

Activation of PKC α is required for migration of C6 glioma cells

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The PKC signaling pathway has been implicated in diverse cellular functions. Here, we sought to investigate the role of PKC α/β in C6 glioma cell migration. We found that both PKC α and PKC β were expressed by C6 glioma cells, but only PKC α was markedly activated in serum-treated C6 cells. Gö6976, a PKC α/β specific inhibitor, was found to cause a dose-dependent reduction of PKC α activation and cell migration induced by serum in C6 cells. These results collectively indicated that the PKC α signaling pathway is necessary for glioma cell migration. Our findings may provide an insight into a better understanding to the malignant progression of gliomas.

Key words: PKC α , activation, C6 glioma cells, migration, signaling pathway

INTRODUCTION

Most primary central nervous system (CNS) tumors are derived from glial cells and collectively referred to as gliomas. Malignant gliomas exhibit a high rate of cell motility and migration that contributes to the invasiveness of the tumors (Amberger et al. 1998, Griscelli et al. 2000, DeAngelis 2001, Bello et al. 2004). Thus, the understanding of the molecular mechanisms which regulate glioma cell motility is very important.

Protein kinase C (PKC) is a member of serine/threonine protein kinase family that are involved in diverse cellular functions, including contraction (Yan et al. 2001), cell motility (Makagiansar et al. 2004), growth (Yokoyama et al. 2005), differentiation (Cannons et al. 2004), proliferation, tumor promotion (Chida et al. 2003), fertilization (Halet 2004), and apoptosis (Baines et al. 2005, Miguel et al. 2008).

Accumulating evidence suggests that there is a potential role of PKC in migration of cells (Nakashima 2002,

Koivunen et al. 2006). PKC α and PKC β are two important members of PKC family, and their activation have been often linked to malignant phenotype. However, PKC α and PKC β have been shown to display variable expression profiles and functions during cancer progression depending on a particular cancer type (Koivunen et al. 2006). The functional role of various isoforms of PKC in gliomas is still poorly understood. Whether PKC α and PKC β are expressed by glioma cells and plays a role in glioma cell migration remains unknown.

In the present study, we provided evidence that PKC α play an important role in migration of C6 cells.

METHODS

Cell line

C6 rat glioma cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained at 37°C in humidified atmosphere at 95% air and 5% CO₂ in DMEM (Invitrogen, Arlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin–streptomycin (Invitrogen).

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Wound migration assay

After C6 cells were grown to confluence in 24-well plates, DMEM-10% FBS was replaced by FBS-free medium for 12 hours. Cells were pretreated with mitomycin C (MMC, 25 μ g/ml; Sigma, St. Luis, MO, USA) for 30 min. A scratch was then made on the monolayer using a sterile 200 μ l pipette tip with 2 mm in width. After rinsed with PBS three times, digital images of the scar were taken using an Olympus BX60 microscope as initiation control. Then the cells were treated with DMEM-10% FBS again for 24 hours. For inhibitor blocking experiment, cells were pre-incubated with DMSO control and PKC α / β inhibitor Gö6976 (Sigma) at different concentration for 45 min in serum-free medium prior to exposure to serum. Finally, the same region of the scar was imaged again after migration for 24 hours. The migration of cells was quantified by measuring the surface area of the scar at 0 and 24 hours using Image J software and migrated effect was calculated by the migrated surface area (initial scar area – scar area after 24 hours) dividing the initiative

scar area. Experiments were done at least three times. Measurements were made in triplicate.

BrdU incorporation assay

To assess the effect of Gö6976 on proliferation of MMC-treated C6 cells, the in situ 5-bromo-2-deoxyuridine (BrdU) incorporation assay was used. Briefly, C6 cells were plated onto coverslips and cultured in DMEM-10% FBS. When cells were grown to confluence, the medium was replaced by FBS-free DMEM. Following being cultured for 12 hours, C6 cells were pretreated with 25 mg/ml MMC for 30 min. After rinsed with PBS three times, cells were pre-incubated with DMSO control or 1 μ M Gö6976 for 45 min in serum-free medium. Following incubation in BrdU (10 μ M; Sigma) in the presence of serum for 16 hours, C6 cells were rinsed and fixed with 4% paraformaldehyde (PFA, Sigma) in PBS (pH 7.4) for 20 min at room temperature. For anti-BrdU immunofluorescence labeling, the cells were treated with 1 N HCl for 40 min at 37°C to denature the DNA prior to the use of primary (mouse anti-BrdU; 1:100; Sigma) and then secondary antibody (rhodamine-conjugated donkey anti-mouse IgG; 1:200; Sigma). After staining, the coverslips were rinsed and mounted with Gel/Mount aqueous mounting media (Biomed Corporation, Foster City, CA, USA) containing Hoechst 33342, a fluorescent nuclear dye (1 μ g/ml; Sigma). The coverslips were examined and photographed using an Olympus BX60 microscope.

