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Enhanced Protein Kinase C Activity Correlates with the Growth Rate of Malignant Gliomas in Vitro

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Direct measurement of protein kinase C (PKC) activity in vitro revealed a significant increase in the activity of the enzyme in all human malignant glioma lines examined and the rat C6 tumor in comparison with control nonneoplastic astrocyte and mixed glial cultures. The total and particulate PKC activity in these cell types correlated strongly [$r = 0.98$ ($P < 0.001$) and 0.94 ($P = 0.002$), respectively] with the maximal growth rates as measured by ^{3}H-thymidine incorporation in each of the samples. An alteration in the growth rate of an individual glioma line (A172) by varying the serum concentration in the growth medium produced comparative changes in the measured PKC activity. The addition of the phorbol ester phorbol-12-myristate-13-acetate to this tumor line under high serum conditions produced down-regulation of the enzyme, which was accompanied by a corresponding reduction in thymidine incorporation. The administration of the PKC inhibitor staurosporine produced a dose-related decrease in the basal proliferation rate of glioma lines A172 and C6, as measured by ^{3}H-thymidine uptake and confirmed by flow cytometry, indicating that the high intrinsic PKC activity is amenable to pharmacological manipulation. Cytofluorometric deoxyribonucleic acid cell cycle analysis of the tumors treated with PKC modulators demonstrated that reduced proliferation rates were caused by an inhibition of entrance into the deoxyribonucleic acid synthesis (S) phase (decrease in proliferative index), supporting the evidence that these modulators are not slowing the tumor growth in a nonspecific cytotoxic manner. The data support the hypothesis that intrinsic high PKC enzyme activity correlates with the rapid proliferation rates observed in malignant gliomas and thereby implicate PKC as an important biochemical and functional marker of neoplastic glia. (*Neurosurgery* 29:880–887, 1991)

Key words: Brain neoplasms, Flow cytometry, Glioma, Phorbol ester, Protein kinase C

INTRODUCTION

Pharmacological manipulations of the protein kinase C (PKC) signal transduction system can alter the growth rates of established human malignant glioma cell lines in vitro (6–8, 16, 23). Following the initial observations that activators of PKC decrease the rate of proliferation of glioma cells, while concordantly increasing their state of differentiation (7, 8), it was hypothesized that glioma cells display inherent high levels of activity of this enzyme that correlate with their rapid growth rates; such high activity would then undergo down-regulation (disappearance of PKC) in the presence of PKC activators such as phorbol esters, resulting in the inhibition of cell growth (8).

In this study, a direct in vitro assay of PKC activity was undertaken to compare endogenous levels of this enzyme in malignant glioma cell lines and nonneoplastic astrocytes and to determine if PKC activity varied with the differential growth rates between these cell types. The PKC system was examined further within an individual tumor by comparing the levels of enzyme activity after manipulations in the growth rate by altering the concentration of serum (with endogenous growth factors) in the culture medium. PKC activity was also measured after the addition of the phorbol ester phorbol-12-myristate-13-acetate (PMA), a PKC activator, to determine if down-regulation of the enzyme occurs under these conditions as postulated. To test the hypothesis that the high intrinsic activity of this enzyme in these tumors may prove useful as a target for inhibition, growth rates of gliomas were measured following the administration of a relatively specific inhibitor of PKC, staurosporine (SP) (25). Flow cytometric analysis of tumor cells after treatment with activators and inhibitors of PKC was performed to gain insight into the mechanism of action of the growth modulation produced by these agents.

MATERIALS AND METHODS

Glioma cell lines and nonmalignant glial controls

The cell lines A172, U178-MG, U373-MG, and U563-MG (courtesy of V. P. Collins, M.D., Ludwig Institute for Cancer Research, Stockholm, Sweden) are characterized human malignant glioma lines established elsewhere (5, 11). The rat glioma C6 (American Tissue Culture Collection, Rockville, Maryland) was also used in these studies. These lines were maintained in Falcon tissue culture flasks (25 m^2), in a medium consisting of Eagle's minimal essential medium supplemented with 10% fetal calf serum (FCS), gentamicin (20 μg/ml), glutamine, pyruvate (1 mmol/L), dextrose (0.1%), and essential amino acids buffered to a pH of 7.0 (all medium constituents were purchased from GIBCO, Grand Island, New York). At confluency, the tumor was passaged regularly by gentle trypsinization (0.05% for 10 min), followed by serum inactivation of trypsin, repeated washes with phosphate-buffered saline (PBS), and replating. Cells were grown at 37% in a humidified 5% CO_2 incubator. All lines in our laboratory were tested routinely for mycoplasma contamination.

