Distinct Protein Kinase C Isoforms Mediate Regulation of Vascular Endothelial Growth Factor Expression by A2A Adenosine Receptor Activation and Phorbol Esters in Pheochromocytoma PC12 Cells

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Vascular endothelial growth factor (VEGF) stimulates angiogenesis during development and in disease. In pheochromocytoma (PC12) cells, VEGF expression is regulated by A2A adenosine receptor (A2AAR) activation. The present work examines the underlying signaling pathway. The adenyl cyclase-protein kinase A cascade has no role in the down-regulation of VEGF mRNA induced by the A2AAR agonist, 2-[4-[(2-carboxyethyl)phenyl]ethylamino]-5′-N-ethylcarboxamidoadenosine (CGS21680). Conversely, 6-h exposure of cells to either phorbol 12-myristate 13-acetate (PMA) or protein kinase C (PKC) inhibitors mimicked the CGS21680-induced down-regulation. PMA activated PKCa, PKCε, and PKCζ, and CGS21680 activated PKCε and PKCζ as assessed by cellular translocation. By 6 h, PMA but not CGS21680 decreased PKCa and PKCε expression. Neither compound affected PKCζ levels. Following prolonged PMA treatment to down-regulate susceptible PKC isoforms, CGS21680 but not PMA inhibited the cobalt chloride induction of VEGF mRNA. The proteasome inhibitor, MG132, abolished PMA- but not CGS21680-induced down-regulation of VEGF mRNA. Phorbol 12,13-diacetate reduced VEGF mRNA levels while down-regulating PKCε but not PKCζ expression. In cells expressing a dominant negative PKCζ construct, CGS21680 was unable to reduce VEGF mRNA. Together, the findings suggest that phorbol ester-induced down-regulation of VEGF mRNA occurs as a result of a reduction of PKCζ activity, whereas that mediated by the A2AAR occurs following deactivation of PKCζ.

Vascular endothelial growth factor (VEGF)† was described initially as a vascular permeability factor (1), and was characterized subsequently as an endothelial cell mitogen (2). VEGF is involved primarily in angiogenesis, the pruning and reorganization of pre-existing vasculature to create new vasculature, and it has a critical role in embryonic development (3). In the adult, VEGF is required for the development and maintenance of the female reproductive cycle (4) and may be cardioprotective during ischemia (5). However, elevated levels of VEGF have been associated with pathologies, such as diabetic retinopathy, endomyocarditis, rheumatoid arthritis, and tumorigenesis (3, 6–8). Many tumors demonstrate elevated levels of VEGF, which can be correlated to disease progression (9–12). This correlation reflects the requirement of an expanding vasculature for tumor growth, and disruption of VEGF signaling regarding cancer progression (13–15).

Several factors, including hypoxia (16–19), various growth factors (20–22), and oncogenic mutations (23–25), up-regulate VEGF and the underlying mechanisms have been extensively examined. Less is known about the factors that down-regulate VEGF: natriuretic peptides (26), N-acetylcysteine (27), somatostatin (28), and certain anti-inflammatory drugs (29, 30). The pathways mediating the down-regulation of VEGF have not been elucidated.

Rat pheochromocytoma (PC12) cells are a frequently employed model for hypoxia-initiated responses and have been used to study VEGF gene regulation, as hypoxia is a potent stimulant of VEGF expression (16–19). Additionally, PC12 cells express A2A and A2B adenosine receptors (AR) (31) and have been employed to study AR signal transduction and physiological activity. This laboratory has shown previously that activation of the A2AAR in PC12 cells results in a substantial reduction of VEGF, which is observed at both the mRNA and protein levels (32). Furthermore, this down-regulation of VEGF mRNA occurs because of an inhibition of VEGF gene transcription (32). The nonselective AR agonist, 5′-[(N-ethylcarboxamido)adenosine, was also reported to down-regulate VEGF expression in PC12 cells (33). Other cell types have been shown to respond to AR agonists with either increases or decreases in VEGF expression (34–36). This differential regulation may exist because of the subtype specificity of various AR ligands, and because of cell-specific variations in the signal transduction cascade to which a distinct AR subtype may be linked.