TUNEL assay

To determine whether Gö6976 affects the survival of MMC-treated C6 cells, the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed using In Situ Cell Death Detection Kit (Mannheim, Germany). Briefly, cells were treated with MMC similar to those described in BrdU incorporation assay. Then cells were incubated with DMSO control or 1 μ M Gö6976 for 24 hours in the presence of 10% serum. The cells were fixed with 4% formaldehyde for 20 min at room temperature (RT), rinsed twice in PBS, and permeabilized with 0.1% Triton X-100 in freshly prepared 0.1% sodium citrate for 2 min on ice. After rinsed twice in PBS, the coverslips were incubated with TUNEL reaction mixture for 1 hour at 37°C. The coverslips were rinsed

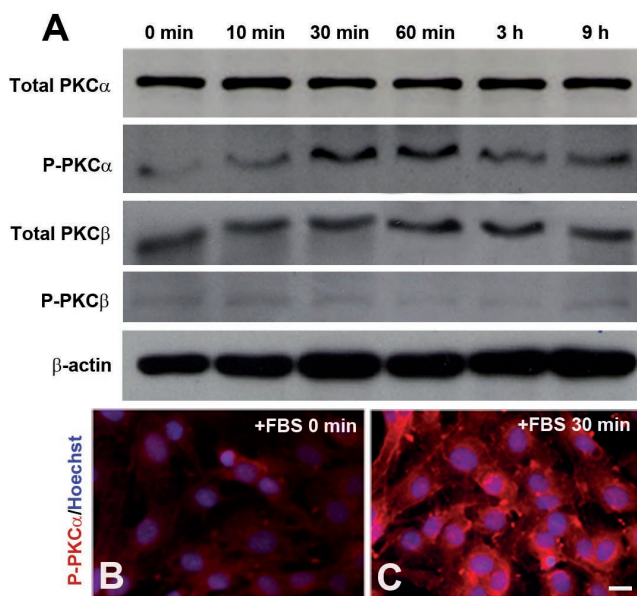


Fig.1 Expression and activation of PKC α in C6 glioma cells.

(A) Western blot analysis showed that both PKC α and PKC β were expressed in C6 glioma cells. PKC α but not PKC β could be phosphorylated by serum treatment. β -actin was used as an internal control. (B, C) Immunofluorescence showed that the fluorescence intensity of phospho-PKC α (P-PKC α) in C6 cells was significantly increased after serum treatment for 30 min compared with FBS-untreated group. Scale bar=50 μ m.

with PBS and mounted with Gel/Mount aqueous mounting media containing Hoechst 33342. The pictures were taken under the BX60 Olympus fluorescence microscope and then the TUNEL-positive cells were counted.

Western blot analysis

To detect expression and phosphorylation of PKC α/β in C6 cells, the cells were collected at various time points after serum administration. For Gö6976 inhibition experiment, cells were pre-incubated with Gö6976 at various different concentrations for 45 min prior to exposure to serum (10% FBS). The cells were washed and lysed with a lysis buffer (50 mM Tris-HCl, 150 mM NaCl buffer, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 4 μ g/ml leupeptin, 1 μ g/ml aprotinin and 100 μ g/ml PMSF; all from Sigma). The supernatant was clarified by centrifugation at 16 000 g for 10 min at 4°C. The protein concentrations were determined using a BCA Protein Assay kit (Pierce, Rockford, IL). For western blotting, the samples containing an equal amount of protein (20 μ g) were electrophoresed on SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride filters (Millipore, Bedford, MA). The filters were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) for 1 hour at RT and then incubated overnight at 4°C with primary antibodies (in TBST-5% BSA) including PKC α and PKC β , phosphorylated PKC α and PKC β (all 1:1000; Cell Signaling Technology, Beverly, MA, USA) and β -actin (1:1000, Sigma). After rinsed with TBST, the membranes were incubated with the appropriate HRP-conjugated secondary antibody (all from KPL, Gaithersburg, MD) for 1 hour at RT. To visualize the immunoreactive proteins, the ECL kit (Pierce, Rockford, IL) was used, following the manufacturer's instructions.

