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HETEROGENEITY OF PROTEIN KINASE C ACTIVITY IN HUMAN U-373 AND G-26 MICE GLIOMA CELLS[1]

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Summary: Protein kinase C (PKC) enzyme activity in a mouse glioma cell line G-26 and a human glioma cell line U-373 were compared at similar cell confluency in-vitro to establish if a G-26 in-vivo mouse model would be useful to examine the role of PKC inhibitors in controlling human glioma growth in-vivo. Original crude cytosolic and membrane PKC fractions of both mouse glioma G-26 and human glioma U-373 cells did not display significant PKC activity compared to partially purified PKC. Partial purification of mouse glioma G-26 and U-373 cytosolic and membrane fractions showed different cytosolic and membrane PKC activity profiles. Total PKC activity was higher (ρ = 0.0001) in human glioma U-373 (7840 picomoles/mg/min) than in mouse glioma G-26 cells (2890 picomoles/mg/min). Thus, results from trials using nude mice human glioma xenografts may be more valid than those obtained from a G-26 in-vivo mouse model for studying the effects of therapeutic drugs on PKC isozymes.

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Abbreviations: PKC, Protein kinase C; DAG, diacylglycerol; EGTA, ethylene glycol bis(ß-aminoethyl ether)- N,N,N',N',-tetraacetic acid; PtdSer, phosphatidylserine; TCA, trichloroacetic acid; DTT, dithiothreitol; PMSF, phenyl-methylsulfonly fluoride.

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The potential therapeutic application of protein kinase C (PKC) inhibitors to alter growth rates of malignant cells has been demonstrated in-vitro and in-vivo (1,2). However, the effects of PKC inhibitors on proliferation of lethal high grade gliomas have not been reported. PKC is a family of eleven known isozymes which are phospholipid dependent serine/threonine kinases found in varying ratios in the cytosolic and membrane fractions of cells depending on the type of tissue and its physiological state (3,4).

The rapid proliferation rates of gliomas have been attributed to inherently high levels of PKC. For example, Couldwell et al. (5) compared growth rates and PKC activity levels in non-neoplastic astrocytes and mixed glial cultures to that of four human malignant glioma lines (A172, U373-MG, U563-MG, U178-MG) and rat C6 cells. Increased PKC activity levels were found in malignant gliomas compared to normal astrocytes and mixed glial cultures in both human and rat cells, suggesting a correlation between PKC activity levels and the cellular growth rate of gliomas.

In this investigation, we determine endogenous cytosolic and membrane phosphorylation PKC activity levels in a mice glioma cell line (G-26) and a human glioma cell line U-373MG (U-373) to establish if G-26 PKC activities are similar to human glioma cells in-vitro. If similar PKC activities exist in both cells, an in-vivo mouse model system (6) may be conducive for in-vivo studies using specific therapeutic drugs that would modify abnormal second messenger (PKC) responses in dysregulated cells.

Materials and Methods

Cell lines and cultures: The human glioma U-373 cell line was obtained from the American Tissue Type Collection. The mouse glioma (G-26) cell line was provided by Dr. Marzenna Wiranowska. Glioma U-373 and G-26 cells were cultured according to Ponten et al. (7). Cells were seeded (1×10^6) on 75 cm^2 flasks containing minimum essential media, 10% fetal calf serum, 2 mM L-glutamine and antibiotics (penicillin 10 U/mL and streptomycin 10 mg/mL). Cells were used at confluency, approximately seven days post subculture.

Subcellular fractionation of cells: Subcellular fractionation of U-373 and G-26 cells was performed by slight modification of published procedures (8,9). Flasks containing cells were placed on ice and washed twice with ice cold Dulbecco's phosphate buffered saline. Monolayers were scraped at 4°C and

sonicated in 2 ml homogenization buffer (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 2mM DTT, 2 mM PMSF, 0.4 mM leupeptin, 0.15 U/mL aprotinin). DTT (2mM) was included in the homogenization buffer to prevent oxidative inactivation of PKC (9). Cell suspensions were centrifuged at 100,000g for 30 min to obtain cytosol and membrane fractions. The latter was resuspended in 2 ml of homogenization buffer with 1% Triton-X-100, sonicated and centrifuged at 100,000 g for 30 min to obtain the soluble membrane fraction.

