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Protein kinase C mediates induced secretion of vascular endothelial growth factor by human glioma cells

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Abstract

To understand how vascular endothelial growth factor (VEGF) production is activated in malignant glioma cells, we employed protein tyrosine kinase (PTK) and protein kinase C (PKC) inhibitors to evaluate the extent to which these protein kinases were involved in signal transduction leading to VEGF production. PTK inhibitors blocked glioma proliferation and epidermal growth factor (EGF)-induced VEGF secretion, while H-7, a PKC inhibitor, inhibited both EGF-induced and baseline VEGF secretion. Phorbol 12-myristate 13-acetate (PMA), a non-specific activator of PKC, induced VEGF secretion by glioma cells, which was enhanced by calcium ionophore A23187, but completely blocked after prolonged treatment of cells with 1 μ M PMA, by presumably depleting PKC. All inhibitors (genistein, AG18, AG213, H-7, prolonged PMA treatment) which inhibited EGF-induced VEGF secretion in glioma cells also inhibited cell proliferation at similar concentrations. However, PKC inhibition only blocked 50% of the VEGF secretion induced by growth factors (EGF, platelet-derived growth factor-BB, or basic fibroblast growth factor). This reserve capacity could be ascribed to a PKC-independent effect, or to PKC isoenzymes not down-regulated by PMA. These findings extend our previous assertion that VEGF secretion is tightly coupled with proliferation by suggesting that activation of convergent growth factor signaling pathways will lead to increased glioma VEGF secretion. Understanding of signal transduction of growth factor-induced VEGF secretion should provide a rational basis for the development of novel strategies for therapy.

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Vascular endothelial growth factor (VEGF) is a potent and specific endothelial cell mitogen in vitro, induces angiogenesis in vivo [1], and produces a profound increase in vascular permeability [2]. It is identical to vascular permeability factor [3,4]. The biological effects of VEGF are mediated mainly by two tyrosine kinase receptors, VEGFR-1 (*Flt-1*) and VEGFR-2 (*KDR/flk-1*), which are expressed almost exclusively in endothelial cells [5]. Two biologic characteristics of VEGF are

unique among angiogenesis factors. VEGF is a direct and specific endothelial cell mitogen. Other angiogenesis factors either support angiogenesis indirectly (epidermal growth factor (EGF), tumor necrosis factor α , transforming growth factor β 1, or angiogenin) or are active on non-endothelial cell types (acid fibroblast growth factor, aFGF; or basic fibroblast growth factor, bFGF) [6]. VEGF is also a secreted protein in contrast to aFGF, bFGF, and platelet-derived endothelial cell growth factor, which lack signal peptides required for extracellular transport. Supposedly, these latter factors are released by leakage from damaged cells or bound to

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secreted extracellular matrix molecules. We have shown that VEGF is detected in glioma cell cultures by ELISA [7] and hypothesize that VEGF is the central mediator of glioma angiogenesis because of its characteristics [8] and its ability to initiate the sequence leading to neo-vascularization [2].

Three of the major histopathologic features of malignant glioma (hypervascularity, tumor necrosis, and peritumoral brain edema) may be related to VEGF. Glioblastoma multiforme thus seems to be the prototype tumor suitable for anti-angiogenic therapy [9]. Definitive studies of regulatory pathways controlling glioma-derived VEGF production may lead to development of new therapeutic strategies for this common malignant brain tumor. We have demonstrated that three tyrosine kinase receptor growth factors, EGF, bFGF, and PDGF-BB, are each capable of inducing VEGF production from glioma cells [7]. The combined effects of these growth factors were additive at low concentrations but reached a maximum stimulatory effect at high concentrations implying a convergent pathway leading to enhanced VEGF secretion.