Immunohistochemistry

C6 cells cultured on coverslips were pre-incubated with or without 1 μ M Gö6976 for 45 min prior to the addition of 10% serum. After incubated with serum-contained medium for 30 min, the cells were rinsed in PBS and fixed with 4% paraformaldehyde (PFA) in PBS for 20 min at RT. Immunohistochemistry was performed according to the method described previ-

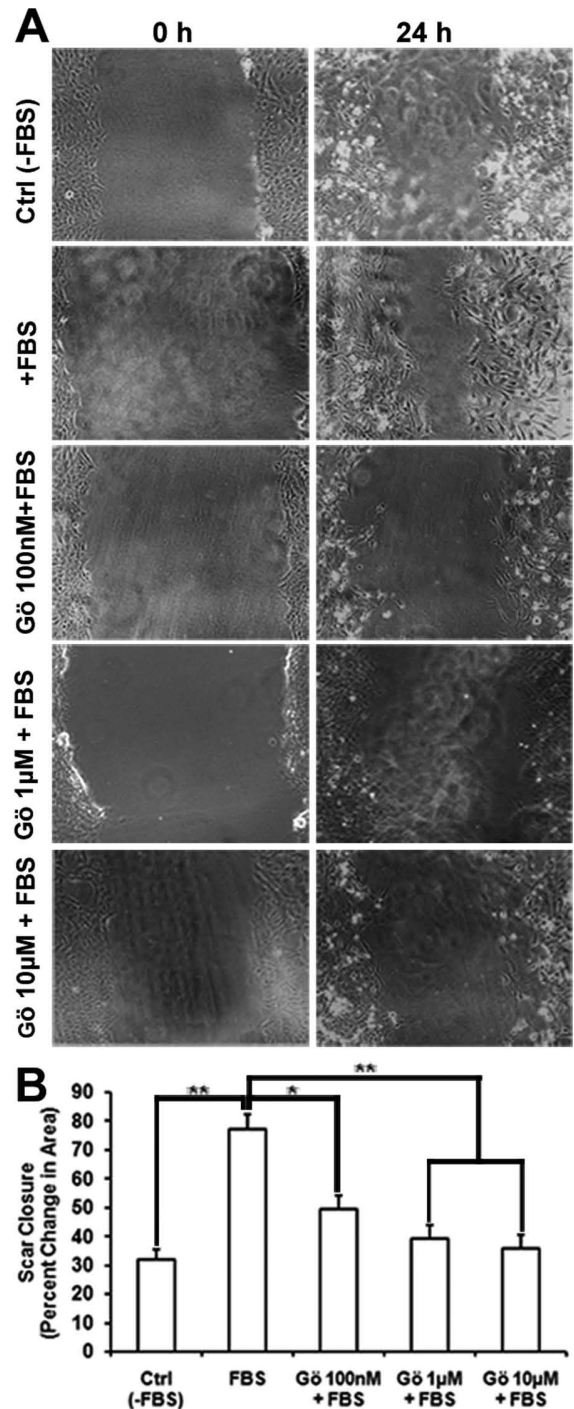


Fig.2 Gö6976, a PKC α/β specific inhibitor, inhibited migration of C6 glioma cells.

(A) Representative photomicrographs of wound migration assay showed that pre-incubation of Gö6976 largely decreased serum-induced migration of C6 cells in a dose-dependent manner. Scale bar=25 μ m. (B) Quantitative comparison illustrating the percent of scar closure in control and Gö6976 pre-treated C6 glioma cells. Data are given as mean \pm SD. (*: $p < 0.05$; **: $p < 0.01$; $n = 5$).

ously (Lü et al. 2009). Briefly, after three rinses in PBS, the cells were incubated with 10% normal goat serum (NGS) in PBS-0.3% Triton X-100 for 1 hour at RT and then with the monoclonal primary antibodies against total or phosphorylated PKC α / β overnight at 4°C. On the second day, the cultures were incubated with rhodamine-conjugated second antibody (1:200; Jackson ImmunoResearch Lab, West Grove, PA) for 1 h at 37°C. After staining, the coverslips were rinsed and mounted with Gel/Mount aqueous mounting media containing Hoechst 33342. The coverslips were examined using an Olympus BX60 microscope. In this experiment, primary antibody omission controls were used to confirm the specificity of the immunofluorescence labeling.

