concentration in serum and brain. After 2-wk administration of caffeine through drinking water (1 mg/mL), animals were sacrificed. Serum and brain tissue contents of caffeine were measured by high-performance liquid chromatography (HPLC) as previously described (19).

Microarray analysis. The microarray analysis was performed on tissue samples of 34 normal brain tissue from lobectomy patients and 51 glioma from human glioblastoma patients. The detailed method is available in Supplementary Materials and Methods.

Results

Ca^{2+} signaling in glioblastoma cells. We first examined the effect of activating RTKs and GPCRs on Ca^{2+} signaling pathway by performing Ca^{2+} imaging experiments from Fura-2 AM–loaded, cultured human glioblastoma cell lines and acutely dissociated glioblastoma cells prepared from surgically removed tissue. A bath application of 100 ng/mL EGF to cultured U178MG and U87MG glioblastoma cell lines induced a robust $[Ca^{2+}]_i$ increases (Fig. 1A). EGF induced Ca^{2+} responses regardless of the status of EGFR—whether it was wild-type or the constitutively active deletion mutant ΔEGFR of U87MG cell line (Fig. 1A). U178MG cells also showed robust Ca^{2+} responses by various GPCR agonists (Fig. 1B). The acutely dissociated glioblastoma cells prepared from surgically removed tissue (Fig. 1C) also showed robust Ca^{2+} responses by TFLLR, EGF, and bradykinin (Fig. 1D). These results show the functional expression of various RTKs and GPCRs, whose activation leads to increases in $[Ca^{2+}]_i$.

We found that an increase in $[Ca^{2+}]_i$ in these cells was contributed in part by a release of Ca^{2+} from intracellular release pools and subsequently by a Ca^{2+} entry through the store-operated channels (Supplementary Fig. S1A–C). The release of Ca^{2+} from intracellular stores was completely inhibited by 1 μmol/L U73122, an inhibitor of phospholipase C (PLC), which produces IP_3 by metabolism of phosphoinositol-4,5-bisphosphate in response to an activation of GPCRs and RTKs (Supplementary Fig. S1D). From these results, we concluded that glioblastoma cells express various surface receptors that are coupled to the common phosphoinositide pathway that leads to Ca^{2+} release from intracellular stores and subsequent Ca^{2+} influx through store-operated channels.

Caffeine inhibits IP_3-mediated Ca^{2+} release. There are two known ion channels primarily responsible for release of Ca^{2+} from intracellular stores: IP_3Rs and RyRs. Caffeine has been classically known to induce a release of Ca^{2+} from intracellular stores by opening RyRs, especially in muscle cells and cardiac myocytes (13). Thus, we tested caffeine along with other agents that enhance or disturb the Ca^{2+} release machinery in various assays for glioblastoma motility, invasion, and proliferation. Contrary to our expectations, we found that caffeine significantly and concentration dependently (1–10 mmol/L) inhibited the motility, invasion, and proliferation of various human glioblastoma cell lines, including U178MG, U87MG, and T98G cells (Fig. 2A–C), while minimally affecting the cell viability at this concentration range (data not shown). This paradoxical effect of caffeine was mimicked by other agents that are known to disturb intracellular concentration of Ca^{2+}, such as 1 μmol/L thapsigargin (Fig. 2A), 10 μmol/L 2-aminoethoxydiphenyl borate, 20 μmol/L cyclopiazonic acid, and 50 μmol/L BAPTA-AM (data not shown). However, 10 μmol/L ryanodine, an agonist of RyRs at this concentration, did not show this inhibitory effect on migration and invasion (Fig. 2A). Furthermore, the blocking concentration of ryanodine at 100 μmol/L was not able to inhibit the bradykinin-induced Ca^{2+} increase (Supplementary Fig. S1E). These results suggested that the mode of action of caffeine might not be related to opening of RyRs.

Caffeine has been shown to inhibit adenosine receptors, $GABA_A$ receptors, phosphodiesterase activity, and G_2 checkpoint for repair of damaged DNA (13). We tested whether modulating these target molecules affects the invasiveness of glioblastoma cells. The A1 adenosine receptor blocker DPCPX (800 nmol/L), $GABA_A$ receptor blocker bicuculline (10 μmol/L), and phosphodiesterase inhibitor IBMX (100 μmol/L) had no significant effect on invasion (Fig. 2B, *right*), suggesting that the mode of action of caffeine might be at other targets.

Caffeine has been also reported to inhibit IP_3Rs without affecting IP_3 binding at millimolar concentrations (20), possibly by competing at the ATP binding site of IP_3Rs (21). Therefore, we tested whether caffeine can inhibit the increase in $[Ca^{2+}]_i$ on activation of GPCRs and RTKs in cultured U178MG cells. We found that caffeine significantly inhibited the bradykinin-induced, EGF-induced, and the PAR1 agonist TFLLR–induced increase in $[Ca^{2+}]_i$ (Fig. 3A and B) in a concentration-dependent manner with half-maximal concentration of 2.45 and 1.81 mmol/L for TFLLR- and EGF-induced responses, respectively (Fig. 3B). This inhibitory action of caffeine was not due to an inhibition of store-operated channels (Supplementary Fig. S2A), a depletion of Ca^{2+} stores (Supplementary Fig. S2B–D), or an activation of

