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Inhibition of growth of established human glioma cell lines by modulators of the protein kinase-C system

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The protein kinase-C (PKC) second messenger system contributes to regulation of cell growth and differentiation. This study was undertaken to examine the effects of modulators of the PKC enzyme system on the state of differentiation and proliferation rates of human gliomas *in vitro*. The administration of the PKC-activating phorbol esters 4-beta-phorbol-12,13-dibutyrate (PDB) and phorbol-12-myristate-13-acetate (PMA) resulted in a dose-related inhibition of growth of human glioma cell lines *in vitro* as measured by ^{3}H-thymidine uptake. The synthetic nonphorbol PKC activator (SC-9) produced an even more pronounced decrease of ^{3}H-thymidine uptake. Diacylglycerol, an endogenous activator of the system, applied externally, transiently decreased the proliferation, in concordance with its short-lived existence *in vivo*. Conversely, the administration of 4-alpha-phorbol-12,13-didecanoate (α-PDD), a phorbol ester that binds but does not activate the enzyme, had no effect on the proliferation rate. At the dosages that maximally decreased proliferation, there was no evidence of direct glioma cell lysis induced by these agents as measured by a chromium-release assay. Immunocytochemical analysis and cytofluorometric measurement of glial fibrillary acidic protein (GFAP) staining in the treated cultures revealed an increase in GFAP staining over control cultures. In contrast to the response of glioma cells, nonmalignant human adult astrocytes treated with the PKC activators responded by increasing their proliferation rate.

The authors postulate that the diametrically opposed effects of PKC activators on nonmalignant astrocytes versus glioma growth may be due to a high intrinsic PKC activity in glioma cells, with resultant down-regulation of enzyme activity following the administration of the pharmacological activators.

KEY WORDS • glioma • astrocyte • cell proliferation • tumor cell growth • protein kinase-C • phorbol ester

INTERNAL signal transduction mechanisms are central in the molecular control of cell growth. One such regulator is known to be the protein kinase-C (PKC) system. This enzyme complex is part of a family of proteins that function in the capacity of translating an externally recognized signal into altered cellular function by systems of regulatory and catalytic proteins, broadly referred to as "second-messenger" systems.[26] In the case of PKC, when a ligand binds to appropriate receptors on the cell surface, through the mediation of G proteins membrane-associated inositol phospholipids are hydrolyzed, producing diacylglycerol and inositol phosphates. It is thought that the primary effect of the diacylglycerol liberated is the activation of PKC which in turn phosphorylates a range of cellular proteins.[18] Protein kinase-C is a calcium and phospholipid-dependent kinase which is very active in central nervous system tissue.[14] Its myriad of effects in different cell types[19] include cell differentiation and process formation in oligodendrocytes[30] and proliferation of normal human fetal astrocytes.[29]

A well-appreciated clinical phenomenon is an acceleration in the growth rate of an existing low-grade glioma *in vivo* coexistent with a change in its histological grade.[22,23] One hypothesis for this change implicates induction of an autocrine growth promotory system in these tumors; in this paradigm, a likely mechanism would be that this occurs by way of a signal transduction mechanism at the cellular level. Previous work in our laboratory has shown that activators of PKC are mitogenic for nonmalignant human fetal astrocytes.[29] As gliomas are of astrocytic origin, this study was undertaken to assess the effects of PKC modulators on the growth and differentiation of established human glioma cell lines *in vitro*. We show here that PKC activators result in a decrease in the proliferation rate

of human glioma cells. In contrast, and paralleling the observation made with nonmalignant fetal astrocytes, human adult astrocytes were induced to proliferate by activators of the PKC system.