The A2AAR is typically coupled via the Gs protein to the stimulation of adenyl cyclase (AC) and activation of protein kinase A (PKA) (31, 37, 38). However, certain effects mediated by the A2AAR have been linked to protein kinase C (PKC) activation (39–42). Based on their requirements for activation, three PKC classes are defined: conventional (α, βI, βII, γ), which are activated by Ca2+ and diacylglycerol (DAG); novel (δ, ε, η, θ), which are Ca2+-independent; and atypical (ζ, λ, τ), which are activated independently of Ca2+ or DAG. PC12 cells have been reported to express PKC isoforms α, βI, βII, δ, ε, η,
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θ, and (43–45). Two specific PKC isoforms, PKC<sub>ζ</sub> and PKC<sub>ε</sub>, have been demonstrated to regulate VEGF expression. For example, increases in PKC<sub>ζ</sub> activity up-regulate VEGF expression in glioblastoma U373 cells (46), and in HT1080 fibrosarcoma and 786-0 renal carcinoma cells (25). Activation of PKC<sub>ζ</sub> has also been implicated in stretch-induced up-regulation of VEGF in retinal capillary pericytes (47). In addition, it has been reported that ischemic preconditioning induces translocation of PKC<sub>ζ</sub> to the nucleus in cardiomyocytes, which causes up-regulation of VEGF expression (48).

The goal of the present study was to elucidate the signal transduction cascade responsible for the down-regulation of VEGF mRNA that is induced by 2-[4-[(2-carboxyethyl)phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS21680), a selective agonist for the A<sub>2A</sub>AR. Our results indicate that stimulation of PKC activity by either CGS21680 or phorbol 12-myristate 13-acetate (PMA) produces an initial up-regulation of VEGF mRNA that is rapidly followed by a marked reduction in VEGF expression. The latter response appears to result from a decrease in PKC activity with specifically PKC<sub>ε</sub> and PKC<sub>ζ</sub> mediating the PMA- and CGS21680-induced response, respectively.

EXPERIMENTAL PROCEDURES

Cell Culture—Rat pheochromocytoma (PC12) cells were grown in complete RPMI medium (RPMI medium 1640 supplemented with 10% fetal bovine serum, 10% equine serum, 1% penicillin-streptomycin-glutamine, and 0.25 μg/ml fungizone), and were maintained in a 5% CO<sub>2</sub>-humidified incubator at 37 °C as previously described (32). Cells were subcultured into collagen-coated six-well dishes, 100-mm dishes, or T-75 flasks for experiments 24 h prior to treatment. Culture medium was replaced with fresh complete RPMI medium or with RPMI 1640 medium, when noted, 30–60 min prior to treatment. The times of treatment with various agonists and inhibitors are provided under “Results.” Control cells were treated with appropriate volumes of dimethyl sulfoxide when appropriate.

Radioimmunoassay of cAMP—Intracellular cAMP levels in PC12 cells were determined with a cAMP-[<sup>32</sup>P]cyclic nucleotide phosphodiesterase assay kit (PerkinElmer Life Sciences). Cells in six-well dishes were treated for the indicated amount of time and washed twice with phosphate-buffered saline (PBS) (1.36% NaCl, 27 mM KCl, 80.5 mM Na<sub>2</sub>HPO<sub>4</sub>·14.7 mM KH<sub>2</sub>PO<sub>4</sub>), scraped, and suspended in 1 ml of EtOH. A 250-μl aliquot of the lysate was then dried at 60 °C for 5 min, and microcentrifuged at 13,000 g for 15 min at 4 °C. Hybridization was conducted overnight with a 600-bp fragment of murine VEGF<sub>50</sub> cDNA random prime-labeled with [<sup>32</sup>P]dCTP. The membrane was sequentially washed and subjected to autoradiography. To normalize total RNA levels, membranes were additionally hybridized with a 1,100-bp fragment of human glyceraldehyde-3-phosphate dehydrogenase cDNA (Clontech, Palo Alto, CA) random primer labeled with [<sup>32</sup>P]dCTP. Autoradiographic signals were quantitated by an AlphaImager 2000 (Alpha Innotech Corp.).