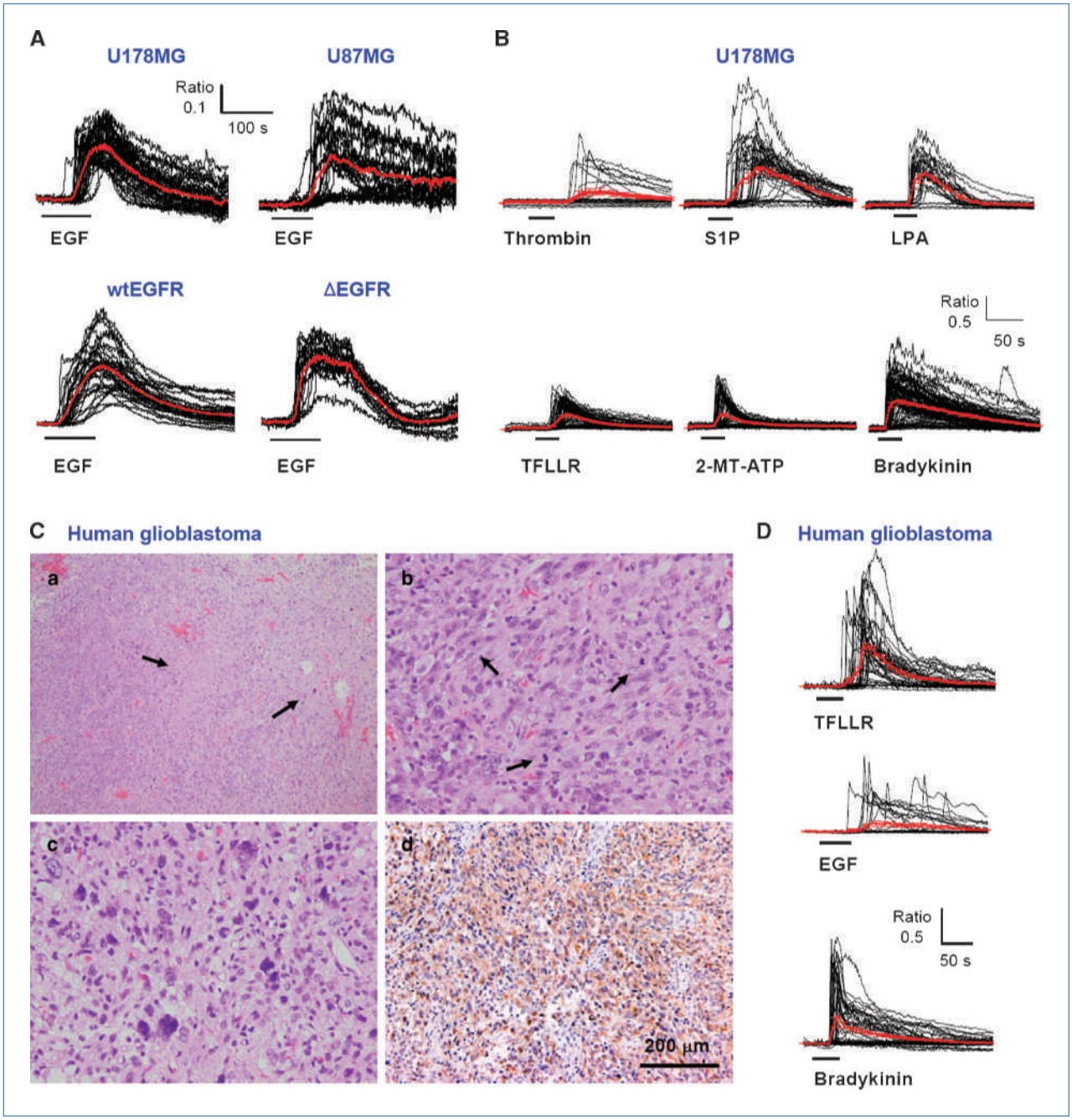

Figure 1. Ca^{2+} responses by various GPCR and RTK agonists. A, traces from Ca^{2+} imaging recordings performed in U178MG, U87MG, wtEGFR-U87MG, or ΔEGFR-U87MG cells. Each trace represents a Ca^{2+} response in one cell (n = 36–83 per cell line). Red lines, average responses. Bars, SE. Black horizontal bars, time and duration of 100 ng/mL EGF application. B, Ca^{2+} responses by 100 nmol/L thrombin, 10 μmol/L S1P, 30 μmol/L LPA, 30 μmol/L TFLLR, 100 μmol/L 2-MT-ATP, and 10 μmol/L bradykinin in U178MG cells. C, a, arrows, low-magnification view of this tumor showing cellular glial tumor with two foci of pseudopalisading necrosis (H&E). b, arrows, the tumor shows frequent mitotic cells (H&E). c, multinucleated pleomorphic nuclei are present (H&E). d, most of the tumor cells are immunoreactive for glial fibrillary acidic protein (glial fibrillary acidic protein immunostaining). D, Ca^{2+} responses induced by GPCR and RTK agonists in the primary human glioblastoma cells.

RyRs (Supplementary Fig. S1E), leaving an inhibition of IP_3Rs as the most likely mode of action by caffeine.

To determine if the inhibitory action of caffeine on Ca^{2+} increase is due to a direct action on IP_3Rs, we performed IP_3 uncaging experiment on U178MG cells that were electroporatically loaded with caged IP_3 and Ca^{2+} indicator dye, Oregon Green 488 BAPTA-2. UV flashing of each cell induced uncaging of IP_3 and subsequent increase in $[Ca^{2+}]_i$. This

effect was significantly inhibited in the presence of caffeine (Fig. 3C), supporting the conclusion that caffeine directly inhibits IP_3R-mediated Ca^{2+} release.