Protein kinase C (PKC) encompasses a complex family of closely related serine/threonine protein kinase isozymes that mediate a wide range of signal transduction processes in cells [10]. Most isoforms of PKC can be physiologically activated by cellular production of diacyl glycerol (DAG) [11]. Free fatty acids also play a role in activating PKC. There is evidence that growth factor-mediated activation of receptor tyrosine kinases (e.g., PDGF or EGF receptors) initiates the cascade of events (PLC γ activation with cleavage of inositol triphosphate producing phosphoinositol phosphate and DAG) which results in activation of PKC [12]. Thus, inhibitors that are directed selectively against PKC may have wide-ranging therapeutic potential [13–15].

To understand how VEGF production is activated in malignant glioma cells, we employed protein tyrosine kinase (PTK) and PKC inhibitors to evaluate whether PTK and PKC are involved in signal transduction leading to VEGF production. The capacity of PMA to down-regulate the DAG-dependent PKC isozymes provided a means to evaluate the relative importance of different PKC isozymes in this process.

Materials and methods

Biochemical and biological reagents. Dulbecco's modified Eagle's medium mixed 50:50 with Ham's nutrient mixture F-12 (DMEM/F12; Mediatech) supplemented to 2 mM L-glutamine and 8% fetal bovine serum (FBS) was used for culture of U-105MG glioma cells. FBS (Intergen, Purchase, NY) was heat inactivated (56°C, 45 min). EGF (human, receptor grade, Collaborative Research, Bedford, MA), recombinant human PDGF-BB (R&D systems, Minneapolis, MN), and bFGF (Promega, Madison, WI) were all diluted to specified concentrations in Dulbecco's PBS (pH 7.2). Culture media and sera were

determined to have <100 pg/ml of endotoxin by the Limulus amoebocyte lysate assay. [3 H]Thymidine was purchased from Amersham (Arlington Heights, IL).

Genistein (GIBCO, Grand Island, NY) was dissolved in dimethyl sulfoxide/ethanol (1:1) at a concentration of 25 mM. Tyrphostin AG18 and AG213 (from Dr. Alexander Levitzki, Hebrew University, Jerusalem, Israel) [16] were dissolved in absolute ethanol at a concentration of 50 mM. The phorbol esters 4 α -P and PMA (Sigma) were dissolved in absolute ethanol at a concentration of 1.6 mM. In addition, H-7, H-8, and HA1004 (Seikagaku America, Rockville, MD) were dissolved in distilled water at a concentration of 10 mM. Chelerythrine (LC Laboratories, Woburn, MA) was dissolved in distilled water at a concentration of 10 mM. All stock solutions were diluted in culture medium or PBS to the desired concentrations at the time of addition.

Cell line. Human glioma cell line U-105MG has been described in detail [17]. The cell line has been passed >200 times and maintained in 150 cm² plastic tissue culture flasks (Falcon Plastics, Lincoln Park, NJ) in complete culture media (DMEM/F12 + 2 mM L-glutamine + 8% FBS) at 37°C and 7.5% CO₂. Antibiotics were not used routinely and U-105MG cells were negative for mycoplasma by two-stage nested primer polymerase chain reaction test (ATCC, Rockville MD).

Antibodies and antisera. A4.6.1 anti-VEGF monoclonal antibody was from Genentech [18]. Polyclonal anti-VEGF antiserum was made in rabbit as has been reported [7,8].

Analysis of dose-response to PTK or PKC inhibitors. U-105MG cells were plated at 1×10^5 cells/well in 24-well plates in 0.5 ml of DMEM/F12 + 2 mM L-glutamine + 0.8% FBS and incubated (72 h, 37°C, 7.5% CO₂) undisturbed. Supernates were aspirated and replaced with 0.5 ml of DMEM/F12 + 2 mM L-glutamine + 0.8% FBS containing various concentrations of inhibitors of PTK or PKC with or without EGF in quadruplicate wells. EGF was added 30 min after adding inhibitors. After 72 h incubation (37°C, 7.5% CO₂), alamarBlue (50 μ l; Alamar Biosciences, Sacramento, CA) was added to each well, the plates incubated for 30 min, and cell viability based on metabolic conversion of the deep blue dye to a pink color was assessed spectrophotometrically in situ. Absorbances of supernates were measured at a wavelength of 562 nm and background absorbances at 590 nm were subtracted using a semiautomatic plate reader (model EL310, Bio-Tek Instruments, Winooski, VT). Conditioned media were subsequently assessed for VEGF by ELISA. Multiple experiments confirmed that alamarBlue did not interfere with detection of VEGF by ELISA [7]. All experiments were repeated a minimum of three times.