Control normal human glia were obtained from patients undergoing surgery to ameliorate intractable epilepsy. Cells were isolated as previously described (26, 27). In brief, brain cubes were incubated with 0.25% trypsin and 20 μg/ml of deoxyribonuclease (DNAse) for 1 hour at 37°C. The dissociated cells were passed through a nylon mesh with a pore size of 130 μm, and the filtrate was centrifuged in Percoll at 15,000 revolutions per minute for 30 minutes. The visible cell layer was collected, washed, and resuspended in a feeding medium as described above for glioma cells. These cells were placed in Falcon flasks with a capacity of 25 cm^2. Twenty-four hours later, floating oligodendrocytes were removed and plated as enriched (>80%) oligodendrocyte preparations. Adherent cells, consisting of astrocytes and microglia, were allowed to develop morphologically. Seven to 10 days later, astrocytes were shaken off (150 revolutions per minute for 5 hr)

to leave behind an enriched microglia preparation of over 90% purity. When human adult cells were used as a mixed glial preparation, these were cells seeded in Falcon flasks after the initial isolation. Because of the low yield of astrocytes in human biopsy specimens (average of less than 300,000 cells per preparation), an enriched astrocyte culture could not be used in the present study.

As controls for the malignant glioma PKC levels in the rat C6 line, nonneoplastic rat astrocytes of over 95% purity were obtained from neonatal brains. Postnatal Day 1 rat brains were dissected into cubes of 1 mm or less and treated with 1% trypsin and 50 µg/ml of DNAse for 10 minutes at 37°C. The cubes were centrifuged at 2000 rpm for 10 minutes to remove the high trypsin concentration and further incubated with 0.1% trypsin and 50 µg/ml DNAse for 30 minutes at 37°C. FCS was added to 10% final concentration to inactivate the trypsin. After centrifugation at 1200 rpm, the dissociated cells were washed twice with a feeding medium and plated onto poly-*l*-lysine-coated Falcon flasks (25 cm^2) at a density of 10×10^6 cells per flask. The cultured cells were allowed to differentiate for a period of 10 days (mostly to astrocytes because of the high serum content). Cell-type characterization at this time indicated that the cells were over 95% astrocytes as determined by immunoreactivity for glial fibrillary acidic protein (GFAP). Other cell types present were oligodendrocytes (galactocerebroside positive; less than 1%), fibroblasts (fibronectin immunoreactivity; 1%), and macrophage/microglia cells (Mac-1 positive; 2%).

Protein kinase C assay

Glioma cells at varying stages of growth (midlogarithmic or approaching confluency) adherent to culture plates (150 mm) were rinsed twice with ice-cold PBS, followed by a rinse with homogenizing buffer containing 50 mmol/L of Tris-HCl, 2 mmol/L of dithiothreitol, 1 mmol/L of phenylmethylsufonylfluoride, and 2 mmol/L of ethyleneglycol-bis-(beta-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid. The cells were scraped off the culture plates into 2 ml of the above solution and homogenized (30 strokes) in a glass homogenizer. The homogenate was centrifuged at $100{,}000 \times g$ for 1 hour. The supernatant was designated the *cytosolic fraction*. The pellet was resuspended in 2 ml of the above buffer containing 1% Triton X-100, homogenized (30 strokes), and mixed slowly for 30 minutes. This resuspension was centrifuged at $100{,}000 \times g$ for 1 hour, and the supernatant designated as the *particulate fraction*. All procedures were performed at 4°C. The enzyme fractions were stored at −70°C before the assay for PKC.

The method used to assay for PKC activity (phosphate transfer into lysine-rich histone) is modified from the procedure of Neary et al. (17), after having determined the correct Michaelis-Menten conditions for all cofactors and substrate. The reaction mixture contains (final concentrations): 50 mmol/L of Tris-HCl, 100 mmol/L of free calcium, 250 µg/ml of lysine-rich Type IIIS histone, 500 µg/ml of phosphatidylserine, 25 µg/ml of diacylglycerol (dioctanoyl-sn-glycerol), 10 µmol of adenosine triphosphate (ATP) (mixed with ^{32}P-ATP, $0.5\text{-}1 \times 10^6$ counts per minute), 10 mmol/L of $MgCl_2$, and 4 µl of enzyme preparation. The pH of the reaction mixture is 7.4 in a final volume of 40 µl. The enzyme preparation (particulate or cytosolic fraction) is preincubated with the reaction mixture minus ATP and $MgCl_2$ at 30°C for 2 minutes. The phosphorylation of histone is initiated by adding ATP and $MgCl_2$ and is allowed to proceed at 30°C for 1 minute. The reaction is terminated by placing a 25 µl aliquot of the mixture onto a phosphocellulose paper (1×2 cm; Whatman P81). Each paper is transferred quickly to a beaker containing 75 mmol/L of phosphoric acid. After three washes in phosphoric acid (5 min each) to remove unreacted ^{32}P-ATP, the strips are dehydrated with absolute methanol, air dried, placed in vials containing 5 ml of Cytoscint (ICN Chemical, Irvine, California), and counted for radioactivity. Negative blanks (non-PKC-dependent reaction) consist of the reaction mixture minus calcium (in the presence of EGTA), phosphatidylserine, and diacylglycerol. The PKC-dependent reaction is calculated as the difference between activity in the presence and absence of calcium, phosphatidylserine, and diacylglycerol. Each enzyme preparation is analyzed in triplicate, and results are expressed as picomoles of ATP transferred per minute per milligram of protein. Variability between triplicates, and between assays, is less than 10% of the mean. All reagents were purchased from Sigma Chemical Co. (St. Louis, Missouri), with the exception of γ-^{32}P-ATP, which was purchased from New England Nuclear (Wilmington, Delaware).