IP_3R3 is required for caffeine sensitivity. We then tested whether the inhibitory action of caffeine on Ca^{2+} responses was also observed in other cell types. We found that a variety of cell types displayed varying degree of inhibition of Ca^{2+} responses by caffeine, with the highest block in U178MG and the lowest block in human astrocytes (Fig. 4A). To see if the degree of block by caffeine is correlated with IP_3R expression, we performed semiquantitative reverse transcription-PCR (RT-PCR) for three subtypes of IP_3R mRNA for each cell type (Fig. 4B). Of the three subtypes of IP_3R, expression of IP_3R3 showed the highest correlation with the percent block of Ca^{2+} responses (coefficient of correlation $r^2 = 0.891$; $P < 0.001$; Fig. 4B), suggesting that the effect of caffeine on Ca^{2+} increase might be linked

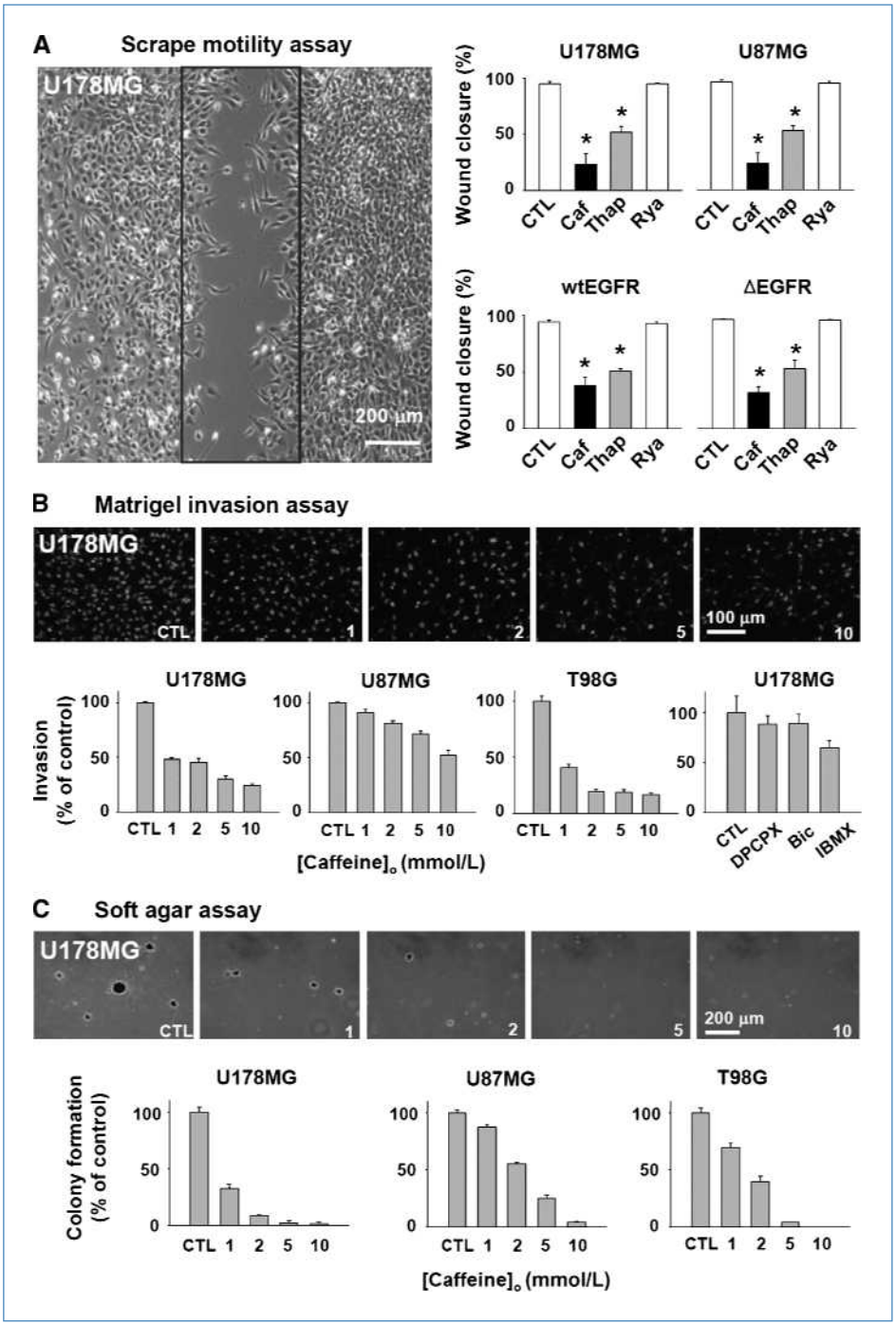

Figure 2. Caffeine slows motility, invasion, and colony formation of glioblastoma cells. A, monolayers of glioblastoma cells were wounded by a scrape (*black box*) and treated with 10 mmol/L caffeine (*Caf*), 1 μmol/L thapsigargin (*Thap*), or 10 μmol/L ryanodine (*Rya*). Columns, mean; bars, SE. *, $P < 0.01$, ANOVA with Newman-Keuls post hoc. B, top, representative pictures of DAPI-labeled cells that invaded through 8-μm holes in the Matrigel inserts in the presence of indicated caffeine concentration; bottom, percentage of invaded cells with respect to the control condition. Right, similar experiment was done with 800 nmol/L DPCPX, 10 μmol/L bicuculline, and 100 μmol/L IBMX, and percent of invasion was plotted. C, caffeine effect on the anchorage-independent growth of glioblastoma cells *in vitro* was tested. Top, representative photographs of colonies grown in indicated caffeine concentration; bottom, percentage of number of colony normalized to the control condition.