Materials and Methods

Glioma Cell Lines

Glioma cell lines U563 MG and A172* were used in this study: they are previously characterized lines established elsewhere.[6,9,21] These lines were passaged regularly (consisting of gentle trypsinization at 0.05% for 10 minutes followed by serum inactivation of trypsin and then repeated washes with phosphate-buffered saline (PBS) and replating) and maintained in 25-sq cm tissue culture flasks, in medium consisting of Eagle's minimum essential medium (MEM) supplemented with 10% bovine fetal serum (FCS), gentamicin (20 μg/ml), glutamine, pyruvate (1 mM), dextrose (0.1%), and essential amino acids buffered to a pH of 7.0.† Cells were grown at 37% in a humidified 5% CO_2 incubator.

Astrocyte Cell Cultures

Nonmalignant human brain tissue was obtained from young adults (mean age 24.5 years) during surgical resection to ameliorate intractable epilepsy. Tissue adjacent to the epileptogenic focus was removed by ultrasonic aspiration.‡ The origin of the tissue was the corpus callosum (one case) or the temporal lobe (three cases). For cell isolation, meninges and visible blood vessels were removed and brain tissue was cut into cubes of 1 cu mm or less. Viable dissociated cells were then obtained using a previously established protocol involving trypsin digestion and Percoll§ centrifugation.[10,30] Cells were suspended in feeding medium and placed in Falcon flasks of 50 ml capacity for 24 hours, after which the floating cells (mostly oligodendrocytes) were removed for other studies. Adherent cells (mostly astrocytes and microglia) were left undisturbed and allowed to differentiate. Seven days later, these cells were removed from their flasks by 0.05% trypsin and seeded on 9-mm plastic coverslips coated with poly-1-lysine‖ at a density of 5×10^4 cells per coverslip. By immunohistochemical identification, the majority of the cells were glial fibrillary acidic protein (GFAP)-positive astrocytes and presumed microglial cells (leu-M5-positive, avid bead ingestion, and MHC class II expression). The feeding medium was Eagle's MEM supplemented with 5% FCS, 20 μg/ml gentamicin, and 0.1% dextrose. The cultures used in this study ranged in age from 2 to 4 weeks postdissociation.

Incubation of Glioma Lines with Protein Kinase-C Modulators

Following passage, the cell lines were seeded at a density of 10^4 cells in 30 μl of medium on 12-mm plastic coverslips placed in 24-well culture plates. The coverslips were previously coated with 10 μg/ml poly-1-lysine to facilitate cell adherence. Twelve hours later, after adequate time for cells to adhere to the coverslips, the wells were flooded with 1 ml of medium. Each modulator* was then added to the wells at predetermined concentrations in replicates of three: 1) 4-beta-phorbol-12,13-dibutyrate (PDB) and phorbol-12-myristate-13-acetate (PMA), which are phorbol esters and activators of PKC, were administered at doses of 1, 10, and 100 nM each; 2) 4-alpha-phorbol-12,13-didecanoate (α-PDD), a phorbol ester which binds but does not activate the enzyme was used at concentrations of 10 and 100 nM; and 3) diacylglycerol (1,2-dioctanoyl-sn-glycerol), an endogenous activator of the PKC enzyme, was used at concentration of 10 μM. The synthetic activator SC-9[17]† was diluted in ethanol and added to supplemented medium to a final concentration of 10 and 50 μM. Medium was changed in each well on the 4th day *in vitro* to prevent nutrient-limited growth.

Tritiated Thymidine Uptake

At specified intervals, wells were pulsed for a period of 5 hours prior to harvest with 1 μCi ^{3}H-thymidine/ml. The coverslips with adherent cells were then washed four times with PBS and placed in vials containing 5 ml of scintillation fluid (Cytoscint), for determination in a beta counter. To demonstrate that uptake of ^{3}H-thymidine into cells is a reliable index of deoxyribonucleic acid (DNA) synthesis, a control experiment was performed in which synthesis of DNA in the glioma cells was stopped by treating the cells with 50 μg/ml of mitomycin-C for 30 minutes; the cells were then washed four times with fresh medium and pulsed as above with ^{3}H-thymidine. Resulting background cell counts were negligible.