To compare reliably the activity between cell types, all samples were run at 1 µg of protein the reaction mixture. When results are expressed as total PKC activity, this represents the sum of the cytosolic and particulate activity in the same cell line.

TABLE 1

Comparative Protein Kinase C Enzyme Levels in Glioma Lines and Nonneoplastic Glia[a]

Cell Type	Cell Fraction	Protein Kinase C Activity (pmol ATP[b] Transferred/min/mg protein)
Human glioma		
A172	Cytosol	2921
	Particulate	5590
	Total	8511
U373-MG	Cytosol	2227
	Particulate	1072
	Total	3299
U563-MG	Cytosol	2018
	Particulate	653
	Total	2671
U178-MG	Cytosol	1424
	Particulate	805
	Total	2229
Human glia		
Mixed	Cytosol	ND
	Particulate	ND
Oligodendroglia	Cytosol	56
	Particulate	ND
	Total	56
Microglia	Cytosol	ND
	Particulate	ND
Rat glioma		
C6	Cytosol	4858
	Particulate	4416
	Total	9274
Rat glia		
Astrocyte (Neonatal)	Cytosol	124
	Particulate	599
	Total	723
Oligodendrocyte (1 month)	Cytosol	41
	Particulate	ND
	Total	41

[a] PKC functional enzyme levels after isolation of cytosolic and particulate fractions of human malignant gliomas A172, U178-MG, U373-MG, U563-MG, and C6 and control nonneoplastic glia (human mixed glia, microglia, and oligodendrocyte; rat astrocyte and oligodendrocyte cultures). Total activity is defined as the sum of cytosol and particulate activity. Glioma cultures were harvested at various times after plating of 1.5×10^6 cells onto 150 mm culture plates; maximal PKC activity (from cultures approaching confluency) are tabulated. Equivalent amounts of protein (1 µg) were assayed in all samples. There were markedly elevated PKC levels present in all the neoplastic glia in comparison with the nonneoplastic controls. At 1 µg of protein in reaction mixture, activity was not detectable (ND) in the human microglia and mixed glial preparations; however, activity was detected at concentrations of 5 µg or more.

[b] ATP, adenosine triphosphate.

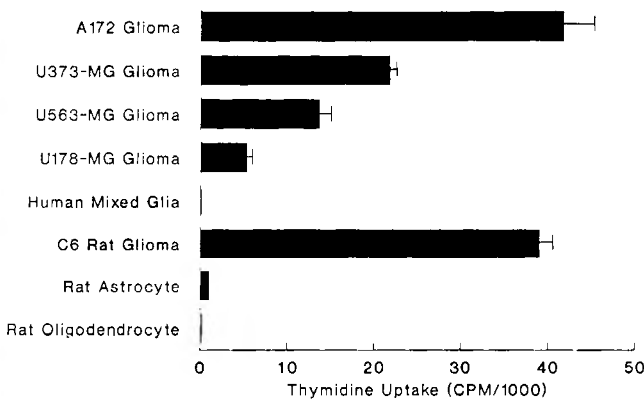

FIG. 1. Comparative maximal proliferation rates among glioma lines and nontransformed glia. ^{3}H-thymidine uptake as a measure of cell proliferation varies among different human glioma lines (A172, U373-MG, U563-MG, and U178-MG), rat glioma (C6), and nontransformed human and rat glia cultures. Values are the mean of triplicate wells + standard error of the mean. Glioma ^{3}H-thymidine uptake values represent maximal counts per minute attained for each tumor during the growth phase. Since nontransformed glia cultures do not proliferate sufficiently to determine growth curve kinetics, these cultures were seeded near confluency to obtain maximal values.