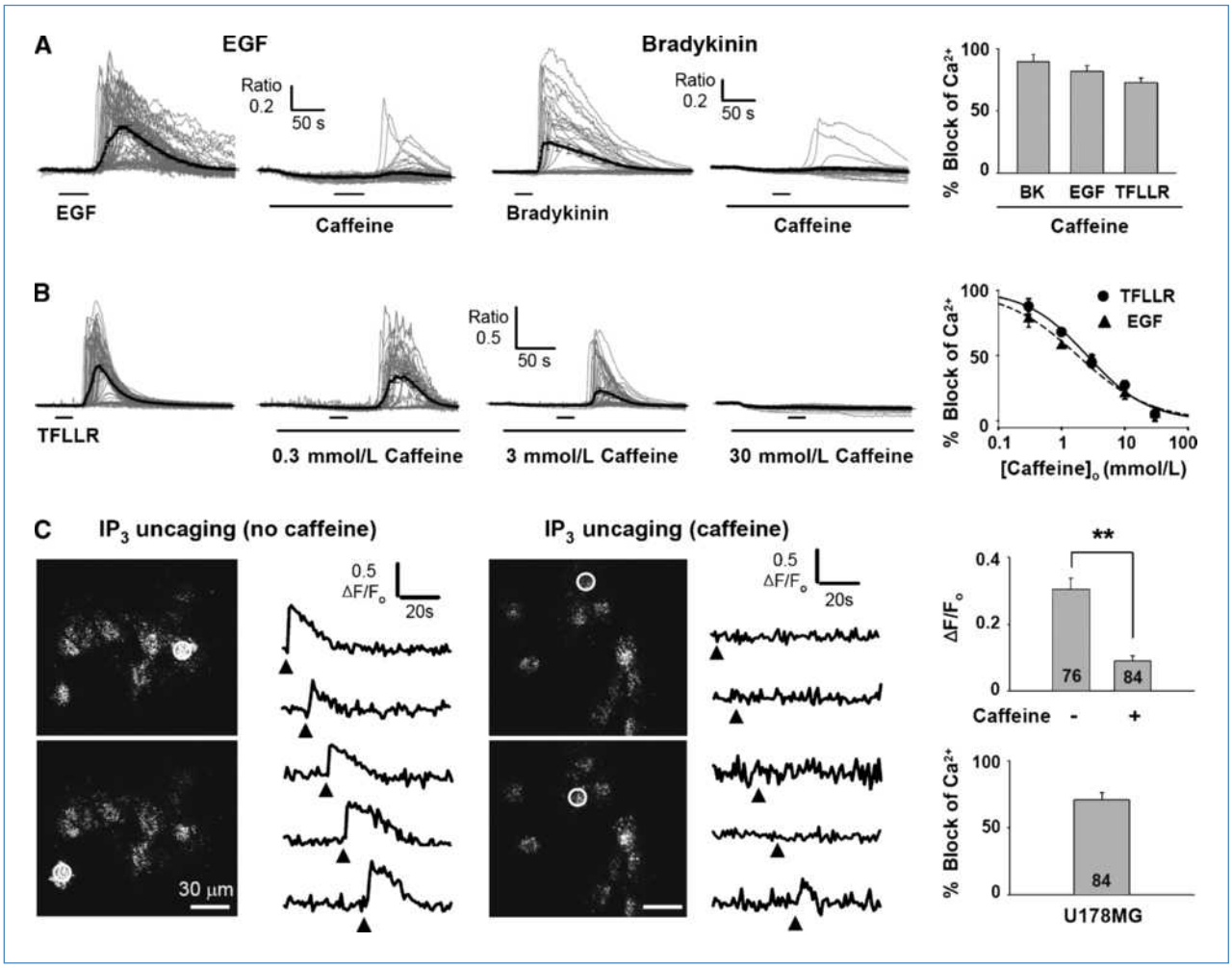

Figure 3. Caffeine reduces GPCR- and RTK-induced Ca^{2+} increase by inhibiting IP_3R. A, EGF- or bradykinin-induced Ca^{2+} responses in the presence or absence of caffeine in U178MG cells. Caffeine (10 mmol/L) was treated 100 s before stimulation with indicated agonist. Percent block of various agonists induced Ca^{2+} release by 10 mmol/L caffeine in U178MG cells. The peak of average Ca^{2+} response trace in the presence of caffeine was divided by the average of Ca^{2+} response in the absence of caffeine ($n = 3$). B, TFLLR-induced Ca^{2+} responses in the presence of 0.3, 3, and 30 mmol/L of caffeine. Concentration-effect curve of Ca^{2+} increase evoked by TFLLR (IC_{50}, 2.45 mmol/L) or EGF (IC_{50}, 1.87 mmol/L). C, left, intensity images of U178MG cells loaded with caged IP_3 and Oregon Green 488 BAPTA-2. White circles, region of interest exposed to a 405-nm laser for uncaging of IP_3. Right traces, representative fluorescence changes on laser stimulation (▲) over time in the absence of caffeine or in the presence of 10 mmol/L caffeine. The average fluorescence intensity changes in the presence or absence of caffeine at the peak of Ca^{2+} transients. **, $P < 0.001$, Student's t test. Summary of IP_3 uncaging experiment. Caffeine blocks 71% of Ca^{2+} release in U178MG cells.

to the expression level of IP_3R3. We also performed semiquantitative RT-PCR for the three subtypes of IP_3R on glioblastoma patient tissue samples and compared with normal tissue samples from lobectomy surgery. We found that glioblastoma tissue displayed on average >2-fold increase in expression of IP_3R3 mRNA compared with the normal tissue, whereas IP_3R2 mRNA was unchanged and IP_3R1 significantly decreased (Fig. 4C). This is consistent with the high percentage of block of Ca^{2+} responses by caffeine in acutely prepared human glioblastoma cells (Fig. 4A). These results indicate that the expression of IP_3R3 is significantly correlated with the inhibitory action of caffeine on Ca^{2+} responses.