Statistical Analysis

The data with two groups were analyzed by Student's *t*-test. The data with three or more groups were analyzed by one-way analysis of variance

(ANOVA) followed by Student–Newman–Keuls tests of multiple comparisons to determine whether there were significant differences between individual groups. All differences were considered significant at $p < 0.05$.

RESULTS

PKC α / β was expressed by C6 glioma cells and PKC α was activated in the presence of serum

Using western blot, we observed expression of PKC α and PKC β in C6 glioma cells. Our results showed that C6 cells expressed both PKC α and PKC β (Fig. 1A). Next, the activation of PKC α and PKC β were examined by analyzing their phosphorylation. We found that treatment of C6 cells with 10% FBS induced an increase in the phosphorylation of PKC α . A kinetic study showed that the phosphorylation of PKC α reached its peak at 30 min and declined at 3 hours after serum treatment (Fig. 1A). But activation of PKC β after expo-

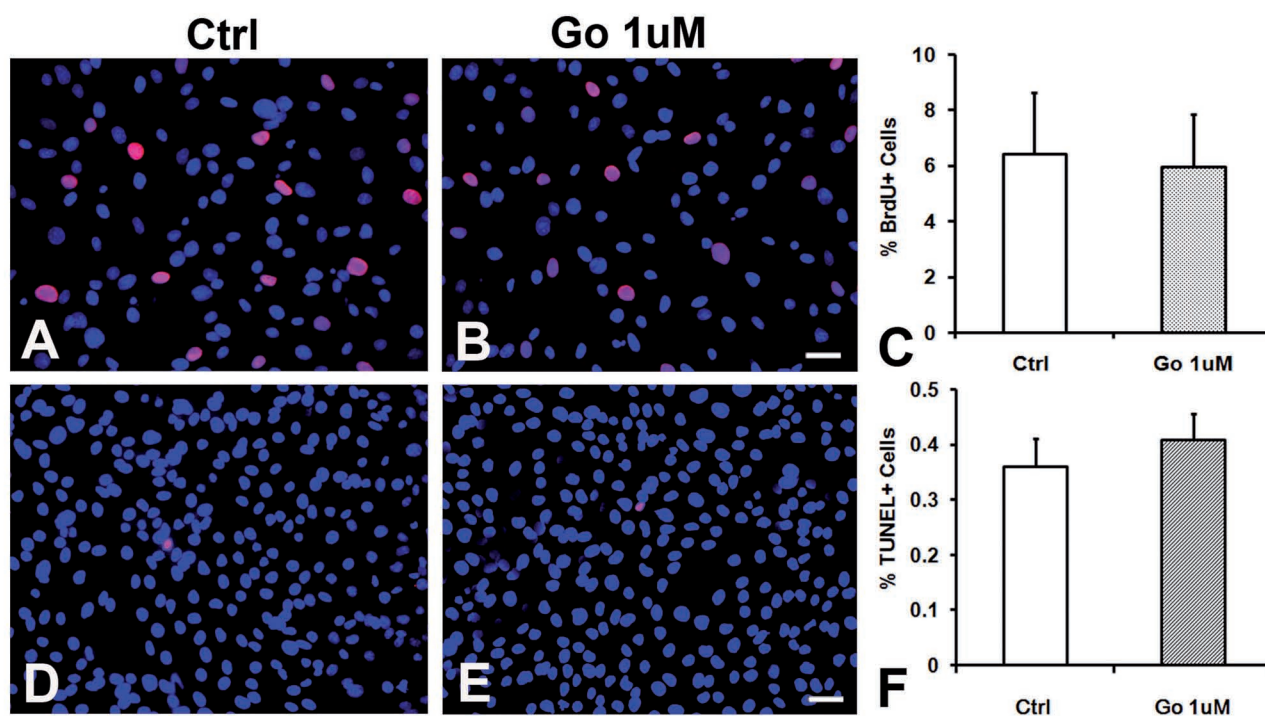


Fig.3 Effects of Gö6976 on the proliferation and survival of MMC-treated C6 glioma cells.