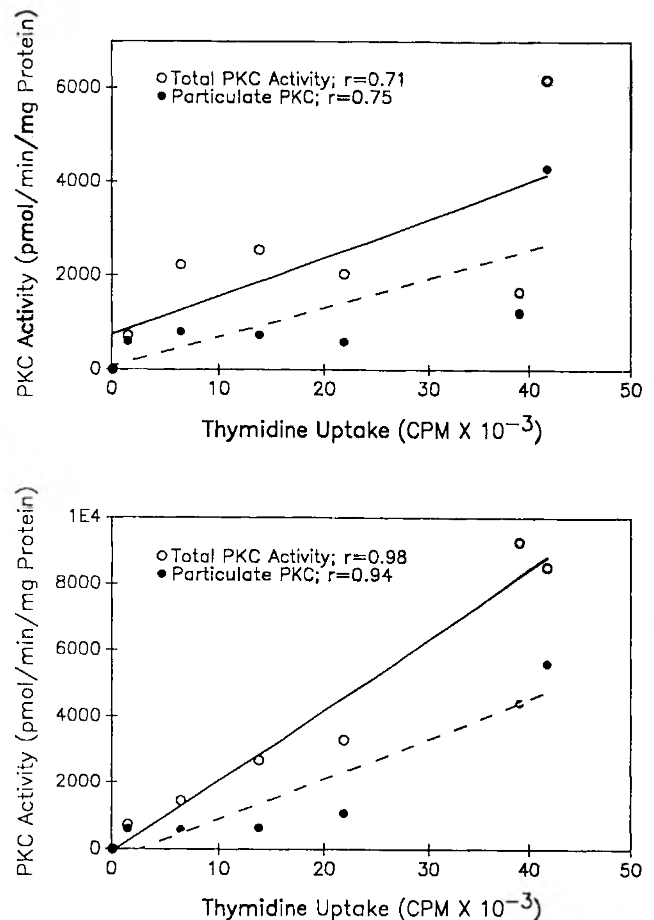

FIG. 2. PKC activity correlates with the proliferation rate. Maximal ^{3}H-thymidine uptake values plotted against total PKC activity measured in the midlogarithmic growth phase (*upper*) and approaching confluency (*lower*) for the individual tumors and glial cultures in the legend to Figure 1. Maximal thymidine uptake correlated strongly with maximal total (*solid line*) and particulate (*dotted line*) PKC activity for the tumor and glial cultures.

In vitro glioma proliferation assay—effects of serum and pharmacological protein kinase C modulators

Our method for determining rates of glioma proliferation has been published previously (8). After passage, the cell lines were seeded at a density of 10^4 cells in 40 μl of medium on glass coverslips (12 mm) placed in culture plates with 24 wells. Coverslips were previously coated with 10 μg/ml of poly-*l*-lysine to facilitate cell adherence. Twelve hours later, after adequate time for cells to adhere to the coverslips, the wells were flooded and washed with PBS. The PBS was removed, and the feeding medium described above, but containing variable quantities of FCS, was added to the wells containing the coverslips. For PKC inhibitor studies, SP was added at predetermined concentrations in replicates of three to wells containing gliomas grown in a range of FCS concentrations (0–10%). At specified intervals, wells were pulsed with 1 μCi of ^{3}H-thymidine/ml for a period of 8 hours before harvest.

For the time-course study of PMA on glioma line A172, the above technique was modified to use a short pulse of ^{3}H-thymidine (30 min) at the concentration above to Petri dishes (35 mm) containing four coverslips. The reason for using this short thymidine pulse period is to attempt growth correlation with the very rapid kinetics of the PKC enzyme as indicated by preliminary studies. Control experiments with bromodeoxyuridine (a thymidine analog) immunofluorescence had indicated that a pulse of 30 minutes was adequate to observe significant incorporation of a proliferation label into glioma cells (data not shown).

To measure thymidine incorporation for both of these methods, the coverslips with adherent cells were washed four times with PBS and placed in scintillation vials containing 5 ml of scintillation fluid for determination in a beta counter. To demonstrate that the uptake of ^{3}H-thymidine into cells is a reliable index of deoxyribonucleic acid (DNA) synthesis, a control experiment was performed in which the synthesis of DNA in the glioma cells was stopped by treating the cells with 50 μg/ml of mitomycin-C (Sigma) for 30 minutes; cells were washed four times with fresh medium and pulsed as above with ^{3}H-thymidine. Resulting background cell counts were negligible.

Cytofluorometry of glioma cultures

To confirm the results of the ^{3}H-thymidine incorporation, cell cycle analysis was performed by propidium iodide staining with analysis by flow cytometry. Cells were plated initially after passage at a density of 5×10^5 cells per tissue culture flask (25 cm^2) in 5 ml of the glioma feeding medium described above containing 10% FCS. After adequate time for cell adherence (12 hr), the medium was changed, and fresh medium containing the PKC modulators (activators and inhibitors) in the concentrations outlined below was added. The cultures were maintained for a period of 96 hours, after which the cells were trypsinized, washed three times in PBS, and fixed by resuspending 1×10^6 cells/ml in a solution of PBS and ice-cold methanol (1:2 final concentration, vortexing the cells to avoid clumping) for a period of 1 hour at 4°C. The staining protocol was adapted from that of Hurley (12). After fixation, cells were centrifuged, the supernatant was decanted, and 0.5 ml of propidium iodide stain solution was added. This stain solution contained 10 mg of propidium iodide (Calbiochem, San Diego, California), 0.1 ml of Triton X-100 (Sigma), and 3.7 mg of ethylenediamine tetraacetic acid (Sigma) in 100 ml of PBS. Immediately after the addition of the stain solution, 0.5 ml of RNAase solution [10 mg of RNAase (Sigma) mixed with 5 ml of PBS and heated to 75°C for 30 min before use] was added to eliminate double-stranded ribonucleic acid, which would interfere with DNA quantification. Samples were analyzed on a fluorescent activated cell sorter (FACScan®, Becton Dickinson, San Jose, California) after a staining period of 1 hour at 37°C in the dark. Cellular DNA events were acquired using CELLFIT software (Becton Dickinson), with an estimation of percentage of cells