To confirm that the inhibitory action of caffeine on Ca^{2+} response is specific to IP_3R3, we either overexpressed IP_3R3 into HEK 293T cells that normally lack IP_3R3 or inhibited the expression of IP_3R3 using shRNA in U178MG cells that highly express IP_3R3. In HEK 293T cells heterologously expressing IP_3R3, the block percentage of TFLLR-induced Ca^{2+} responses by caffeine was significantly increased compared with the IP_3R1- or IP_3R2-overexpressing HEK 293T cells (Fig. 5A). To silence the IP_3R3 mRNA in U178MG cells, we first developed a shRNA that is specific to IP_3R3 mRNA by screening several candidate hairpin-forming oligomers, one of which (candidate 3) selectively reduced the IP_3R3 mRNA expression without affecting mRNA levels for the other IP_3R subtypes (Supplementary Fig. S3A). Subsequent testing of the shRNA on U178MG cells revealed that gene silencing of IP_3R3 in U178MG suppressed the caffeine sensitivity of Ca^{2+}

responses in these cells (Fig. 5C; Supplementary Fig. S3B) but not by the control vector containing GFP only (Fig. 5B; Supplementary Fig. S3B). Interestingly, gene silencing of IP_3R3 in U178MG caused a general reduction in TFLLR- or bradykinin-induced Ca^{2+} responses (Fig. 5C), suggesting that IP_3R3 is the major Ca^{2+} release channels in these cells. These results indicate that IP_3R3 is critical for the inhibitory action of caffeine. Moreover, glioblastoma cells show aberrant increase in expression of this subtype (Fig. 4C), providing a potential target for treatments aimed at altering glioblastoma migration and invasion.

Next, we examined whether gene silencing by shRNA for IP_3R3 in U178MG cells would make these cells less sensitive to caffeine in Matrigel invasion assay. As expected, gene silencing of IP_3R3 by shRNA rendered these cells significantly less sensitive to caffeine treatment in Matrigel invasion assay compared with the GFP vector–expressing cells ($P < 0.01$, t test; transfection efficiency, ~30%; Fig. 5D). These results indicate that IP_3R3 confers caffeine sensitivity on invasion of U178MG cells.

Caffeine inhibits invasion and increases survival rate. To translate the results from the *in vitro* experiments, we examined the effect of caffeine on brain slices and in *in vivo* animal models, in which local microenvironments could compromise the effect of caffeine. In mouse brain slices in culture, we placed 1 μL of DiI-labeled U178MG cells (~5,000 cells) in hippocampal region after 6 days in culture and examined the radial progression of these cells to neighboring regions 5 days after the placement. We found that the invasion of DiI-labeled U178MG cells was significantly lower