Chromium-Release Assay

Following passage, cells were plated at 2×10^4 per well in replicates of four in a 96-well flat-bottomed plate. After 24 hours, the wells were gently washed with PBS and ^{51}Cr was added in a concentration of 1 μCi/100 μl of culture medium to each well. The cells were then incubated for a period of 12 hours to allow for adequate incorporation of ^{51}Cr, after which the wells were gently washed three times with medium to remove the free label. The cells were then left for the first 30 minutes for spontaneous release to occur. Medium was removed, and the modulators were added to the wells

* Glioma cells provided by courtesy of V. P. Collins, M.D., Ludwig Institute of Cancer Research, Stockholm, Sweden.

† All medium constituents obtained from GIBCO, Grand Island, New York.

‡ Ultrasonic aspirator manufactured by Cavitron Surgical Systems, Inc., Stamford, Connecticut.

§ Percoll manufactured by Pharmacia, Uppsala, Sweden.

‖ Poly-1-lysine supplied by Sigma Chemical Co., St. Louis, Missouri.

* PDB, PMA, α-PDD, and diacylglycerol obtained from Sigma Chemical Co., St. Louis, Missouri.

† SC-9 obtained from Seikagaku America Inc., Rockville, Maryland.

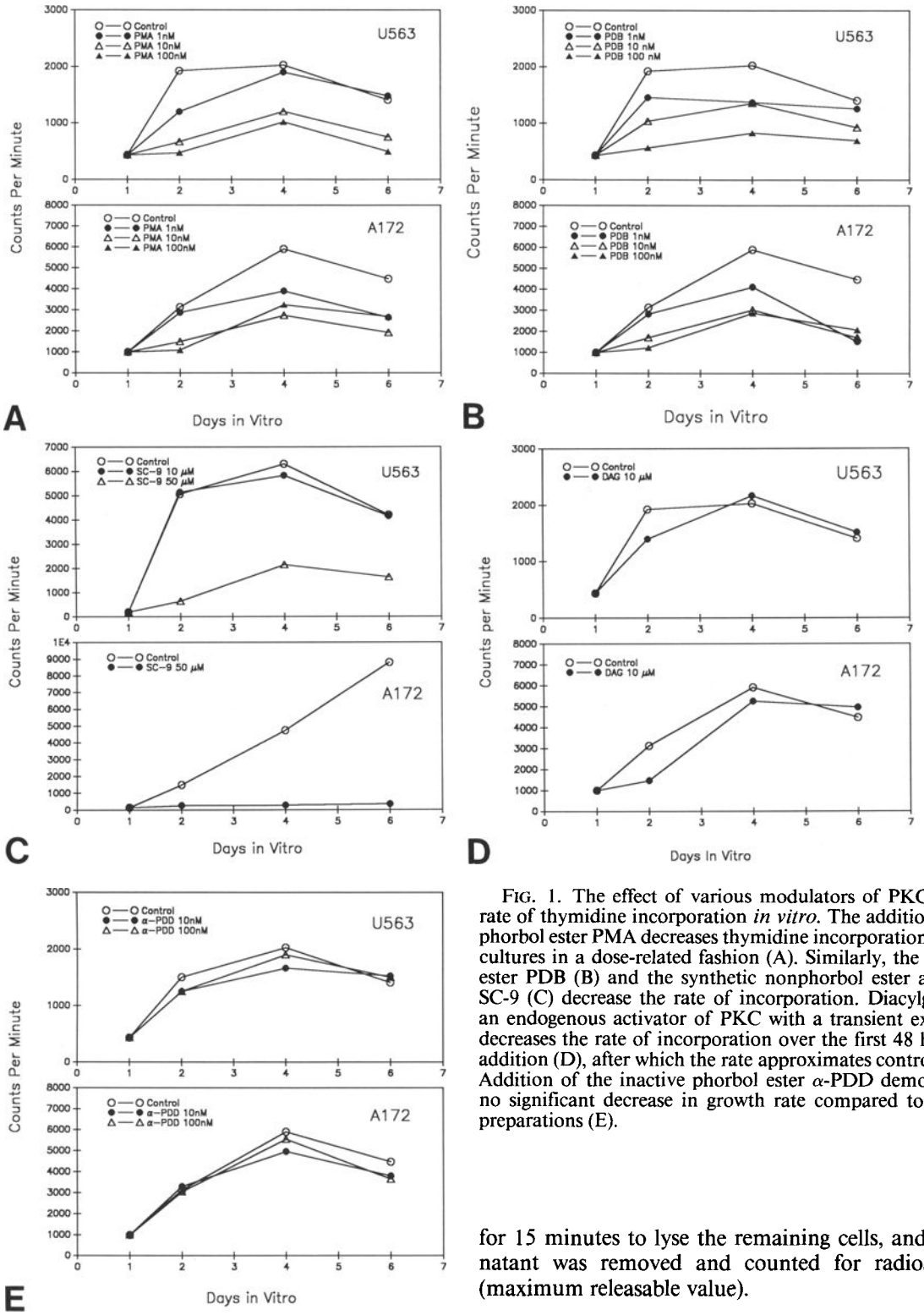

FIG. 1. The effect of various modulators of PKC on the rate of thymidine incorporation *in vitro*. The addition of the phorbol ester PMA decreases thymidine incorporation in both cultures in a dose-related fashion (A). Similarly, the phorbol ester PDB (B) and the synthetic nonphorbol ester activator SC-9 (C) decrease the rate of incorporation. Diacylglycerol, an endogenous activator of PKC with a transient existence, decreases the rate of incorporation over the first 48 hours of addition (D), after which the rate approximates control levels. Addition of the inactive phorbol ester α-PDD demonstrates no significant decrease in growth rate compared to control preparations (E).