Western Blot Analysis—Total protein from PC12 cells was isolated and determined to analyze AKT protein levels. Cells were treated for the indicated amount of time and lysed in 250 μl of lysis buffer (125 mM Na<sub>2</sub>HPO<sub>4</sub>·1/2H<sub>2</sub>O, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 0.5 μg/ml trypsin, 0.1 μg/ml aprotinin) for 30 min, followed by a Dounce homogenization with 10 strokes, and lysates were microcentrifuged at 500 g for 5 min. Protein concentrations were determined with Bio-Rad Protein Assay, and equal amounts of protein were electrophoresed on 8% polyacrylamide gels. Protein was then transferred to nitrocellular membranes and blocked for 1 h with Blotto (5% nonfat dry milk, 0.2% Triton X-100, 0.05% thimerosal, in PBS), prior to being incubated overnight at 4 °C with the appropriate primary antibody at a 1:1000 dilution in Blotto. The following antibodies were employed: cPKC<sub>α</sub> (C-20), nPKC<sub>δ</sub> (C-15), and aPKC<sub>ζ</sub> (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed three times for 5 min with Blotto and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody at 1:10,000 dilution for 1 h at room temperature. Membranes were then washed three times for 5 min in Blotto and two times for 5 min with PBS prior to being developed with ECL Western blotting detection reagents (Amersham Biosciences) and being exposed to x-ray film. Signals were analyzed with an AlphaImager 2000.

Nuclear, Cytosolic, and Membrane PKC Analysis—PC12 cells were treated with the appropriate agonists for 5 min. After treatment, subcellular fractions were isolated as previously described (49), with minor modifications. Briefly, cells from 100-mm dishes were scraped in 100 μl of extraction buffer (20 mM Tris, pH 7.6, 2 mM EDTA, 5 mM EGTA, 10 mM β-mercaptoethanol (β-ME), and protease inhibitors including 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.5 μg/ml aprotinin). Cells were then Dounce homogenized with 10 strokes, and lysates were microcentrifuged at 500 g for 10 min. The supernatant (plasma membrane and cytosolic fractions) was removed and centrifuged at 100,000 g for 60 min, and the resulting pellet (plasma membrane) was resuspended in 50 μl of suspension buffer (10 mM Tris, pH 7.6, 5 mM MgCl<sub>2</sub>, 5 mM β-ME, and protease inhibitors as described above) by sonication. The supernatant was collected as the cytosolic fraction. The cytosolic fraction was then resuspended in suspension buffer, boiled for 5 min, and microcentrifuged for 15 min at 13,000 g prior to protein concentration determination by Bio-Rad protein assay. Equal amounts of protein were run on 8% polyacrylamide gels as described above. Nuclear sample purity was demonstrated with the histone H1 (AE-4) antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Adenoviral Vector Expression of PKC<sub>ζ</sub>—PC12 cells were infected with previously characterized replication-deficient adenoviral vectors containing either wild-type or kinase-deficient mutant (dominant negative) forms of PKC<sub>ζ</sub>, or a vector containing β-galactosidase as a control (50, 51). Each recombinant adenovirus was prepared as previously described (52). The vectors were expanded in HEK293 cells, and the resulting plaque-forming units for each were determined to infect at an equal multiplicity of infection (m.o.i.). The wild-type PKC<sub>ζ</sub> adenoviral vector was infected at an m.o.i. of 600, whereas the dominant negative PKC<sub>ζ</sub> adenoviral vector was infected at an m.o.i. of 800. PC12 cells were infected for 8 h in 4 ml of RPMI 1640 medium supplemented with 2% fetal bovine serum with gentle shaking in a 5% CO<sub>2</sub>-humidified 37 °C cell incubator. Complete RPMI medium was then added to the cells overnight, and cells were either treated for 24 h with 1 μg PMA prior to a 6-h treatment with the appropriate agonist in fresh complete medium, or cells were directly given fresh complete RPMI medium and treated for 6 h with the appropriate agonist. Cells were then scraped in 1 ml of PBS, and a 150-μl aliquot was microcentrifuged at 13,000 g for 5 min, and the resulting pellet was lysed in 100 μl of lysis buffer. Protein concentrations of samples were assessed by Western blot as described above. The remaining cell suspension was spun at 200 × g, and the resulting pellet was used for RNA isolation. VEGF mRNA levels were assessed by Northern blot analysis as described above.