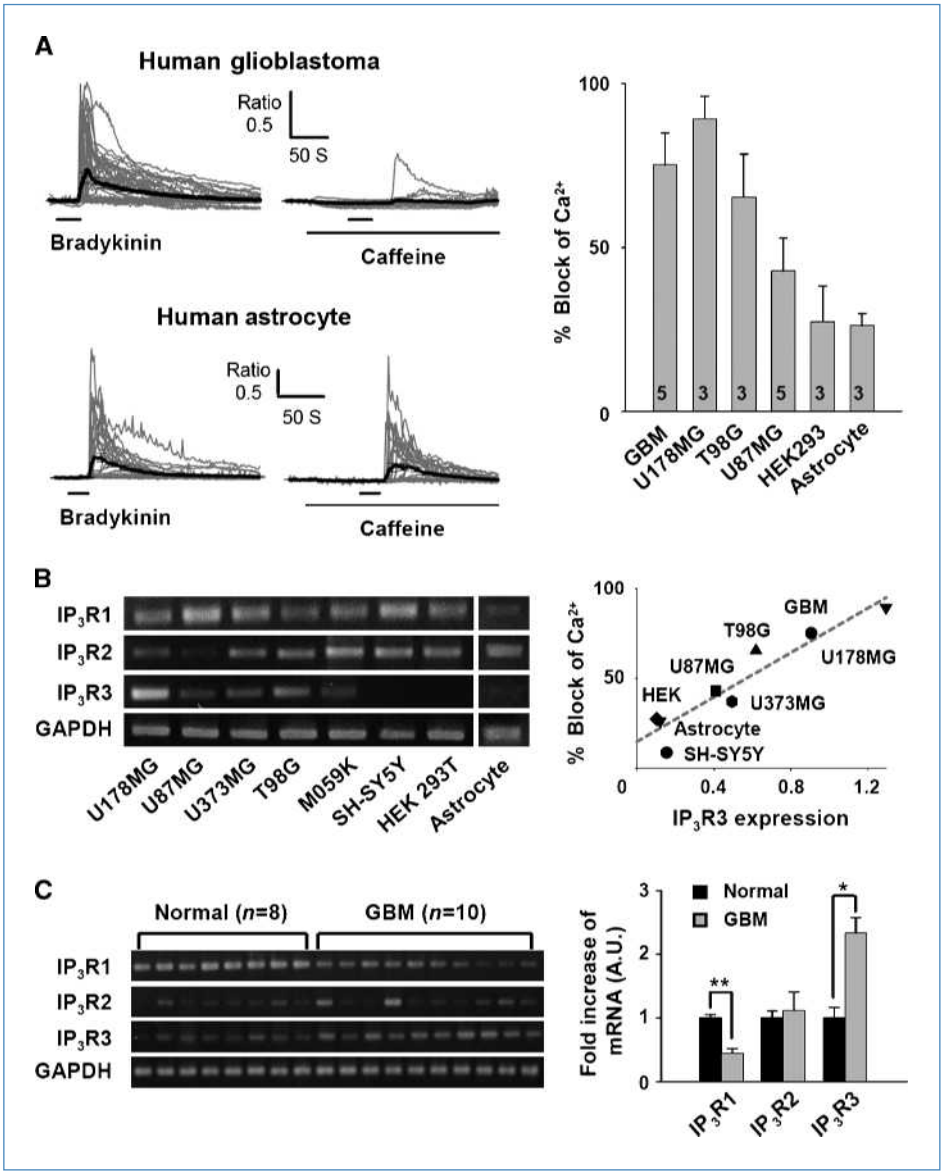

Figure 4. Correlation between IP_3R3 and block of Ca^{2+} release by caffeine. A, block of bradykinin-induced increase in $[Ca^{2+}]_i$ by caffeine on the primary human glioblastoma cells and astrocytes. Summary of block of GPCR agonist-induced Ca^{2+} responses by caffeine on various cell types. B, mRNA expression of IP_3Rs and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was tested by semiquantitative RT-PCR in various human glioblastoma cell lines (U87MG, U178MG, U373MG, T98G, and M059K), human neuroblastoma cell line (SH-SY5Y), human embryonic kidney cell line (HEK 293T), and human astrocyte. Correlation between expression of IP_3R3 normalized to GAPDH in each cell type and caffeine block of agonist-induced Ca^{2+} responses ($r^2 = 0.891$, $P < 0.001$). C, semiquantitative RT-PCR of IP_3R subtypes in normal human brain and human glioblastoma tissue samples. Averages of densitometric measurement of IP_3R mRNA expression in human samples, normalized to the normal human brain tissue sample. *, $P < 0.001$; **, $P < 0.0001$, Student's t test.