Partial purification of PKC: Membrane and cytosolic fractions were chromatographed on DEAE-Sephacel (Pharmacia, Piscataway, NJ) according to Gopalakrishna et al. (10). Partial purification of PKC by chromatography on DEAE-Sephacel was performed to remove extrinsic inhibitors and activators of PKC (11).The DEAE-Sephacel column (1.0 ml bed) was equilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM DTT). Cytosolic or membrane fractions (2 ml) were applied to the column and the column washed with 4 ml of buffer A. Bound PKC was subsequently eluted with 2 ml of 0.1 M NaCl in buffer A. Proteolytically activated PKC (M-kinase) and/or oxidatively modified PKC remained bound to the column until eluted with 0.25 M NaCl in buffer A (10).

Assay for PKC: Original and partially purified cytosolic and membrane PKC phosphotranferase activity was assayed by its ability to transfer ^{32}P from [γ-^{32}P] ATP into histone as described previously (12). Briefly, 100 μl of cytosolic or membrane fractions containing approximately 20 μg of protein were incubated in a 250 μl standard reaction mixture consisting of 20 mM Tris-HCl, pH 7.5, 200 μg/ml histone type III-S (Sigma), 5nmole of [γ-^{32}P] ATP (1.5 x 10^6 cpm/nmol), 6mM magnesium acetate, and various combinations of PtdSer (5 μg), DAG (0.2 μg) and $CaCl_2$. Reactions proceeded for 3 min at 30°C and were terminated by addition of 1 ml of ice-cold 25% TCA. Precipitates were collected on Millipore filters (0.45 μm HAWP) by vacuum filtration, washed twice with 2 ml of ice-cold TCA and counted for ^{32}P using liquid scintillation spectroscopy. Activity was measured as picomoles of ATP/mg/min. Basal kinase activity was measured in varying $CaCl_2$ concentrations, in the absence of $CaCl_2$ and phospholipid and in the presence of EGTA. Basal kinase activity was subtracted from the total incorporated ^{32}P in order to calculate total specific PKC activity. Data are from two separate experiments and are expressed as the means ± SEM of six replicated determinations. Statistical determination was by Student's T test using Minitab program (Minitab Inc. State College, PA.).

Protein assay: Protein concentration was determine as described by Bradford (13).

Results

Original crude U-373 cytosolic (Fig. 1A) and membrane (Fig. 1B) PKC fractions did not display significant PKC activity compared to partially purified PKC. Optimal Ca^{2+} concentration for partially purified U-373 PKC activity in the cytosol was 1.0 mM and 0.5 mM in the membrane. Partially