at the maximum concentrations used in this experiment. The agents were incubated for 5 hours, after which supernatant was removed and radioactivity was measured on a gamma counter (measurement of cytotoxic cell lysis). Then 100 μl of 5 N NaOH was added for 15 minutes to lyse the remaining cells, and supernatant was removed and counted for radioactivity (maximum releasable value).

Immunohistochemistry

Glial Fibrillary Acidic Protein Staining. Live cells were seeded on 12-mm plastic coverslips and prepared as outlined above. The cells were first fixed with 95% ethanol-5% acetic acid mixture at −20°C for 15 minutes and, after rehydration with PBS, were incubated with

rat anti-GFAP monoclonal antibody (2.2B10)[16] for 30 minutes at room temperature. Following this, coverslips were washed with PBS and then incubated with goat anti-rat immunoglobulin conjugated to rhodamine‡ for another 30 minutes. The coverslips were again washed with PBS and wet-mounted on glass coverslips with glycerol-PBS. Negative control tests for staining were performed on the glioma cells by omitting the primary antibody step; positive control tests for the GFAP-specific monoclonal antibody were the simultaneous staining of nonmalignant astrocyte cultures. The slides were viewed with a fluorescence microscope.

Ki-67 Staining. The Ki-67 antibody was utilized to confirm the modulation of glioma growth as measured by ^{3}H-thymidine incorporation. Ki-67 is a murine monoclonal antibody directed against a nuclear antigen expressed in proliferating cells in the G_1, S, G_2, and M phases of the cell cycle and is shown to correlate with the proliferation rate of malignant gliomas.[31] The cells were plated and fixed following the aforementioned protocol. After fixation, the Ki-67 antibody (diluted 1:50)§ was placed in 25-μl volumes on the coverslips for 30 minutes at room temperature. The coverslips were then washed and incubated with a secondary goat anti-mouse antibody conjugated to rhodamine. The coverslips were mounted and viewed as described above.