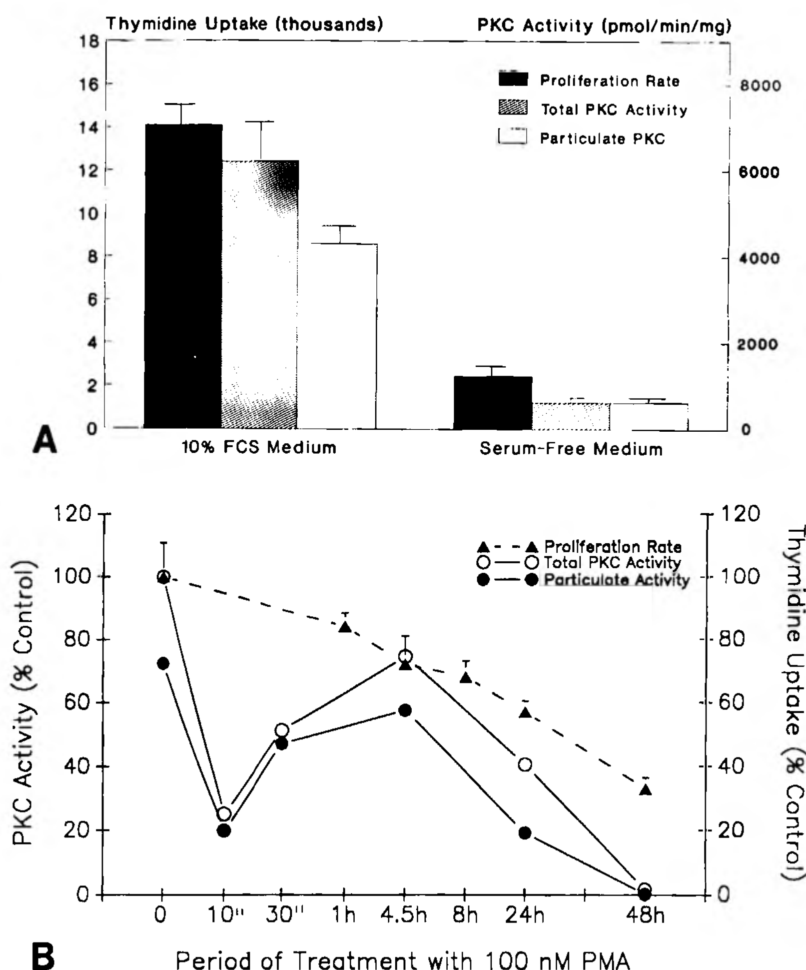

FIG. 3. Alterations in the growth rate produce corresponding PKC activity changes within glioma line A172. *A*, removal of FCS from the growth medium reduces the proliferation rate of glioma line A172, accompanied by a proportional reduction of total and particulate PKC activity. Values are the mean of triplicates + standard error of the mean. *B*, administration of the phorbol ester PMA (100 nmol/L) to the tumor produces a rapid decrease in proliferation. Thymidine values are the mean of triplicate wells + standard error of the mean. PKC activity decreases in the controls within the first time point measured (10 min), following a time course similar to that of the proliferation rate.

in particular phases performed by the SFIT model. Proliferation indexes (1, 10), defined by the sum %S + %G_2/M, were calculated for each of the various PKC treatments. For flow cytometric analysis of cell cycle kinetics, the inhibitor of DNA replication cytosine arabinoside (10^{-5} mol/L; Sigma) was used as a control (to arrest the tumor in the S-phase).

Incubation of glioma lines with protein kinase C modulators

The following agents were used in the PKC assay or the growth measurement assays described above. The relatively selective PKC inhibitor SP (Calbiochem, La Jolla, California) (25) was dissolved in ethanol and further diluted in PBS; it was used over a range of concentrations from 1 to 100 nmol/L. PMA (Sigma), a phorbol ester and activator of PKC (2, 14), was administered at a dose of 100 nmol/L. A non-phorbol synthetic activator of PKC, SC-9 (Seikagaku America Inc., Rockville, Maryland) (18), was diluted in ethanol and added to the feeding medium to a final concentration of 50 µmol/L. We previously reported that, at these concentrations, the PKC modulators were not directly cytotoxic to glioma cells as measured by chromium-51 release (8) and flow-cytometric DNA analysis (9). Furthermore, in the absence of serum, we have found that glioma proliferation was increased by 100 nmol/L of PMA, further suggesting that this concentration was not toxic to cells. For the purposes of this study, we therefore chose to employ relatively high concentrations (e.g., PMA at 100 nmol/L) to amplify the results.

RESULTS

Protein kinase C activity in nontransformed and malignant glia

The direct measurement of intrinsic PKC activity was determined in malignant glioma and nonneoplastic glia controls (human mixed glial cultures, enriched oligodendrocyte cultures, and rat astrocytes and oligodendrocytes). Maximal PKC activity in A172, U178-MG, U373-MG, U563-MG, and C6 cells during the logarithmic growth phase approaching confluency revealed high levels of activity discernible in both particulate and cytosolic fractions (Table 1). Activity in nonneoplastic glia under the same test conditions was at least an order of magnitude less compared with that of each homologous tumor.

PKC activity correlates with proliferation rates

Growth rates of the tumor lines as measured by ^{3}H-thymidine incorporation was performed over the entire growth phase to confluency. Maximal counts obtained for each tumor were determined from the individual growth curves (attained approaching confluency). The corresponding thymidine uptake was measured in the nontransformed control glia cultures (the same glial cultures as in Table 1) seeded near confluency and was found to be substantially lower than that of malignant glia (Fig. 1).