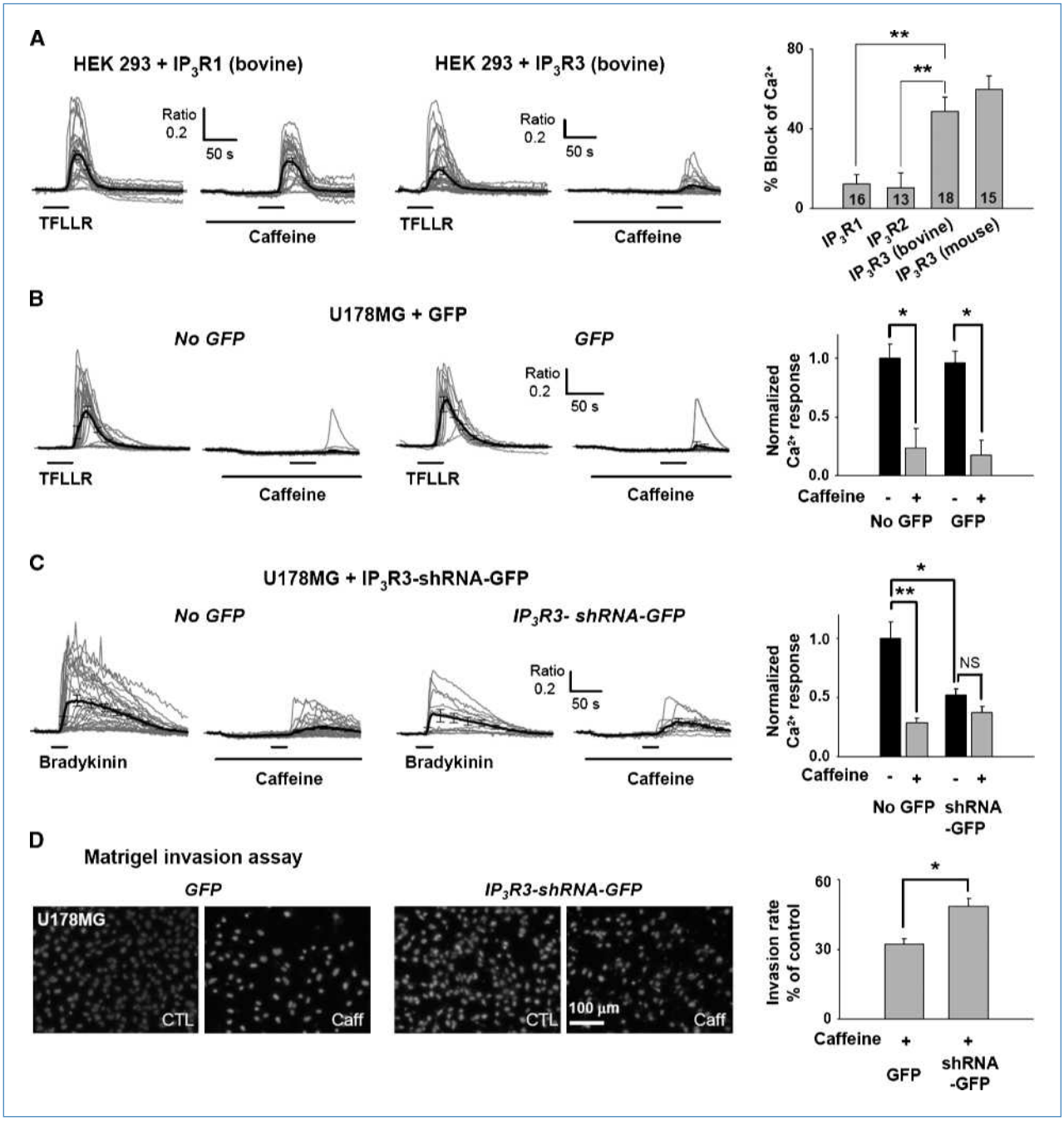

Figure 5. IP_3R3 is required for caffeine sensitivity. A, block of TFLLR-induced increase in $[Ca^{2+}]_i$ by 10 mmol/L caffeine in HEK 293T cells transfected with IP_3R1 (bovine) and IP_3R3 (bovine). Summary of percent block by caffeine in HEK 293T cells transfected with IP_3R1 (bovine), IP_3R2 (bovine), IP_3R3 (bovine), or IP_3R3 (mouse). **, $P < 0.001$, Student's t test. B, Ca^{2+} responses of GFP-negative and GFP-positive cells on U178MG transfected with vector containing only GFP. Ca^{2+} responses in each condition are normalized to Ca^{2+} responses in GFP-negative without caffeine treatment. *, $P < 0.05$, Student's t test. C, Ca^{2+} responses of GFP-negative and GFP-positive cells on U178MG transfected with vector containing IP_3R3 shRNA and GFP. Ca^{2+} responses in each condition are normalized to Ca^{2+} responses in GFP-negative cells without caffeine treatment. *, $P < 0.05$; **, $P < 0.001$, Student's t test. D, results of the Matrigel invasion assay on IP_3R3 shRNA–expressing cells and GFP-expressing cells with or without caffeine treatment. Summary of the Matrigel invasion assay with IP_3R3 shRNA–expressing cells shows significantly less inhibition of invasion by caffeine. *, $P < 0.01$, Student's t test.

in the brain slices that were treated with various concentration of caffeine compared with the untreated slices (Fig. 6A and B). Next, in a skin xenograft model, U87MG cells that are known to exhibit high tumorigenicity were injected into skin of nude mice and progression of tumor mass was followed. We found that those mice supplied with caffeine containing drinking water [1 mg/mL (equivalent to 5 mmol/L), starting from 7 days after implantation] showed significantly reduced

tumor mass compared with control mice at 35 days after implantation (Fig. 6C). In an intracranial xenograft model of glioblastoma using U87MG cells, the same dose of caffeine improved the survival of tumor-bearing mice (mean survival time, 38 days) compared with control (mean survival time, 28 days; Fig. 6D). It has been reported that when caffeine (0.44 mg/mL) was treated in the drinking water for 2 weeks, serum concentration in mice was near 6 μg/mL (22), which was approximately the same concentration in people drinking two to five cups of coffee per day (23). Because the brain concentration of caffeine was highly correlated with serum concentration (19) and our experimental mice received 1 mg/mL drinking water for >2 weeks, we predicted that the brain concentration should be at least 6 μg/mL (equivalent to 0.03 mmol/L). We then directly measured the serum and brain content of caffeine after caffeine treatment using HPLC as previously described (19). The measured caffeine concentrations were not very different from the predicted values (serum content, 7.134 ± 1.089 μg/mL; brain content, 6.135 ± 0.368 μg/mL).

Molecules in Ca^{2+} signaling pathway are upregulated in glioblastoma. To see if the molecules in the phosphoinositide and Ca^{2+} signaling pathway in glioblastoma are changed, we have performed genome-wide DNA chip microarray analysis on 51 tissue samples from glioblastoma patients and compared with 34 normal tissue samples from lobectomy surgery (Supplementary Fig. S4A). We found that most of molecules that are in the signaling pathway of phosphoinositide production and Ca^{2+} release showed an average of >2-fold increase in mRNA expression in glioblastoma patient samples. These include PAR1 (7.7-fold increase), EGFR (3.1-fold increase), PLCβ3 (1.4-fold increase),