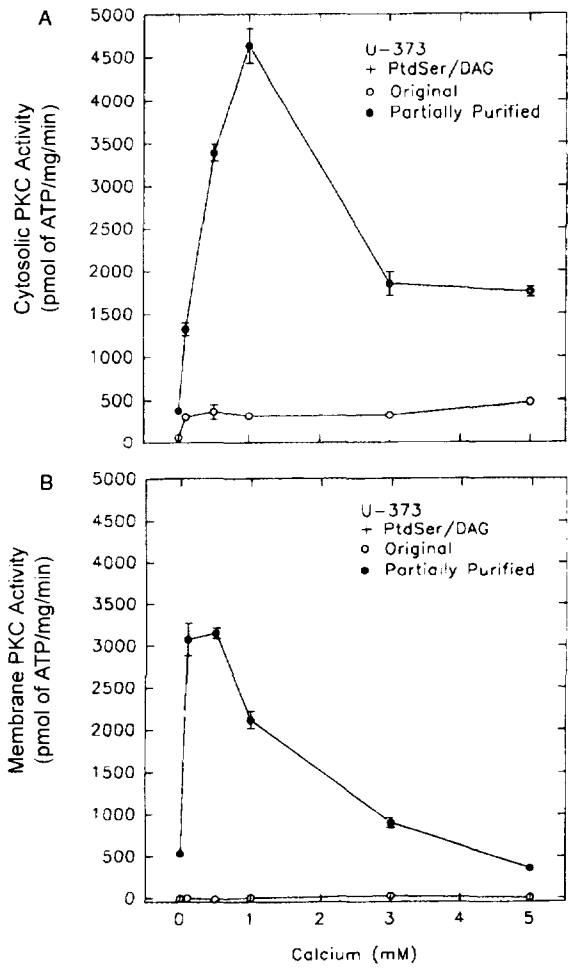

Fig. 1. Protein kinase C activities in human U-373 glioma cells. (A) Original ○ and partially purified ● cytosolic PKC. (B) Original ○ and partially purified ● detergent-solubilized membrane PKC. PKC activity was determined as described in Materials and Methods. Data are expressed as the mean ± SEM of two separate experiments with six replicated determinations.

purified U-373 PKC activity was distributed 59% in the cytosol and 41% in the membrane fraction when compared at their respective optimum Ca^{2+} concentration. Mouse G-26 cells contained 70% of their PKC activity in the cytosolic fraction (Fig. 2A) and 30% in the membrane (Fig.2B). The total amount of PKC activity present in U-373 (7840 picomoles/mg/min) is 2.7 fold more (ρ = 0.0001) than that present in G-26 cells (2890 picomoles /mg/min).

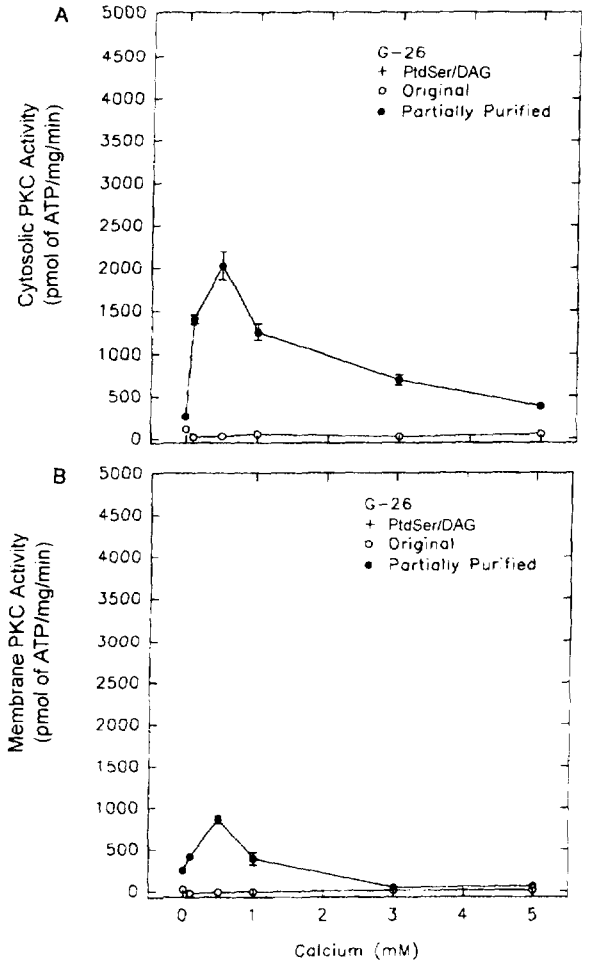

Fig. 2. Protein kinase C activities in mouse G-26 glioma cells. (A) Original ○ and partially purified ● cytosolic PKC. (B) Original ○ and partially purified ● detergent-solubilized membrane PKC. Data are expressed as the mean ± SEM of two separate experiments with six replicated determinations.