Effect of PMA pre-treatment on growth factor- and PMA-induced VEGF secretion. U-105MG cells were plated in 24-well plates as described above and incubated (37°C, 72 h) either with PMA (1.6 μ M) or 4 α -P (1.6 μ M) to down-regulate conventional and novel isoforms of PKC. Supernates were aspirated, cells gently washed twice with warm PBS buffer, and incubated (37°C, 72 h) with fresh medium containing various concentrations of PMA, EGF, PDGF-BB, or bFGF. The degree of inhibition of induced VEGF secretion was calculated by the following formula:

$$100\% - (C_{ig} - C_i)/(C_g - C_0) \times 100\%,$$

where C_{ig} is the VEGF concentration with PMA (or inhibitor) pre-treatment and growth factor (or inducer), C_i is the VEGF concentration with PMA pre-treatment but without growth factor, C_g is the VEGF concentration without PMA pre-treatment but with growth factor, and C_0 is the baseline VEGF concentration without PMA pre-treatment or growth factor.

ELISA. A semiquantitative capture ELISA was used to detect amounts of VEGF in supernates as previously reported [7]. Briefly, plates were coated with monoclonal anti-VEGF antibody A4.6.1, blocked, incubated (with intervening wash cycles) sequentially with: known or unknown samples, polyclonal anti-VEGF antiserum, biotinylated goat anti-rabbit antiserum, and avidin-biotinylated alkaline phosphatase complex (Vector Labs, Burlingame, CA). Polyclonal

antibody binding to captured VEGF was detected by addition of *p*-nitrophenyl phosphate substrate (Sigma Chemical, St. Louis, MO) with absorbance (405 nm) determined using a semiautomated plate reader. VEGF amounts in samples were extrapolated from standard curves generated in each assay [7].

[³H]Thymidine incorporation. Proliferation of U-105MG cells was determined by [³H]thymidine incorporation. Cells were seeded in 96-well plates at a density of 1×10^4 cells/well in 90 μ l of DMEM/F12 + 2 mM L-glutamine + 0.8% FBS and incubated (37 °C, 72 h, 7.5% CO₂) undisturbed. Various concentrations of inhibitors of PTK or PKC were then added and incubated for 6 h. Cells were incubated with [³H]thymidine (specific activity 41 Ci/mmol; 0.5 μ Ci/well) for an additional 15 h. Cells were detached by 30 min incubation at 37 °C in cell release solution (trypsin 0.1%, EDTA 2.15 mM, sucrose 0.4 M, pH 7.5, 40 μ l/well). Cells were then harvested with a semi-automatic cell harvester (Skatron, Sterling, VA) to scintillant-impregnated Ready Filter (Beckman, Fuller, CA). Filter discs were transferred to scintillation vials and radioactivity measured in a liquid scintillation spectrometer (LKB Rackbeta 1211, Finland). Experiments were performed with at least four replicates and results were expressed as mean counts per minute (cpm).