When thymidine uptake values were plotted against the PKC activity at the midlogarithmic growth phase for each of

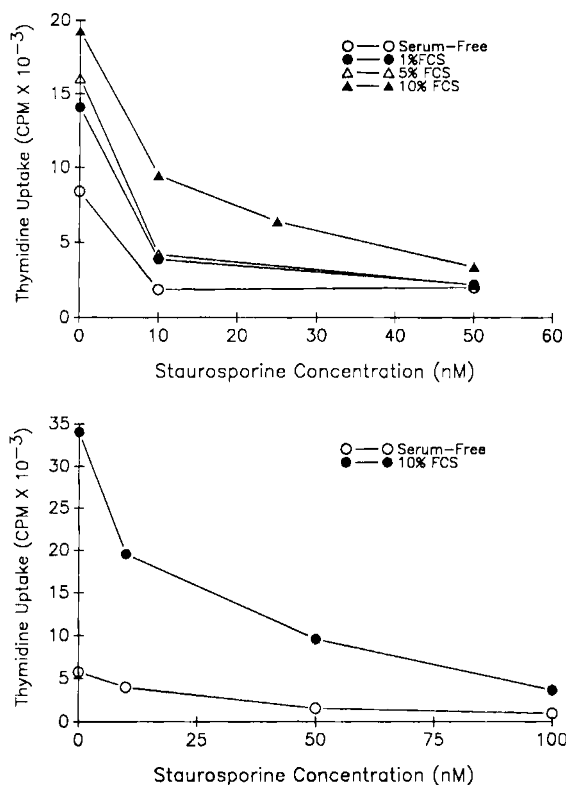

FIG. 4. Administration of PKC inhibitor reduces the basal proliferation rates of glioma lines A172 and C6. *Top*, addition of increasing concentrations of FCS to the culture medium produces an increase in basal proliferation of the tumor A172. SP, a PKC inhibitor, reduced the ^{3}H-thymidine uptake in all media tested in a dose-dependent manner. The inhibition was facilitated in lower medium serum concentrations. *Bottom*, rat glioma C6 also was inhibited by SP. *Points* represent the mean for triplicate wells, with the standard error of the mean less than 5% of the mean in all cases.

the tumor and control glia lines (Fig. 2; *upper*), correlation coefficients of $r = 0.71$ ($P = 0.073$) and 0.75 ($P = 0.055$) were obtained for total and particulate fractions, respectively. When the proliferation rate was plotted against total or particulate PKC activity after tumors were harvested approaching confluency (Fig. 2; *lower*), the corresponding correlation coefficients were 0.98 ($P < 0.001$) and 0.94 ($P = 0.002$), respectively.

Effects of serum and protein kinase C modulators on growth rate and protein kinase C activity

To ascertain if PKC activity varies with the growth rate of an individual tumor, the glioma line A172 was grown in either the absence or presence of serum. The removal of serum from the feeding medium reduced both the growth rate and PKC activity (Fig. 3*A*).

The PKC activity in glioma line A172 was measured at several time points after the addition of 100 nmol/L of the PKC activator PMA (Fig. 3*B*). To determine if down-regulation of the enzyme occurs in the face of persistent stimulation by phorbol esters, and if this is correlated to the reduced growth rates observed in these cells, the thymidine uptake was determined over the same intervals. Both the particulate and total activity diminished markedly, following a time course similar to that of the measured thymidine counts.

Administration of the PKC inhibitor SP produced a dose-related decrease in A172 glioma growth, in all serum concentrations tested (Fig. 4*A*). The C6 line was also inhibited by SP in a dose-dependent manner (Fig. 4*B*). The inhibition produced by the lower doses of SP was more effective in low serum concentrations, indicating that the enzyme may be easier to inhibit under these conditions. For both the A172 and C6 lines, the proliferation rate at maximal SP-induced inhibition was similar regardless of the serum concentrations and the differences in initial growth rates.

Cytofluorometric analysis of cell cycle and proliferation rates

Figure 5 shows DNA profiles of glioma line A172 grown in 10% FCS-containing medium alone or in the presence of PKC modulators for a period of 96 hours. Proliferation indexes are noted on the corresponding DNA histograms. In accordance with the ^{3}H-thymidine assay results, SP produced a dose-dependent decrease in the proliferation index of the tumor compared with the untreated control. The PKC activator SC-9 also reduced the proliferation index.

DISCUSSION

The results from the PKC enzyme assay in the present study indicate very high PKC activity in all the glioma lines tested when compared with PKC levels in nonmalignant astrocytes, oligodendrocytes, and mixed glial cultures, both in human and rat systems.

Levels of PKC activity in different tumors and normal glia correlated strongly with their differential proliferation rates as measured by thymidine uptake. Moreover, within an individual tumor, manipulation of the serum concentration produced corresponding changes in both the growth rate and PKC activity. While both the total and particulate (membrane associated) PKC activity correlated with proliferation in this study, the particulate fraction most likely is the critical factor in determining functional intracellular activity (13, 19, 20). The cytosolic fraction may be considered a reserve, ready to be translocated to the membrane after an appropriate signal. The total fraction may therefore be considered as the potential of PKC activity within the cell.