When U-373 kinase activity were assayed in the absence of both PtdSer and DAG to determine phospholipid-independent kinase activity, U-373 cytosolic kinase activity was minimal compared to partially purified membrane kinase activity which increased 12 fold at 0.5 mM Ca^{2+} (Fig. 3A and 3B). In contrast, G-26 kinase activity (Fig. 4A and 4B) was present in both partially purified G-26 cytosol (47%) at 1 mM Ca^{2+} and membrane (53%) at 0.1 Ca^{2+}.

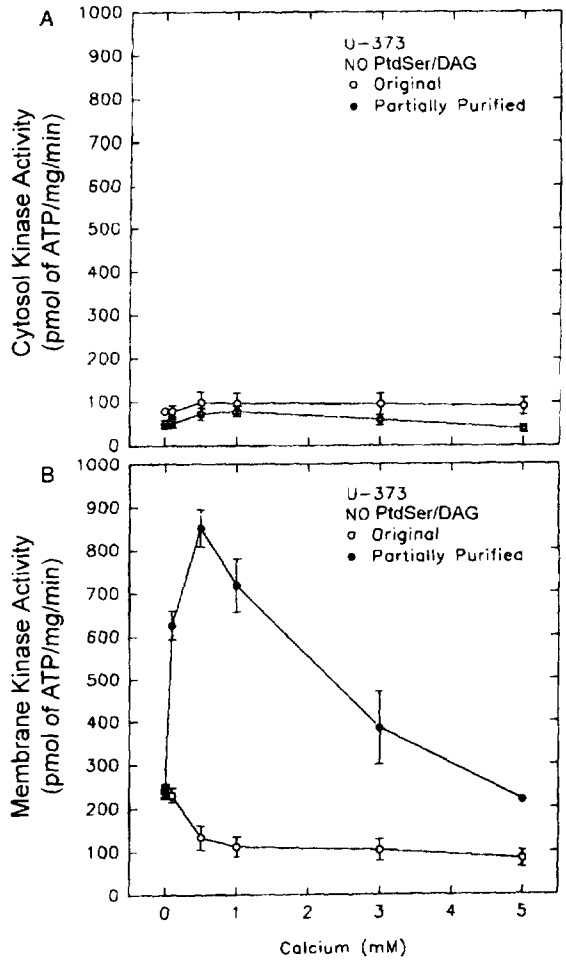

Fig. 3. Protein kinase activities in human U-373 glioma cells. (A) Original ○ and partially purified ● cytosolic PKC. (B) Original ○ and partially purified ● detergent-solubilized membrane PKC. Protein kinase activity was determined in the absence of PtdSer/DAG. Results shown are the mean ± SEM of two separate experiments with six replicated determinations.

Discussion

Couldwell *et al.* (5) reported moderate U-373 PKC activity for total crude cytosolic and membrane fractions (3299 pmol of ATP/mg/min) compared to the other glioma cell lines they studied. However, we found that crude U-373 cytosolic and membrane PKC fractions had minimal PKC activity (364 pmol of ATP/mg/min) and required partial purification by DEAE-Sephacel to obtain high PKC activity levels (7840 pmol ATP/mg/min).