(A, B) Representative photomicrographs showed BrdU-positive cells (pink in the merged image) in MMC-treated C6 cells pre-treated without (A) or with (B) Gö6976 (1 μM). (D, E) Representative photomicrographs showed TUNEL-positive cells (pink in the merged image) in MMC-treated C6 cells pre-treated without (D) or with (E) Gö6976 (1 μM) for 24 hours. Cells in A, B, D, and E were counterstained with Hoechst 33342 (blue), a nuclear dye. Scale bar=50 μm. (C, F) The statistical data showed no statistically significant difference in the percent of BrdU-positive cells (C) and TUNEL-positive cells (F) between the Gö6976-treated and non-treated groups. Data are given as mean \pm SD ($p < 0.05$; $n = 4$ /group).

sure to serum was not observed (Fig. 1A). To confirm above results, the phosphorylation of PKC α was further examined by immunofluorescence staining. As a result, the fluorescence intensity of phospho-PKC α (P-PKC α) was very low in FBS-untreated C6 cells (Fig. 1B). While in FBS-treated cells, the stronger positive immuno-staining of phospho-PKC α was observed (Fig. 1C).

Gö6976, the PKC α / β specific inhibitor, inhibited migration of MMC-treated C6 glioma cells

To examine whether PKC signaling is involved in migration of C6 cells, Gö6976, a PKC α inhibitor, was applied in wound migration assay. To avoid the effect of cell proliferation, the C6 cells were treated with MMC before migration experiment. As shown in Fig. 2, there was a greatest migration of C6 cells in the presence of 10% serum. However, the migration was inhibited by pre-incubation with Gö6976 in a dose-dependent manner, and reached its peak at the concentration of 1 μ M. This result suggests that PKC α is required in the serum-induced C6 migration.

Gö6976 did not affect proliferation and survival of MMC-treated C6 glioma cells

Although the above results demonstrated that Gö6976 inhibited migration of C6 cells, it remained unclear whether the inhibition resulted from its effect on cell proliferation and/or survival. To exclude these possibilities, we examined the effect of Gö6976 on proliferation of MMC-treated C6 cells using BrdU incorporation assay. As a result, there was no statistically significant difference in the percent of BrdU⁺ cells between Gö6976-treated and non-treated groups (Fig. 3A-C). We next examined the effect of Gö6976 on survival of MMC-treated C6 cells using TUNEL assay. Results showed that there was still no statistically significant difference in the percent of TUNEL-positive cells between the two groups at 24 hours (Fig. 3D-F).

Serum-induced activation of PKC α was inhibited by Gö6976 in C6 glioma cells

To observe the effect of Gö6976 on serum-induced activation of PKC α in C6 glioma cells, western blot analysis was used. As expected, we found that the

phosphorylation of PKC α in serum-treated C6 glioma cells was significantly inhibited by Gö6976 in a dose-dependent manner (Fig. 4A). Immunochemistry results also showed that the fluorescence intensity of phospho-PKC α in C6 cells was decreased by pre-treatment with Gö6976 and returned to the basal level at the concentration of 1 μ M (Fig. 4B-D). Taken together, these results strongly demonstrated that the activation of PKC α is necessary for C6 cell migration.

DISCUSSION

PKC isoenzymes have been shown to display variable expression profiles depending on a particular cancer type (Koivunen et al. 2006). The studies have shown that PKC α highly express in high grade urinary bladder, prostate, and endometrial cancers (Langzam et al. 2001, Koren et al. 2004, Varga et al. 2004), whereas low grade tumors and normal epithelia of the respective organs show significantly lower expression (Koivunen et al. 2006). In contrast, breast, colon, hepatocellular, and basal cell cancers display the downregulation of PKC α (Tsai et al. 2000, Ainsworth et al. 2004, Kerfoot et al. 2004, Koivunen et al. 2006). PKC β has been shown to be upregulated in colon and