Results

PTK inhibitors genistein and tyrphostin inhibited EGF-induced VEGF secretion

Genistein, a broad action protein tyrosine kinase inhibitor, inhibited EGF-induced VEGF secretion (Fig. 1) in a dose-dependent manner with IC₅₀ (concentration causing a 50% inhibition) estimated to be about 25 μ M. Tyrphostin AG18 and AG213, which are effective blockers of EGF receptor kinase but extremely poor inhibitors of serine/threonine kinases [19,20], also inhibited EGF-induced VEGF secretion with IC₅₀ for each estimated to be about 15 μ M (Fig. 2A). Baseline secretion of VEGF was not inhibited by either tyrphostin. AG18 and AG213 also inhibited cell proliferation as assessed by [³H]thymidine incorporation (Fig. 2B); cell viability was not affected.

Fig. 1. Genistein inhibited EGF-induced VEGF secretion (closed circle) in U-105MG cell line with an IC₅₀ around 25 μ M. alamarBlue assay (triangle symbols) revealed decreased cell viability or cell metabolism at concentrations above 25 μ M.

Fig. 2. PTK inhibitors inhibited VEGF secretion and cell proliferation in U-105MG glioma cells. (A) PTK inhibitor tyrphostins AG18 and AG213 inhibited EGF-induced VEGF secretion (closed symbols) in dose-dependent fashion with IC₅₀ values around 15 μ M. Baseline secretion of VEGF (open symbols) was not inhibited. Representative of three experiments. (B) AG18 and AG213 inhibited cell proliferation as assessed by [³H]thymidine incorporation. Each data point represents mean $\pm$ SD of quadruplicate cultures and these experiments were repeated three times with similar results.

PKC inhibitor H-7 inhibited EGF-induced VEGF secretion as well as baseline level of VEGF secretion

PKC inhibitor H-7 began to inhibit EGF-induced VEGF secretion at a concentration of 10 μ M and inhibited baseline secretion of VEGF (Fig. 3A). HA1004 did not inhibit either EGF-induced or baseline secretion of VEGF at the same or higher concentrations. HA1004 strongly inhibits PKA and PKG, but weakly inhibits PKC with a Ki 7-fold higher than H-7 [21]. H-7 also inhibited cell proliferation as assessed by [³H]thymidine incorporation, while HA1004 did not (Fig. 3C). alamarBlue dye conversion revealed decreased cell metabolism at H-7 concentrations above 25 μ M (Fig. 3A). To determine whether this inhibitory effect was due to a cytotoxic action of the inhibitor, U-105MG cell viability was assessed by trypan blue exclusion and found to be 96% and 90% at 10 and 25 μ M, respectively. However, cell viability was observed to decrease to 61% at 50 μ M.

H-8, a PKG/PKA inhibitor, did not inhibit VEGF secretion at concentrations below 20 μ M, but inhibited EGF-induced and baseline secretion of VEGF at concentrations above 30 μ M. H-8 also inhibited cell proliferation at higher concentration (50 μ M) but did not

Fig. 3. PKC inhibitors inhibited both baseline and EGF-induced VEGF secretion in U-105MG cells as well as DNA synthesis. (A) PKC inhibitor H-7 at 10 μM inhibited both EGF-induced and baseline secretion of VEGF (circles). AlamarBlue dye conversion revealed decreased cell metabolism at concentrations above 25 μM (triangles). HA1004, a negative control compound for H-7, had no effect on secretion of VEGF at the same or higher concentrations (squares). Representative of three experiments. (B) H-8 inhibited both EGF-induced and baseline secretion of VEGF at concentrations above 30 μM (circles) but did not interfere with cell metabolism as assessed by alamar blue (triangles). (C) H-7 and H-8 inhibited cell proliferation as assessed by $[^3\text{H}]$ thymidine incorporation, while HA1004 did not. Each data point represents mean $\pm$ SD of quadruplicate cultures. These experiments were repeated three times with similar results.

interfere with cell metabolism as assessed by alamarBlue dye reduction (Figs. 3B and C). H-8 has been reported to be >1 log more effective at inhibiting PKG (0.48 μM) and PKA (1.2 μM) than PKC (15 μM) [22].