Data Analysis—All experiments were performed a minimum of three times, and in duplicate when noted. Results are expressed as mean ± S.E. Statistical analysis was performed by one-way analysis of variance followed by a Newman-Keuls post-test. A p value < 0.05 was considered significant.

Reagents—The following compounds were purchased from Calbiochem (La Jolla, CA): forskolin, N-[2-[(p-bromocinnamyl)aminomethyl]-5-isouquinolinesulfonamide (H-89), protein kinase A inhibitor 14-22 amide (PKI), PMA, MG-132, bisindolylmaleimide IX (Ro-31-8220), and bisindolylmaleimide I (GFX). Epidermal growth factor (EGF) and all cell culture reagents were obtained from Invitrogen. The following compounds were purchased from Sigma: 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), CGS21680, and phorbol 12,13-diacetate (PDA).
RESULTS

Examination of the Involvement of PKA in the A2AAR-mediated Down-regulation of VEGF—As stimulation of the A2AAR is linked to AC activation (31, 37, 38), the signaling pathway mediating the down-regulation of VEGF mRNA induced by CGS21680, a selective agonist for the A2AAR, was initially explored using activators and inhibitors of PKA (Fig. 1A). As previously described (32), treatment of PC12 cells for 6 h with 1 μM CGS21680 reduced the VEGF mRNA level to 27.0 ± 8.1% of that in control cells. Forskolin (5 μM), an activator of AC and 8-Br-cAMP (1 μM), a cell-permeable cAMP analogue, did not alter VEGF mRNA levels (Fig. 1A). The PKA inhibitors, H-89 (5 μM) and PKI (10 μM), had no effect on VEGF mRNA and were unable to reverse the CGS21680-induced down-regulation of VEGF mRNA (Fig. 1A). To further examine the role of cAMP in CGS21680-induced down-regulation of VEGF mRNA, whole cell accumulation of cAMP was assessed in response to forskolin (5 μM) or CGS21680 (1 μM) (Fig. 1B). Radioimmunoassay of cAMP content showed that forskolin elicited a greater increase in cAMP levels than CGS21680 at all time points examined during a 6-h time course. As forskolin increased cAMP levels significantly more than CGS21680, but had no effect on VEGF mRNA levels, the data strongly suggest that cAMP is not involved in the A2AAR-mediated down-regulation of VEGF. Overall, the sum of these data indicates that pathways involving cAMP and/or PKA are not involved in the observed down-regulation of VEGF following the stimulation of the A2AAR in PC12 cells.