Cytofluorometry

For purposes of determination of cellular GFAP content, cells were propagated in Petri dishes for a period of 3 days with the corresponding PKC modulator (100 nM PDB or 100 nM PMA). At this time, the cells were removed using gentle trypsinization (0.05% for 10 minutes) and washed three times in PBS after inactivation of the trypsin with FCS. The cells were fixed for a period of 30 minutes in a solution of 95% ethanol-5% acetic acid at −20°C. Immunocytochemical GFAP staining was then performed in 1.8-ml Ependorf tubes using 30 μl of rat anti-GFAP primary antibody (volume to achieve saturation of antigen) followed with equivalent volumes of goat anti-rat secondary antibody conjugated with fluorescein isothiocyanate. Each stain was followed by three washes with PBS. The samples were analyzed with a Fluorescent Activated Cell Sorter flow cytometer to quantitate the amount of immunofluorescence specifically related to GFAP staining. Gating for acquisition of single cells was performed and 5000 events were acquired from each culture for GFAP fluorescence analysis.

Analysis of Proliferation of Nonmalignant Astrocytes

Since the nonmalignant cultures contained a mixture of cell types, ^{3}H-thymidine incorporation would not have yielded information as to the cell type that incorporated the label. For this reason, a double immunofluorescence technique that allows direct visualization of the cell type that has incorporated the proliferation label (bromodeoxyuridine or BUdR) was used. Cells were incubated with test agents (PDB, PMA, and α-PDD) for 4 days, and 10 μM BUdR was added during the last 48 hours to allow proliferating cells to incorporate this label. The cells were then immunostained using antibodies to GFAP and BUdR by a method that has been described in detail elsewhere.[27,28] The results from the four primary astrocyte cultures were pooled.

Results

Effects of PKC Modulators on Thymidine Uptake in Glioma Lines

In the tumor lines U563 MG and A172, after 48 hours in culture with PDB, the rate of proliferation as assessed by ^{3}H-thymidine uptake decreased to 29% and 46% of controls, respectively (Fig. 1). This decreased uptake persisted throughout the duration of the study (6 days), after which the control cell cultures were monolayers and exhibited density-dependent reduced growth rates. Similarly, the administration of another biologically active phorbol ester, PMA, produced a dose-related reduction of proliferation to 24% and 35% of controls. The synthetic nonphorbol ester activator (SC-9) exhibited a more pronounced decrease in thymidine incorporation, to 12% and 18% of controls in the same period. Diacylglycerol, an endogenous activator of PKC, transiently decreased the proliferation, in concordance with its short-lived existence *in vivo*. Conversely, the addition of a phorbol ester, α-PDD, which binds but does not activate the enzyme, had no effect on the proliferation rate.

Immunocytochemistry performed with the monoclonal antibody Ki-67 revealed a marked diminution in fluorescent staining of both A172 and U563 MG following incubation with PMA for a period of 36 hours, supporting evidence that the decrease in thymidine incorporation with treatment truly reflected a decrease in cell proliferation.

PKC Modulators and Cytotoxicity

A chromium-release assay was performed to determine if the diminution of thymidine uptake observed was due to direct cytolysis of glioma cells induced by modulators of the PKC system. Results of the assay revealed that, at the dosages that decreased proliferation, there was no direct cytotoxicity of these agents (that is, no increase in percent specific lysis compared to media control). Ethanol 30% was used as a positive assay control.

PKC Activity and GFAP Staining

Four days following the administration of the phorbol esters PDB and PMA, the intensity of GFAP im-

‡ Rhodamine obtained from Cappel, West Chester, Pennsylvania.

§ Ki-67 antibody supplied by Dakopatts, Copenhagen, Denmark.

munolabeling of glioma cultures was qualitatively compared with the control preparations. These results show that the immunostaining of GFAP appeared to increase in the U563 MG cells exposed to the phorbol ester (Fig. 2).