Chelerythrine inhibited baseline and EGF-induced VEGF secretion only at concentrations of 30 μM or higher but also inhibited cell metabolism and cell proliferation in the same range (30 and 20 μM, respectively; Figs. 4A and B). Trypan blue exclusion revealed that most of these cells were not alive at 30 μM, and therefore, this was judged to be a toxic, rather than a specific inhibitory, effect.

Fig. 4. Inhibition of EGF-induced VEGF secretion by chelerythrine is due to a non-specific cytotoxic effect. (A) Chelerythrine inhibited baseline and EGF-induced VEGF secretion at concentration of 30 μM. Representative of three experiments. (B) Chelerythrine also inhibited cell proliferation and cell metabolism at about the same concentration (20 and 30 μM, respectively) Each data point represents mean $\pm$ SD of quadruplicate cultures. These experiments were repeated three times with similar results.

Response of cultured U-105MG cells to PMA

The phorbol ester PMA, activates conventional and novel forms of PKC, caused morphologic changes in U-105MG glioma cells. Within several hours of treatment, cells changed from a fusiform bipolar appearance to a more flattened, polygonal cell shape with multiple short processes. Later, cells became larger and cytoplasmic vacuoles became evident. These morphologic changes were induced by as little as 3.2 nM PMA, whereas no noticeable changes in morphology were induced by 4α-P (a phorbol ester that does not activate PKC) under identical conditions.

PMA-induced VEGF secretion in a dose-dependent manner with a significant increase of VEGF secretion at 3.2 nM ($P < 0.001$, by ANOVA and Bonferroni method). VEGF secretion reached peak levels at about 50 nM PMA and declined slightly as the dose of PMA was increased (Fig. 5A). The control compound, 4α-P, did not modulate VEGF secretion within the same range (Fig. 5A). Pretreatment of cells with 1.6 μM PMA for 3 days, which down-regulates PKC in other cell types [23,24], completely blocked a subsequent increase in VEGF secretion after stimulatory challenge with PMA (Fig. 5A). After pretreatment with PMA, however, baseline secretion of VEGF was significantly elevated above that without PMA pretreatment or that with 4α-P pretreatment ($p < 0.01$, unpaired Student's t test).

Fig. 5. Down-regulation of PKC by PMA pretreatment blocked PMA-induced VEGF secretion in U-105MG cells. (A) PMA induced VEGF secretion in a dose-dependent manner achieving a plateau response at 64 nM (closed squares). The control compound, 4α-phorbol (4α-P), had no effect within the same range (closed circles). Pretreatment of the cells with PMA 1.6 μM for three days completely down-regulated the stimulatory effect of PMA on VEGF secretion (open squares). (B) As little as 3.2 nM PMA significantly inhibited DNA synthesis in U-105MG cells (closed squares). After three days of pretreatment (1.6 μM PMA), U-105MG DNA synthesis recovered modestly and was refractory to PMA inhibition (open squares). 4α-P had no significant effect on U-105MG DNA synthesis, even at a concentration of 1.6 μM (closed circles). Each data point represents mean ± SD of quadruplicate cultures; representative of three experiments.

Incorporation of [³H]thymidine, used as an index of cell proliferation, demonstrated that as little as 3.2 nM PMA significantly inhibited DNA synthesis acutely in U-105MG cells to 26% of baseline (Fig. 5B; $p < 0.001$, by ANOVA and Bonferroni method). Higher concentrations of PMA exhibited no additional effect.

However, after 72 h exposure to 1.6 μM PMA, cellular proliferation appeared to have recovered as indicated by the modest rate of [³H]thymidine uptake. Despite these effects, U-105MG cells remained viable at high PMA concentration as demonstrated by alamarBlue assay (Fig. 5A) and trypan blue exclusion. By comparison, 4α-P exhibited no significant effect on U-105MG cell DNA synthesis, even at 1.6 μM (Figs. 5A and B).