PKC Activation in Response to Stimulation of the A2AAR—As activation of the A2AAR has been linked to PKC stimulation in certain systems (39–41), including PC12 cells (42), the role of PKC in the regulation of VEGF mRNA was explored. For the initial analysis, cells were serum-starved for 14 h prior to a 6-h agonist treatment, as growth factors found in serum can stimulate PKC activity and/or up-regulate VEGF expression (21, 32). Under serum-free conditions, CGS21680 (1 μM), PMA (100 nM), and EGF (10 ng/ml), a growth factor known to activate PKC (21, 53), similarly regulated VEGF mRNA in a biphasic manner over a 6-h time period (Fig. 2A). At 1 h of treatment, all three compounds induced an initial increase in VEGF mRNA levels, followed by a down-regulation of VEGF mRNA by 6 h. The similarity of the PMA-, EGF-, and CGS21680-induced responses indicated that PKC may be involved in the A2AAR-mediated regulation of VEGF. To explore this possibility, the up-regulation of VEGF mRNA induced at 1 h by CGS21680 and PMA was examined for sensitivity to chemical inhibitors of PKC. As shown in Fig. 2B, administration of GFX (5 μM) and Ro-31-8220 (5 μM) blocked the increase in VEGF expression that was induced by CGS21680 and PMA. Under these conditions, the PKC inhibitors alone had no effect on VEGF mRNA.

Based on the above findings, the ability of CGS21680 and PMA to activate various PKC isoforms was assessed by examining the translocation of PKC isoforms in the nuclear, cytosolic, and membrane fractions as translocation to the membrane...
or the nucleus is an indicator of enzyme activation (54–56). PKCα, PKCe, and PKCζ isoforms were studied, as each represents one of the three PKC classes: conventional, novel, and atypical, respectively. As shown in Fig. 3, PMA promoted the nuclear translocation of PKCα, PKCe, and PKCζ by 710.8 ± 181.8%, 520.5 ± 107.2%, and 397.7 ± 98.9%, respectively. CGS21680 induced the nuclear translocation of PKCe and PKCζ by 358.7 ± 86.3% and 416.0 ± 146.1%, respectively. This correlates with previous data indicating activation of the PKC pathway following stimulation of the A2AAR (39–42).

Role of PKC Inhibition in the Down-regulation of VEGF mRNA—To further explore the role of PKC activity in the down-regulation of VEGF, modulation of VEGF mRNA expression by a 6-h treatment with CGS21680 (1 μM) or PMA (100 nM) was examined for sensitivity to GFX (5 μM) and Ro-31-8220 (5 μM). These experiments were conducted in cells maintained in complete growth medium. As shown in Fig. 4A, the CGS21680- or PMA-induced down-regulation of VEGF mRNA was unaltered in the presence of GFX and Ro-31-8220. Interestingly, however, when these PKC inhibitors were administered alone, GFX and Ro-31-8220 lowered VEGF mRNA to 44.8 ± 8.7% and 40.7 ± 7.8% of control, respectively. To characterize this decrease in VEGF mRNA elicited by GFX and Ro-31-8220, time-course studies for these PKC inhibitors were performed. As shown in Fig. 4B, both compounds significantly reduced VEGF mRNA levels at 30 min of treatment (to 55.0 ± 17.2% by Ro-31-8220 and to 48.3 ± 0.7% by GFX) and this down-regulation remained throughout 6 h.

The above data suggest that prolonged exposure of PC12 cells to CGS21680 or PMA may decrease VEGF mRNA content via an inhibition of PKC activity that may result from a down-regulation of PKC levels. Such a regulation of novel and conventional PKC isoforms is well documented in cells exposed for extended periods of time to PMA (57, 58). To examine this possibility, PC12 cells were treated with CGS21680 (1 μM) or PMA (100 nM) for 6 h and whole cell lysates were analyzed for PKC content (Fig. 5). Relative to control cells, PMA reduced PKCα and PKCe expression by 55.7 ± 4.5% and 91.2 ± 1.7%, respectively. PMA did not significantly alter PKCζ levels. Treatment of PC12 cells with CGS21680 for 6 h caused no significant change in the expression of any examined PKC isoform.