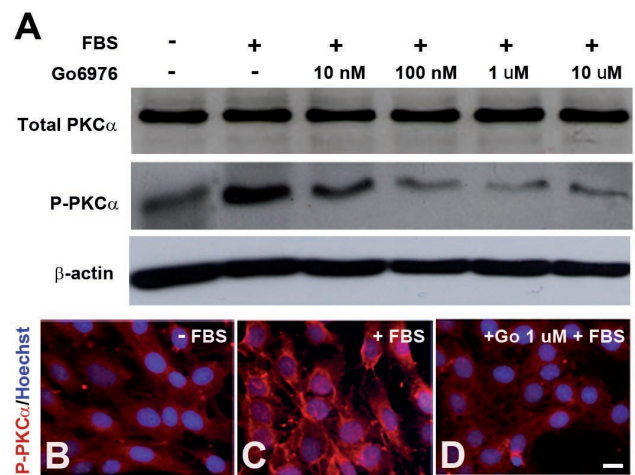


Fig.4 Effects of Gö6976 on phosphorylation of PKC α in C6 glioma cells treated with serum for 30 min.

(A) Western blot analysis showed that the serum-induced phosphorylation of PKC α in C6 glioma cells could be significantly inhibited by pre-treatment with Gö6976. β -actin was used as an internal control. (B-D) Immunochemistry showed that the fluorescence intensity of phospho-PKC α in C6 cells was decreased by pre-treatment with 1 μ M Gö6976. Scale bar=50 μ m.

prostate cancers (Koren et al. 2004, Koivunen et al. 2006) and downregulated in bladder cancer (Langzam et al. 2001, Varga et al. 2004). In the present study, we observed substantial expression of both PKC α and PKC β in C6 glioma cells. Since PKC α and PKC β activity is regulated by phosphorylation, we next examined the activation of PKC α and PKC β in C6 cells. We showed that there was less phosphorylated PKC α in the absence of serum. However, the PKC α was markedly activated in C6 cells after treatment with serum for 30 min. But we have not found the phosphorylation of PKC β in both presence and absence of serum. These results suggest that PKC α , but not PKC β , may play an important role in activation of C6 cells.

It has been reported that the PKC α activity is associated with the motility and invasion in several cancer cells, including urinary bladder carcinoma cells (Koivunen et al. 2004), colon carcinoma cells (Masur et al. 2001), renal cell carcinoma (Engers et al. 2000) and multiple myeloma cells (Podar et al. 2002). The mechanisms include inhibition of adherens junctions and desmosomes (Masur et al. 2001, Koivunen et al. 2006), inhibition of β 4-integrin mediated hemidesmosomes (Koivunen et al. 2004, Rabinovitz et al. 2004), and changes in β 1-integrin mediated cell-matrix junctions (Ng et al. 1999, Koivunen et al. 2004). Recently, it was reported that PKC α mediates an essential pro-mitogenic and pro-survival signal in glioma cells (Cameron et al. 2008). However, it is still unknown whether PKC α also plays a role in migration of C6 glioma cells. In this study, to examine whether PKC signaling is involved in migration of C6 cells, we applied Gö6976, a PKC α inhibitor, to investigate the migration of C6 cells in wound migration assay. To exclude the effect of cell proliferation, we used MMC to inhibit proliferation of C6 cells. The BrdU incorporation assay demonstrated that the MMC-treated C6 cells have no proliferation ability. The migration assay showed that pre-treatment of Gö6976 decreased migration of C6 cells in a dose-dependent manner. This result strongly supports the possibility that PKC α is involved in serum-induced C6 cell migration. However, it is also possible that the inhibition resulted from the effects of Gö6976 on proliferation and survival of C6 cells. To exclude these possibilities, we examined the effects of Gö6976 on proliferation and survival of MMC-treated C6 cells with BrdU incorporation and TUNEL assay, respectively. The results suggest that Gö6976 has no effect on proliferation and survival of

MMC-treated C6 cells. PKC α is activated through phosphorylation. Thus, phosphorylation of PKC α is a prerequisite for regulating C6 cell migration. In the present study, we provide evidence that the phosphorylation of PKC α could be significantly inhibited by Gö6976, the specific PKC α / β inhibitor, in a dose-dependent manner. Taken together, the results strongly demonstrated that PKC α plays a key role in serum-induced migration of C6 glioma cells.

CONCLUSION

In conclusion, our results demonstrated that PKC α is expressed by C6 glioma cells and its activation is important for glioma cell migration. Our findings may provide an insight into a better understanding to the malignant progression of gliomas.

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