Calcium ionophore A23187, which activates some isoforms of PKC by increasing intracellular calcium concentration, enhanced VEGF secretion as effectively as PMA (80 nM) at a concentration of 1.25 μM (Table 1). A23187 also potentiated PMA-induced VEGF secretion.

Down-regulation of PKC with PMA partially blocked growth factor-induced VEGF secretion

We have shown previously that EGF, bFGF, or PDGF-BB each are able to induce VEGF secretion in U-105MG [7]. Here, we demonstrate that down-regulation of PKC by pretreating the cells with PMA (1.6 μM for 3 days) blocked VEGF secretion induced by EGF (50 ng/ml), bFGF (50 ng/ml), or PDGF-BB (50 ng/ml) by 48%, 53%, or 47%, respectively (Fig. 6). In comparison, PMA pretreatment eliminated the PMA induction of VEGF secretion almost completely ($97 \pm 7\%$). While PMA pretreatment did not eliminate growth factor-induced VEGF secretion completely, the differential inhibition of PMA-induced and growth factor-induced VEGF secretion was statistically significant ($p < 0.01$, by ANOVA and Bonferroni method). An analogous relation could also be demonstrated in that the PKC inhibitor, H-7 (25 μM), inhibited PMA-induced VEGF secretion ($83 \pm 9\%$) to a much greater extent than it did EGF-induced VEGF secretion ($45 \pm 4\%$) (Fig. 7, $p < 0.01$).

Discussion

We have previously demonstrated that EGF, bFGF, or PDGF-BB each are capable of inducing VEGF secretion in U-105MG cells [7]. Receptors for EGF, bFGF, or PDGF-BB are themselves protein tyrosine kinases [25,26] and stimulation of these receptors with

Table 1
Effects of calcium ionophore A23187 and PMA on VEGF secretion by U-105MG

Treatment condition	VEGF (ng/ml)		Alamar blue assay (absorbance)	
	Mean ± SD	(% of Control)	Mean ± SD	(% of Control)
Control	9.0 ± 0.6	(100)	0.33 ± 0.02	(100)
A23187 (1.25 μM)	22 ± 1.9	(240)	0.29 ± 0.02	(88)
PMA (80 nM)	18 ± 2.0	(200)	0.33 ± 0.04	(100)
A23187 + PMA	28 ± 1.0	(310)	0.36 ± 0.04	(110)

Fig. 6. Down-regulation of PKC by PMA pretreatment blocked growth factor-induced VEGF secretion in U-105MG cells. U-105MG cells, incubated (72 h) either with (closed symbols) or without (open symbols) PMA (1.6 μ M), were washed and incubated (72 h) with fresh media containing various concentrations of EGF (A), bFGF (B), or PDGF-BB (C). Supernates, assessed for VEGF by ELISA, revealed that down-regulation of PKC by PMA pretreatment blocked EGF-, bFGF-, or PDGF-BB-induced secretion of VEGF significantly, but did not eliminate growth factor-induced VEGF secretion completely. Cell viabilities were unaffected by PMA pretreatment (triangles). Representative of three experiments.

Fig. 7. PMA pretreatment (PKC depletion) blocked EGF-, bFGF-, or PDGF-BB-induced VEGF secretion to 48%, 53%, and 47% of control, respectively. H-7, a specific PKC inhibitor, inhibited EGF-induced VEGF secretion to 45% of control, while it blocked PMA-induced VEGF secretion to 83%. PMA pretreatment almost completely blocked (97%) PMA-induced VEGF secretion.