For purposes of quantitation of the change in GFAP immunostaining observed, cytofluorometric analysis was performed 4 days after the addition of the PKC stimulators. These results are summarized in Fig. 3. Incubation in 100 nM of the phorbol ester PMA produced a significant increase in the anti-GFAP immunofluorescence over controls in glioma line U563 MG. Similarly, PDB produced a smaller increase in the same cell line. In glioma line A172, the quantitative increase in anti-GFAP staining following treatment with 100 nM PMA as reflected by increased immunofluorescence was more pronounced.

PKC Modulators and Proliferation of Nonmalignant Astrocytes

In contrast to the results obtained with the glioma cells, phorbol esters that activate PKC caused increased proliferation rate of nonmalignant astrocytes. As shown in Fig. 4, PDB exerted a dose-dependent effect such that, at 100 nM, a proliferation index of 7 was obtained (that is, a sevenfold increase over controls was found). At 100 nM, PMA was similarly effective, while the inactive phorbol ester α-PDD did not elicit a proliferative response. An example of nonmalignant astrocytes incorporating BUdR is shown in Fig. 5.

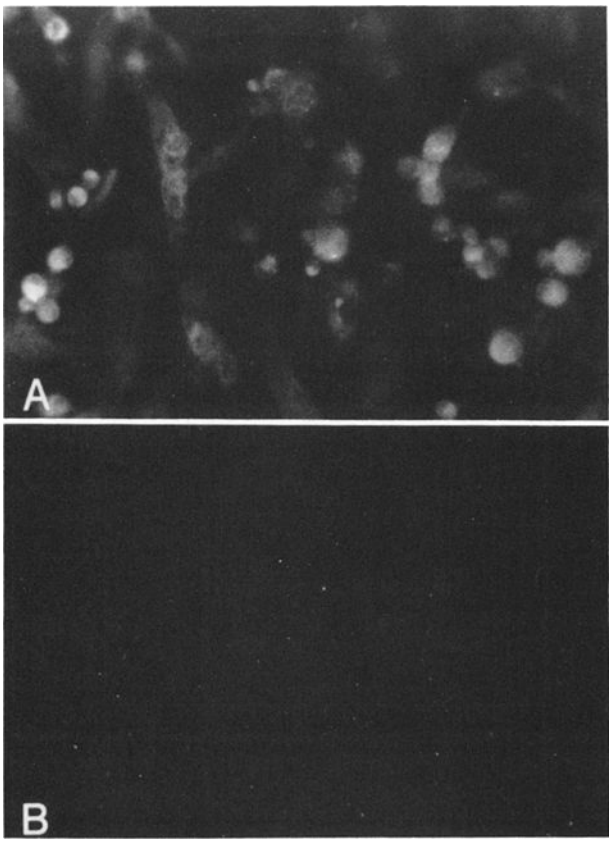

FIG. 2. Immunocytochemical staining of glial fibrillary acidic protein (GFAP), × 550. The culture of U563 MG following 96 hours in culture with 100 nM PMA (A) demonstrates markedly increased staining of GFAP in comparison to the control preparation (B).

Discussion

Phorbol esters are the classic pharmacological activators of PKC.[3,15,19] In addition to their historical role as second-stage tumor promoters,[1,2] they are mitogenic for many cell types,[8] including normal human fetal astrocytes,[29] and (as shown in Fig. 4) for biopsy-derived nonmalignant human adult astrocytes. In contrast, glioma cells demonstrate a dose-related decrease in proliferation after treatment with PKC activators. That the effects of PMA and PDB on glioma cells are mediated by the PKC system is supported by the ability of the nonphorbol PKC activator SC-9 to decrease the proliferation rate and by the failure of α-PDD (which binds but does not stimulate PKC) to duplicate this effect. Further support comes from the time course of action of diacylglycerol, which decreased the ^{3}H-thymidine incorporation transiently in concordance with its more rapid metabolism to inactive compounds.