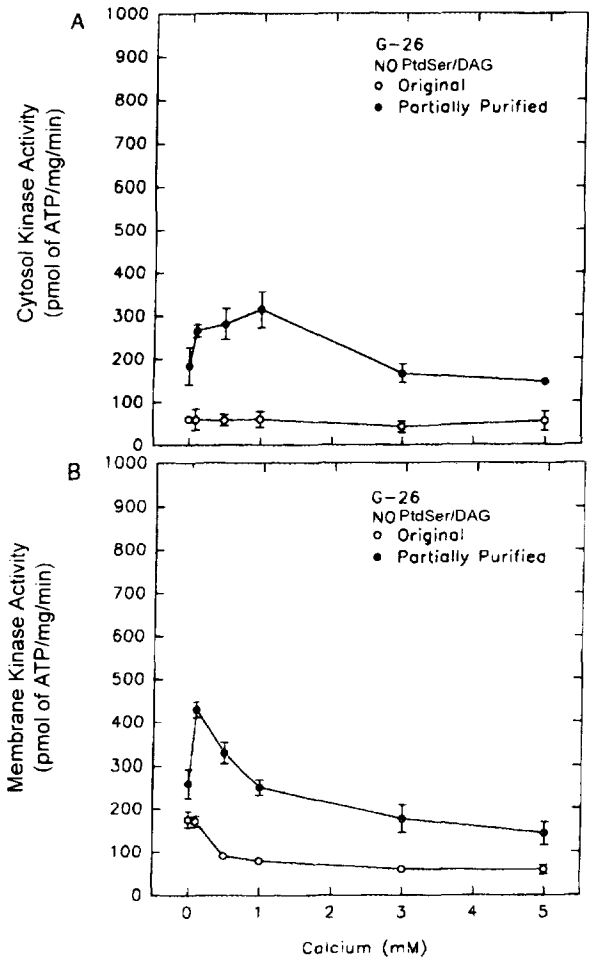

Fig. 4. Protein kinase activities in mouse G-26 glioma cells. (A) Original ○ and partially purified ● cytosolic PKC. (B) Original ○ and partially purified ● detergent-solublized membrane PKC. Protein kinase activity was determined in the absence of PtdSer/DAG. Results shown are the mean ± SEM of two separate experiments with six replicated determinations.

Differences between our findings and those of Couldwell *et al.* (5) may be due to the techniques used to measure PKC activity.

Our data also demonstrated that PKC enzyme activity at varying Ca^{2+} concentration is different in G-26 cells compared to U-373 glioma cells. These results raise questions about the validity of using G-26 glioma cells as a model for testing treatment modalities which affect human glioma PKC. Since variation in glioma PKC enzyme activities and isozyme content

exist within tumor grades and between species (3,5,14,15), a glioma tumor model which uses nude mice human glioma xenografts may be more suitable for investigating the use of PKC inhibitors to control proliferation of human gliomas.

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References

1. Meyer. T., Regenass, U., Fabbro, D., Alteri, E., Rosel, J., Muller, M., Caravattii, G., and Matter, A. (1989) Int. J. Cancer 43, 851-856.

2. Akinagaka, S., Gomi, K., Morimoto, M., Tamaoki, T., and Okabe, M. (1991) Cancer Res. 51, 4888-4992.

3. Stabel, S., and Parker, P.J. (1991) Protein kinase C. Pharmac. Ther. 51, 71-95.

4. Selbie, L.A., Schmitz-Peiffer, C., Sheng, Y., and Biden, T. (1993) J. Biol. Chem. 268, 24296-24302.

5. Couldwell, W., Uhm, J.H., Antel, J.P. and Yong, V.W. (1991) Neurosurgery 29, 880-887.

6. Wiranowska, M., Gonzalvo, A.A., Saporta, S. and Prockop, L.D. (1992) J. Neuro-Oncol. 44, 225-236.

7. Ponten, J., and MacIntyre (1968) Act. Pathol. Microbiol. Scand. 74, 465-486.

8. Acevedo-Duncan, M., Cooper, D.R., Standaert, M.L. and Farese, R.V. (1989). FEBS Lett. 244, 174-176.

9. Gundimeda, U., Hara, S.K., Anderson, W.B., and Gopalakrishna, R. (1993) Arch. Biochem. Biophys. 300, 526-530.

10. Gopalakrishna, R., Chen, Z.H. Gundimeda, U., Wilson, J.C., and Anderson, W.B. (1992) Anal. Biochem. 206, 24-35.

11. Thomas, T.P., Gopalakrishna, R., and Anderson, W.B. (1986) Meth. Enzymol. 141, 399-411.

12. Davis, J.S., and Clark, M.R. (1983) Biochem. J. 214, 569-574.

13. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.

14. Todo, T., Shitara, N., Nakamura, H., Takakura, K., Tamonaga, M., and Ikeda, K. (1991) Neurosurgery 29, 399-404.

15. Reifenberger, G., Deckert, M. and Wechsler, W. (1989) Acta Neuropathol 78, 166-175.