their cognate growth factors rapidly leads to receptor autophosphorylation on tyrosine residues. Ligand-stimulated autophosphorylation of tyrosine enhances subsequent tyrosine kinase activity of the receptors and creates binding sites for recruitment of specific enzymes, which include phosphatidylinositol (PtdIns) 3-kinase, *ras*-GTPase activating protein, PtdIns-specific phospholipase C γ , and pp74^{c-raf} [27]. Ligand-activated PDGF receptors associate with all four enzymes. Ligand-activated EGF receptors appear to associate with at least two of these four targets (PtdIns 3-kinase and PtdIns-specific PLC γ) and may associate with *ras*-GTPase activating protein. Activated bFGF receptors also associate with PLC γ [25]. Activation of PtdIns-specific PLC γ would stimulate canonical PtdIns turn-

over pathways, leading to elevation of cytosolic calcium and activation of PKC [28]. Thus, tyrosine kinase-type growth factor receptors transduce signals through PKC-dependent and PKC-independent pathways. While most signaling pathways have yet to be completely defined, it may be possible to block a major signaling pathway if the specific enzymes involved are known. PTK and PKC mediate a wide range of signal transduction processes and are considered more selective targets for chemotherapy than known targets of inhibitors of DNA or RNA synthesis, or agents causing disruption of the cytoskeleton [29,30]. In this study, we used inhibitors for both PTK and PKC as probes to explore control points in signal transduction of growth factor-induced VEGF secretion. Given that induction of glioma cell proliferation and growth factor-induced VEGF secretion are apparently inextricably linked, these control points may lead to rational development of novel chemotherapeutic strategies for malignant glioma.

Selective inhibition vs. cytotoxicity

Most compounds used to investigate regulatory roles of PKC lack selectivity for PKC over several other kinases [31]. For example, H-7 inhibits PKC, PKA, or PKG at 6.0, 3.0, or 5.8 μ M, respectively [32], and therefore, is not a PKC-specific inhibitor. In order to compensate for this lack of specificity, HA1004 was used as a “negative” control compound for H-7. HA1004 inhibits PKA/PKG with comparable potency (2.3 and 1.3 μ M, respectively) to that of H-7 but is about 1 log less effective in PKC inhibition (40 μ M) [21]. In this study, we showed that H-7 inhibited EGF-induced VEGF secretion, while HA1004, used at similar concentrations, did not (Fig. 3A). We propose that the inhibition was most likely due to an effect on PKC. Although viability of U-105MG cells was still above 90% at 25 μ M, it decreased to 61% at an H-7 concentration of 50 μ M. Further, H-7 inhibited cell metabolism

significantly at concentrations above 25 μM as shown by alamarBlue assay (Fig. 3A). Thus, the inhibitory effect of H-7 occurred at a concentration just below that which caused a cytotoxic effect.

As a further control for the inhibitory effects of H-7 on PKA and PKG, we tested H-8, which inhibits PKG (0.48 μM) and PKA (1.2 μM) much more effectively than it inhibits PKC (15 μM) [21]. As observed with HA1004, H-8 did not inhibit EGF-induced VEGF secretion at concentrations (10 μM) at which it should have inhibited PKA and PKG. However, by increasing the concentration above 20 μM , an inhibitory effect was observed which could be attributable to PKC inhibition. Like HA1004, H-8 did not interfere with cell metabolism at concentrations up to 50 μM .

While H-7 and H-8 are thought to inhibit PKC via competition for the ATP binding site on PKC [33] (Fig. 8), chelerythrine is a non-competitive inhibitor with respect to ATP [34]. It inhibits the catalytic domain of PKC, but not the regulatory domain [34] (which binds DAG and PMA; Fig. 8). Chelerythrine is a potent and specific inhibitor of PKC in vitro and showed potent cytotoxic effects against L-1210 tumor cells [34]. Our data demonstrated that chelerythrine inhibited both EGF-induced and baseline VEGF secretion, as well as metabolism at the same concentration (20 μM), which we presume to represent a non-selective cytotoxic effect, rather than specific inhibition of PKC.