Several different mechanisms could potentially account for the observed PKC-regulated growth of gliomas. If intrinsic PKC activity of gliomas were elevated compared to nonmalignant glia, persistent stimulation of PKC by phorbol esters could down-regulate such elevated enzyme activity, as reported in other cell types.[4,5,20,24] This hypothesis may help to explain the paradoxical effect of PDB and PMA on the proliferation of slower growing normal astrocytes compared to the suppression of thymidine incorporation by glioma cells in this study.

Another interpretation would be that the response of glioma cells to phorbol esters represents a state of increased differentiation of these cell lines, with secondary slowing of the rate of cell division. That an increased state of differentiation of glioma lines occurs after treatment of phorbol esters in this study is supported by the observed increase in GFAP content as analyzed by immunohistochemistry and cytofluorometry. An intermediate filament, GFAP is characteristic of astrocytes and tumors of astrocytic lineage. The quantity of GFAP staining in astrocytic tumors is often inversely proportional to the degree of anaplasia, and indeed the highly malignant glioblastoma multiforme may be GFAP-negative.[12,13] Gliomas often lose their expression of GFAP with repeated passages in tissue culture.[6] In further support of glioma cell differentiation is the report by Colombatti, *et al.*,[7] of growth inhibition and concordant decrease in surface tumor-associated antigen in one glioma cell line after phorbol ester treatment. Phorbol esters have been shown to induce differentiation of a murine reticulum cell sarcoma line to phe-

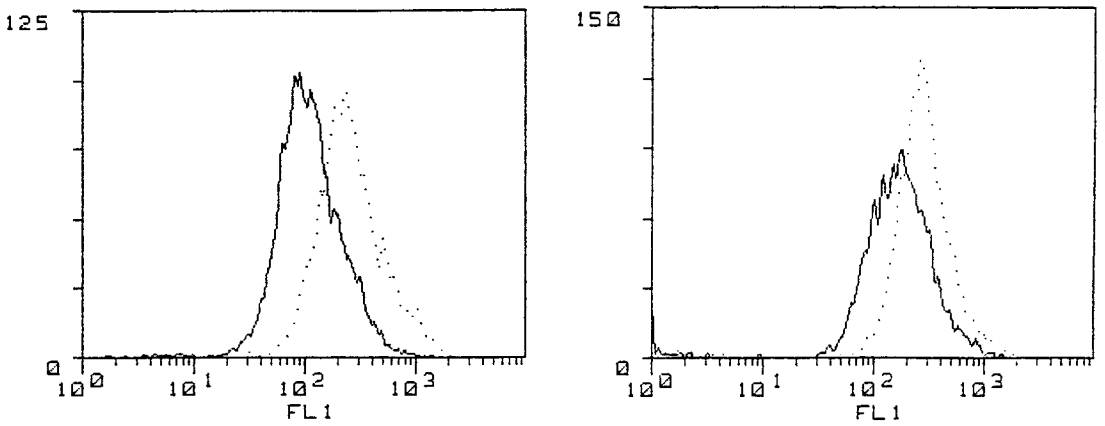

FIG. 3. Histogram plots showing fluorocytochemical analysis of glial fibrillary acidic protein (GFAP) staining. x axis: logarithmic fluorescence units. A: Culture A172 control *(solid line)* and after incubation with 100 nM PMA *(dotted histogram)* demonstrating a shift in fluorescent intensity related to increased GFAP staining. B: Similar display of the U563 MG control and after incubation with the phorbol ester showing increased immunofluorescence to a lesser magnitude.

notypically mimic macrophages,[25] increase neurite formation in neuroblastoma (an index of neuronal differentiation),[11] and facilitate process development in human adult oligodendrocytes.[30] Our findings of a decrease in proliferation rate of gliomas after treatment with the PKC activators as early as 30 minutes (earliest time point measured) after drug administration (unpublished results), would favor the fact that the increased differentiation of these cells is secondary to a decrease in proliferation.