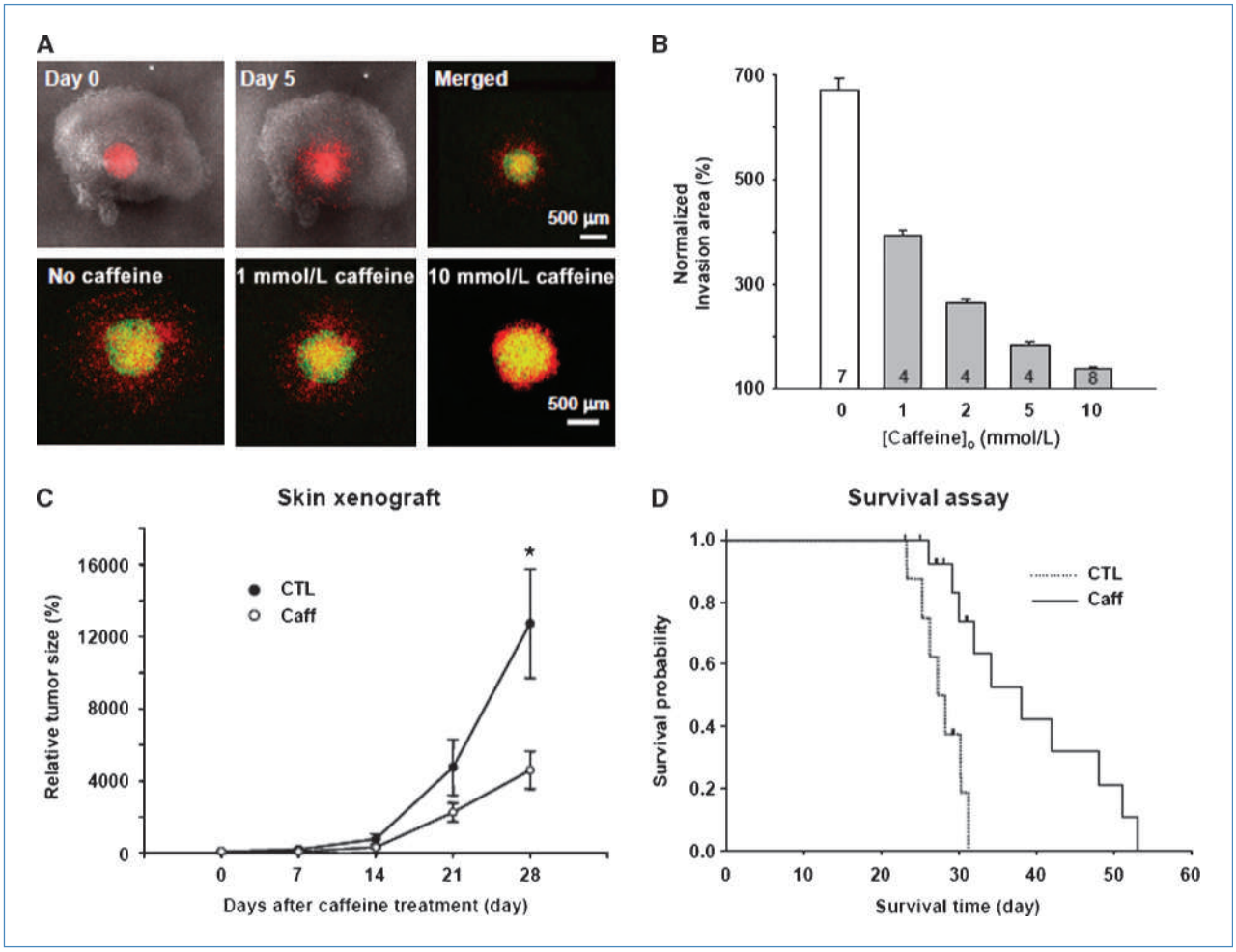

Figure 6. Caffeine inhibits invasion and increases survival rate. A, DiI-stained U178MG cells were placed on the surface of slices in the presence or absence of caffeine (0–10 mmol/L) 6 d after slice preparation. The first two merged DIC and red fluorescence images show the hippocampal brain slice and DiI-stained U178MG cells. After 1 h (green) and 120 h (red), movement of the glioblastoma cells in the slices was detected with an inverted confocal laser scanning microscope. B, invasion area (%) was calculated from the following formula: (area of DiI-stained cells at 120 h/area of DiI-stained cells at 1 h) × 100. C, effect of caffeine on the growth of U87MG cells in *in vivo* skin xenograft model. *, $P < 0.01$. D, Kaplan-Meier survival curves of nude mouse bearing intracranial U87MG tumors. $P = 0.001$, log-rank test, control versus caffeine.

PLCγ1 (1.4-fold increase), IP_3R3 (2.3-fold increase), and TRPC6 (2.1-fold increase). However, RyRs did not show any significant increase. The gene tree analysis also shows clusters of genes related to upstream or downstream signaling pathway whose expression is significantly increased (Supplementary Fig. S4B). The enhanced Ca^{2+} signaling provides a source of Ca^{2+} that is sensitive to caffeine and critical for motility, invasion, and possibly cell division of glioblastoma cells (Supplementary Fig. S4C).

Discussion

We have initially started with a simple hypothesis that inhibiting the Ca^{2+} signaling pathway would inhibit the invasive and motile behavior of glioblastoma cells. Similar to normal glial cells, glioblastoma cells show robust Ca^{2+} increases on activation of various GPCR and RTK agonists (Fig. 1). The major source of Ca^{2+} increase was found to be the release of intracellular Ca^{2+} stores through IP_3Rs and subsequent trigger of influx through the store-operated channels on depletion of the intracellular stores (Supplementary Fig. S1A and B). Of the three subtypes of IP_3R, we found that IP_3R3 is overexpressed in various glioblastoma cell lines and glioblastoma patient samples, whereas normal astrocytes showed virtually no expression of IP_3R3 (Fig. 4B and C). On the other hand, IP_3R1 was significantly decreased in glioblastoma tissues (Fig. 4C), making IP_3R3 as the major contributor of Ca^{2+} signaling in these cells.

IP_3Rs are difficult to study especially due to the lack of suitable inhibitors and subtype-specific blockers. We found that caffeine paradoxically inhibited IP_3R-mediated Ca^{2+} responses in a subtype 3–specific manner (Fig. 5). Using caffeine as a tool to inhibit IP_3R3-mediated Ca^{2+} release, we have shown that inhibiting IP_3R3 effectively reduced the migration, invasion, and survival of glioblastoma cells (Fig. 2). The gene silencing of IP_3R3 by shRNA also effectively reduced the caffeine sensitivity of Ca^{2+} signaling and invasiveness in the Matrigel invasion assay (Fig. 5). Our results are the first to show the involvement of IP_3R3 in glioblastoma Ca^{2+} signaling and invasion. Furthermore, we suggest that IP_3R3 can be specifically targeted for therapeutic intervention in glioblastoma patients with minimal influence on normal glial as well as neuronal functions.

Whether caffeine can directly affect the gating of IP_3R3 channels is still unknown. However, according to previous studies showing that caffeine can compete with ATP binding to IP_3Rs (21) at millimolar concentrations (20), caffeine could selectively bind to IP_3R3 and affect the gating of IP_3R3. Further work is required to investigate the direct role of caffeine on IP_3R3 gating in comparison with other subtypes of IP_3R.

In summary, our study provides IP_3R3 as a novel therapeutic target for glioblastoma treatment. Our study also provides new insights into the detailed molecular mechanism of caffeine action on migration and invasion of glioblastoma. The apparent beneficial effect of caffeine suggested by our study should trigger future investigations of the therapeutic potential for caffeine to treat this deadly disease that otherwise has no cure.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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