Identification of PKC Isoforms Involved in the CGS21680- and PMA-induced Down-regulation of VEGF mRNA—To further define a role for PKC and to identify the specific PKC isoform(s) that may be involved in the CGS21680- or PMA-induced down-regulation of VEGF mRNA, PC12 cells were treated with 1 μM PMA for 24 h to remove conventional and novel PKCs. Cells were then treated with cobalt chloride (50 μM) with or without CGS21680 (1 μM) or PMA (100 nM) for 6 h. Application of cobalt chloride mimics hypoxia and has been demonstrated to elevate VEGF mRNA expression (16). As shown in Fig. 6, in control cells and cells treated for 24 h with PMA, cobalt chloride induced VEGF expression by 3.1 ± 0.5- and 4.9 ± 1.3-fold, respectively. In control cells, this cobalt chloride-induced up-regulation of VEGF mRNA was inhibited
by 71.0 ± 9.5% by CGS21680 and 124.9 ± 13.8% by PMA, i.e. PMA reduced VEGF mRNA to a level lower than that observed in cells not treated with cobalt chloride. In cells treated for 24 h with PMA, CGS21680 similarly produced a 66.7 ± 7.5% inhibition of the cobalt chloride-induced response. Conversely, the ability of PMA to block the cobalt chloride-induced up-regulation of VEGF mRNA was nearly abolished, with an observed inhibition of 20.3 ± 9.1%. These data indicate that CGS21680-induced down-regulation of VEGF mRNA is not dependent on conventional and/or novel PKC isoforms, but that it may depend on PKCζ, as CGS21680 can further down-regulate VEGF mRNA when PKCζ remains available.

Use of a Proteasomal Inhibitor and a Selective Phorbol Ester to Identify the Specific PKC Isoform That Is Involved in the PMA-induced Response—The present findings suggest that the PMA-induced reduction in VEGF mRNA occurs as a result of the ability of this phorbol ester to down-regulate susceptible PKC isoforms. The down-regulation of PKC isoforms frequently results from targeting of activated PKCs for degradation through proteasome pathways (58–60). Thus, PMA- and CGS21680-induced down-regulation of VEGF mRNA was analyzed for sensitivity to MG-132, a chemical inhibitor of proteasomal degradative activity. As shown in Fig. 7, treatment with MG-132 (500 μM) alone increased VEGF mRNA levels to 215.0 ± 29.0% of control. It may be speculated that MG-132 inhibits the degradation of HIF-1α, a transcription factor known to up-regulate VEGF mRNA and to be subject to constitutive proteasomal degradation (61). MG-132 abolished the ability of PMA to down-regulate VEGF mRNA while having no effect on the reduction of VEGF mRNA elicited by CGS21680. To confirm the ability of MG-132 to inhibit the PMA-induced down-regulation of PKC isoforms, whole cell lysates of PC12 cells were analyzed for PKC expression by Western blotting following treatment with PMA in the absence or presence of MG-132 (Fig. 7, inset). The PMA-induced down-regulation of both PKCα and PKCε was completely reversed by MG-132. MG-132 itself had no effect on PKC expression. These data indicate a possible role for PKCα and/or PKCε in PMA down-regulation of VEGF mRNA, while also supporting the above described findings (Fig. 6) that indicate these PKC isoforms are not involved in the mechanism by which CGS21680 down-regulates VEGF mRNA.

To further support the role of PKC down-regulation as the mechanism by which PMA reduces VEGF mRNA, PDA was explored for its ability to regulate PKC isoform expression and VEGF mRNA levels. PDA has been reported to activate but not promote the degradation of PKCζ in rat brain cortical slices (62). As demonstrated in Fig. 8, a 6-h treatment of PC12 cells with 10 μM PDA promoted a down-regulation of VEGF mRNA nearly identical to that observed with 100 nM PMA. Although producing an 85.2 ± 4.3% reduction in PKCε expression, 10 μM PDA had no significant effect on PKCζ levels relative to untreated cells. As observed with PMA, PDA did not regulate PKCζ expression. The sum of these findings indicates a role for PKCε in the phorbol ester-mediated down-regulation of VEGF mRNA.