PMA binds to the DAG-specific regulatory domain of PKC and activates PKC. An alternative technique to

assess the role of PKC in cell function relies on the observation that prolonged treatment of cells with PMA activates and eventually depletes PKC. Though PMA significantly inhibited the growth of U-105MG cells acutely, DNA synthesis was able to proceed at a modest rate (Fig. 5B). These cells remained viable and metabolically active after treatment with a high PMA concentration (1.6 μM) for 3 days (Figs. 5A and B). PMA acutely increased secretion of VEGF and this effect could be blocked completely either by PMA pretreatment to deplete PKC or by inhibition of PKC with H-7, thus supporting the notion that induced secretion of VEGF is PKC dependent.

Growth factor-induced VEGF secretion depends at least in part upon the activation of PKC

While down-regulation of PKC by prolonged treatment of cells with PMA completely blocked PMA-induced VEGF secretion, it did not completely block EGF-, bFGF-, or PDGF-BB-induced VEGF secretion. This implies that more than one signaling pathway is involved in growth factor-induced VEGF secretion. Notably, PMA pretreatment blocked growth factor-(EGF, bFGF, or PDGF-BB) induced VEGF secretion to about the same degree, i.e., about 50% of the induced effect was PKC dependent. The remaining capacity could be due to so called “PKC-independent” mechanisms, or simply due to PKC isozymes that are not down-regulated by PMA [35].

Co-regulation of VEGF secretion and cell proliferation by growth factors

Axiomatically, PTK and PKC exert major roles in cell proliferation and differentiation. In this study, all of the inhibitors which attenuated EGF-induced VEGF secretion also diminished cell proliferation at similar concentrations. PMA also inhibited proliferation of cells in U-105MG, but this effect was probably due to down-regulation of PKC instead of activation of PKC, because PMA-induced DNA synthesis inhibition could not be blocked by PKC inhibitors H-7 and H-8 (data not shown). These findings suggest that regulation of VEGF secretion is tightly coupled with cell proliferation, and further support the concept that normal tissue growth and neovascularization are co-regulated by normal growth signals, while tumor growth and tumor angiogenesis are co-regulated by the inappropriate presence of growth signals.

In summary, we have determined that growth factor-induced VEGF secretion by U-105MG human glioblastoma cells in vitro depended in part upon activation of PKC, could be blocked by inhibitors of PKC or by broad spectrum down-regulation of conventional or novel isoforms of PKC. It appears, however, that

Fig. 8. Schematic representation of protein kinase-C family members. PKC is currently comprised of a family of at least 11 isoenzymes thought to play critical roles in control of many cellular processes. These serine/threonine kinase isozymes are broadly divided into three classes: the “conventional” Ca²⁺-dependent forms (α , β , and γ), the “novel” forms that lack calcium binding C₂ domain (δ , ϵ , η , and θ) and do not require Ca²⁺ for activation, and the “atypical” forms that lack the Ca²⁺ binding domain and are not activated by DAG/PMA (ζ and τ). V₁–V₅ are variable or nonconserved regions of the PKC molecule. C₁–C₄ are conserved regions of the PKC molecule. The C₁ region contains two sets of cysteine-rich sequences and is required for phorbol ester binding. PKC ζ has only one set of cysteine-rich sequences and does not translocate or down-regulate in response to phorbol esters or diacylglycerol derivatives. Different PKC isoenzymes probably have distinct functions in the processing and modulation of a variety of physiological and pathological responses to external signals. The existence of PKC isoenzymes can, in part, explain why activation of PKC produces varied, and at times disparate, cell-specific responses.

polypeptide growth factors (EGF and PDGF) and PMA may utilize common elements of a signal transduction pathway leading to the induction of cell proliferation and VEGF secretion. This suggests that both glioma cell growth and angiogenesis mediated by VEGF secreted from proliferating glioma cells might be susceptible to pharmacologic agents that interfere with these signal transduction pathways. Further elucidation of these important pathways and identification of control points may allow the development of focused therapies that will be toxic to tumor cells but relatively benign with respect to normal cells.

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