Down-regulation is a well recognized phenomenon within the PKC system, characterized by the disappearance of enzyme activity after persistent stimulation, presumably by a rapid degradation of the activated enzyme complex (3, 19, 20). The elevated PKC activity in these tumors is particularly susceptible to down-regulation by the persistent stimulation of a pharmacological activator such as PMA, as demonstrated in this study. Of interest, and unlike other cell types in which phorbol esters produce an initial increase in particulate fraction activity followed by a steady decline in both the particulate and cytosolic enzyme levels (3, 19, 20), glioma cells in this study did not exhibit the initial rise in particulate PKC activity; this may be related to the nature of the glioma cells themselves, that is, the initial early rise may have been missed by the earliest time point measured (10 min). The kinetics of down-regulation of the enzyme followed a similar temporal course with the changes in the proliferation rate as measured by ^{3}H-thymidine incorporation, supporting a strong correlation between the dynamic changes in PKC activity and the proliferation rate within a given tumor. That the PKC system is selectively involved in these growth rate changes is further supported by the observation that phorbol esters known to activate PKC (PMA and 4β-phorbol-12, 13-dibutyrate) alter the rates of growth, but that a phorbol ester that binds but does not activate PKC (4α-phorbol-12,13-didecanoate) (2, 14) does not (8).

In reviewing the literature, we note that Pollack et al. (23) have reported that PMA produces an unequivocal rise in thymidine uptake of two glioma cell lines. The difference between our studies is that these authors have employed a serum-free