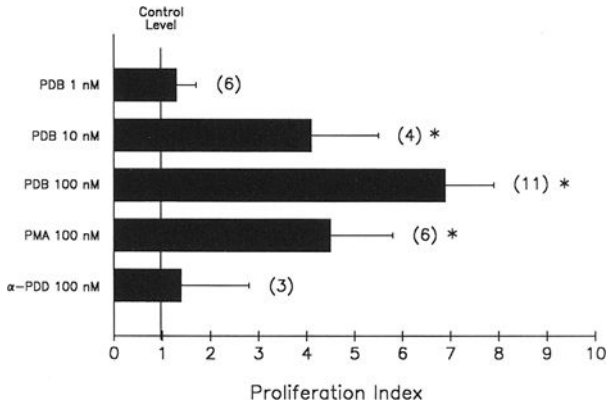

FIG. 4. Response of nonmalignant human adult astrocytes to protein kinase-C modulators. Values are means + standard error of the mean, with the number of coverslips analyzed shown in parentheses. An average of 100 glial fibrillary acidic protein (GFAP)-positive astrocytes were counted on each coverslip. The percent of astrocytes that incorporated the bromodeoxyuridine (BUdR) in each test culture was divided by results from appropriate controls to give the proliferation index (therefore, a value of 1 indicates no proliferative response). On average, over the 48-hour pulse with BUdR, 3% to 5% of control astrocytes incorporated BUdR. Asterisks indicate values significantly different from control cultures using a one-way analysis of variance with Duncan's multiple comparison, $p = 0.05$. All analyses were performed blind on coded specimens.

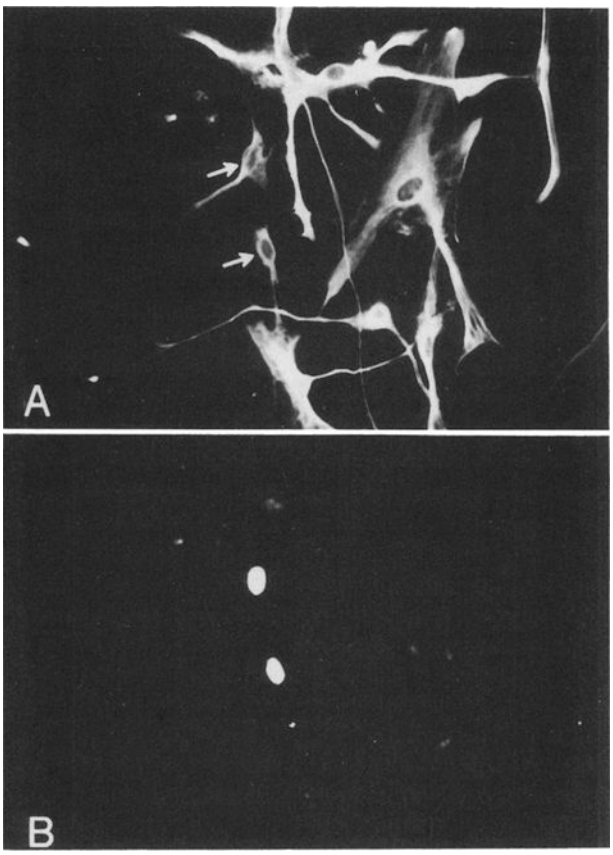

FIG. 5. Immunohistochemical technique of determining proliferating astrocytes, × 540. Double labeling using glial fibrillary acidic protein (GFAP) alone (A) or with bromodeoxyuridine staining (B) enables quantitation of GFAP-positive cells that are actively dividing within the same field. *Arrows* demarcate two such double-labeled cells.

In conclusion, in contrast to the response of nonmalignant human fetal or adult astrocytes, glioma cells exhibited a decrease in the proliferation rate when treated with activators of the PKC system. We are presently attempting to correlate this response to the intrinsic PKC enzyme activity of glioma cells and to ascertain if a particular isoform of the PKC enzyme is present within such cells.

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