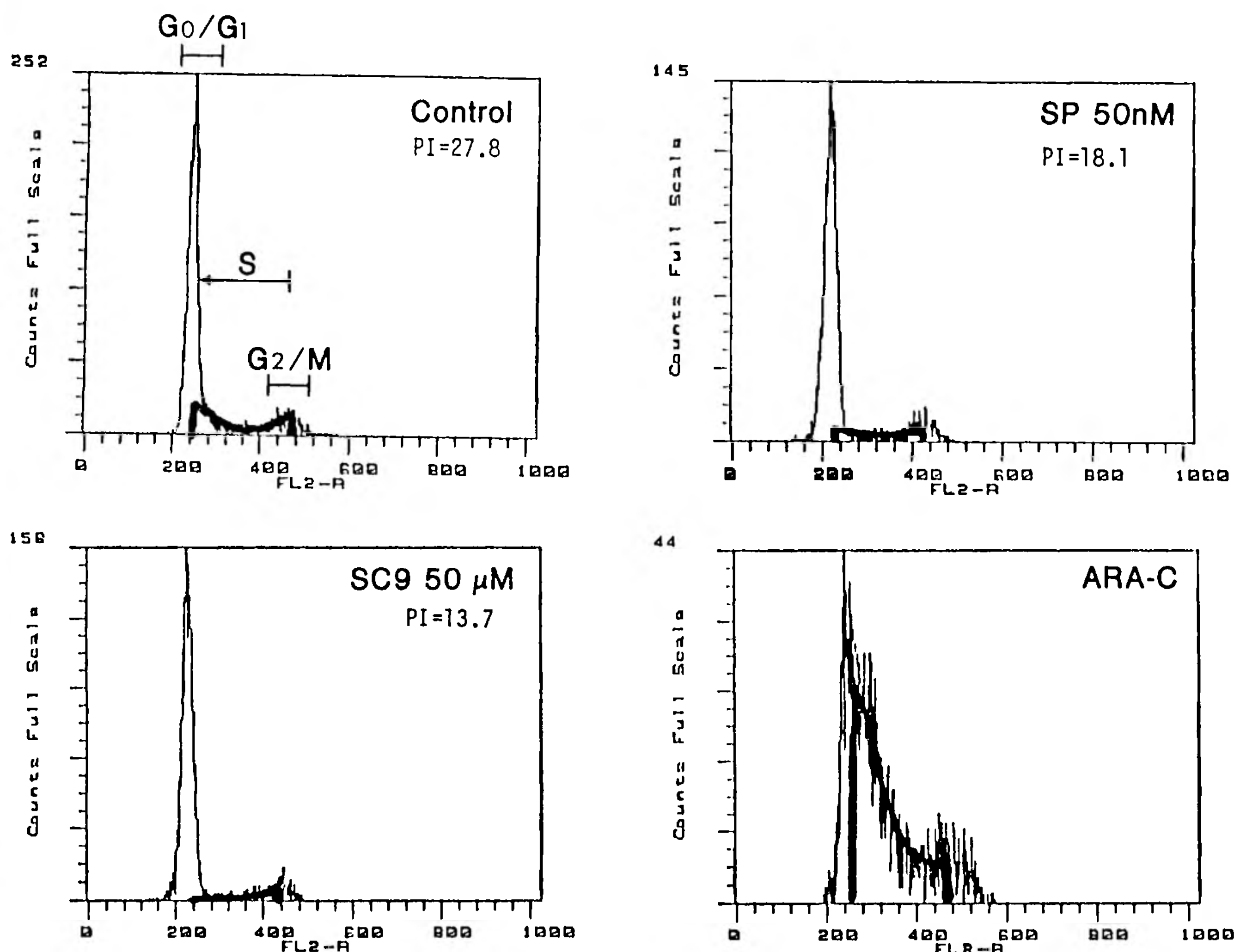

FIG. 5. PKC inhibitors block the entrance of the cell into the S-phase. Flow cytometric analysis of cell cycle kinetics after the administration of PKC modulators in a medium containing 10% FCS. G_0/G_1 and G_2/M peaks are defined on the control DNA histogram; the S-phase is defined by the *outlined area* in all plots. Cell percentage estimates of the S-phase and the proliferation index (defined as the sum %S + %G_2/M phases) were determined using the SFIT model from the DNA histograms. Treatment with PKC activators and inhibitors reduces the calculated proliferation index as measured cytofluorometrically. Both S-phase and G_2/M percentages are reduced when the cells are treated with either an activator (SC-9 50, μmol/L) or inhibitor (SP 50, nmol/L) of PKC for a period of 96 hours. The reduction in proliferative rates results from the blockage of the cell entrance into the S-phase. In contrast, cultures treated with DNA synthesis inhibitor cytosine arabinoside display a high percentage of cells arrested in the S-phase.

medium, whereas our conditions contained serum. We have, however, performed experiments under variable concentrations of serum and also have found initial mitogenic activity of phorbol esters on some cell lines (A172 and U87-MG) under serum-free conditions (9).

The high PKC activity, which correlates with the rapid proliferation in these tumors, offers a natural target for the inhibition of tumor growth. This possibility is illustrated by the ability of the PKC inhibitor SP (25) to reduce markedly the proliferation rate of glioma line A172 and C6 as measured by ^{3}H-thymidine uptake and confirmed by flow cytometric DNA analysis.

The precise mechanism by which PKC modulation alters growth rates in gliomas remains to be established. Our flow cytometric studies indicate that the decrease in the proliferation index observed after treatment with PKC modulators was the result of a direct growth-inhibiting effect on gliomas. Analysis was performed with cytometer gating to analyze specifically only intact cells that previously been adherent to the culture plate (thriving cells); this, considered together with the results of the chromium release assay performed in the presence of these agents (7, 8), argues against a nonspecific cytotoxic effect of PKC modulators on the tumor cells. The flow cytometric observations support the postulate that growth inhibition occurs by way of signaling through the nucleus (21), in that a reduction of the proliferation index indicates that the point of arrest of the cell growth cycle is before the entrance into the S-phase; however, limitations of the technique are that the specific stage (G_0–G_1 or G_1–S transition) may not be resolved. Additional support for the ability of PKC modulators to alter nuclear transcriptional events that may affect growth is found in the observations of Press et al. (24) of enhanced expression of c-*sis* oncogene messenger ribonucleic acid [in which the B chain portion of platelet-derived growth factor is encoded (4)] in glioma line A172 after treatment with PMA, the report of transformation of a fibroblast line by transfection with a PKC-I cDNA expression vector (22) and the ability of PKC to phosphorylate the *jun* "regulator" protein, which, in association with *fos* protein, may bind to specific DNA pro-

motor sequences, thereby activating (or inactivating) adjacent genes (15).

In conclusion, results from this study indicate that PKC activity of malignant gliomas is increased markedly in comparison with that of normal glia, indicating an enhanced expression of this enzyme, which may thereby function as a biochemical marker of malignancy in these astroglial tumors. Further, the PKC activity of gliomas in vitro is correlated strongly to their proliferation rates, although it must be cautioned that a high correlation does not indicate a causal relationship. Altering enzyme activity by down-regulation or direct inhibition reduces the tumor growth rate significantly. Pharmacological inhibitors of PKC may constitute a novel chemotherapeutic approach to the treatment of gliomas. The molecular basis for the overexpression of the enzyme in these tumors is currently under study.

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COMMENTS

This paper establishes an important correlation between protein kinase C (PKC) production and activity with the proliferative capacity of human malignant glioma cell lines. The authors further show that the activity of PKC in the malignant glioma cell can be up- or down-regulated. This regulation is related to the inhibition of the entrance into the S-phase of division. The elegance of the control data on human astrocytes demonstrates that this increased activity of PKC in transformed astrocytes is unique to the glioma cell in this in vitro model. The isolation of the increased PKC activity in glioma cells and not in normal astrocytes is of great value because of

the possibility that therapeutic regulation of PKC may be important in the clinical management of malignant gliomas and astrocytomas.

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The interest in PKC is linked to the role played by this enzyme in the growth rate of glioma cells. In this study, Couldwell et al. have shown convincingly a high in vitro correlation between PKC production and deoxyribonucleic acid synthesis in several glioma cell lines. Similarly, deoxyribonucleic acid synthesis was reduced substantially with the addition of staurosporine, a PKC inhibitor.

Whether the use of a PKC inhibitor in humans will lead to equally encouraging results remains to be demonstrated. At a recent national meeting, the authors have shown that, in a glioma animal model, staurosporine was moderately effective in prolonging the survival of the animals. This indicates that the PKC pathway might escape effective inhibition in vivo or perhaps that the PKC pathway is not an essential link in the chain of events leading to tumor growth.

Another puzzling finding in this paper relates to the rapid inhibition of PKC activity after the addition of a PKC activator to a glioma cell line. This paradoxical phenomenon is explained insufficiently on the basis of the "down-regulation" principle described in the text. The authors nevertheless have developed a tissue culture model that allows them to further our understanding of the role of the PKC pathway in the biology of gliomas.

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