Application of Adenoviral Vectors Expressing Either Wild-type or Dominant Negative PKCζ to Alter the CGS21680-induced Response—To study the putative role of PKCζ in constitutive VEGF expression in PC12 cells and more specifically the A2aAR-mediated down-regulation of VEGF mRNA, we employed adenoviral vectors that directed expression of either wild-type PKCζ or a dominant negative form of PKCζ (DNPKCζ). Control cells were infected at the same m.o.i. with a β-galactosidase adenoviral construct. 24 h after infection, cells were treated with 1 μM PMA for 24 h prior to treatment with CGS21680. The addition of long term PMA allowed for the specific analysis of PKCζ activity, as novel and conventional PKC isoforms were removed. As shown in Fig. 9A, 1 μM CGS21680 reduced VEGF mRNA to 45.0 ± 12.1% of that observed in untreated cells expressing β-galactosidase. In cells in which there was a 3.9 ± 0.9-fold overexpression of wild-type PKCζ (Fig. 9A, inset), CGS21680 was unable to induce a significant decrease in VEGF mRNA. In a separate set of experiments, cells were infected with the β-galactosidase or DNPKCζ adenoviral constructs at the same m.o.i. (Fig. 9B). The addition of CGS21680 to cells expressing the β-galactosidase construct reduced the VEGF mRNA level to 40.7 ± 6.4% of that in
untreated cells. In cells expressing DNPKCζ (Fig. 9B, inset), VEGF mRNA levels were 43.7 ± 1.3% of that observed in β-galactosidase-expressing cells. However, 1 μM CGS21680 did not further down-regulate VEGF mRNA in cells expressing DNPKCζ.

**DISCUSSION**

Research examining the signal transduction cascade linking A2AAR activation to physiologic responses has typically demonstrated a critical role for the AC-PKA pathway (31, 37, 38). Therefore, the initial focus of this study was the role of AC and PKA in the A2AAR-mediated down-regulation of VEGF mRNA. Forskolin and 8-Br-cAMP did not decrease VEGF mRNA, and PKA inhibitors did not modulate the CGS21680-induced down-regulation of VEGF. As there is evidence for PKA-independent, but cAMP-dependent events in signal transduction (63), changes in cellular cAMP were evaluated. Forskolin elevated cAMP levels to a greater degree than CGS21680 throughout a 6-h time course, indicating that CGS21680-induced VEGF down-regulation is not cAMP-dependent. Thus, multiple results strongly imply that AC and PKA are not involved in the A2AAR-mediated down-regulation of VEGF mRNA.

Subsequent focus was placed on the PKC pathway, as there have been reports linking A2AAR stimulation to PKC activation (39–42). Under serum-free conditions, CGS21680, PMA, and EGF initially up-regulated VEGF mRNA, and this was followed by a down-regulation at 6 h. The ability of two well described activators of PKC, PMA and EGF, to mimic the CGS21680-induced response suggested that the A2AAR may be linked to PKC stimulation. Indeed, the up-regulation of VEGF mRNA produced by CGS21680, PMA, and EGF was blocked by two different chemical inhibitors of PKC. Moreover, CGS21680 induced the translocation of PKCe and PKCζ to the nucleus whereas PKCα directed nuclear translocation of PKCα, PKCe, and PKCζ. Recently, Huang and co-workers (42) demonstrated the rapid nuclear translocation of PKCζ following A2AAR activation in PC12 cells and associated this response with the anti-apoptotic effects of CGS21680. It should be noted that, although insensitivity to direct activation by DAG has served to define atypical PKC isoforms, stimulation of PKCζ by PMA in various cell types including PC12 cells has been observed (64, 65). Several studies have demonstrated that translocation of PKC to the nucleus is concurrent with activation (42, 44, 48, 55). This facet of PKC activity is particularly relevant to VEGF mRNA regulation, as PKC-mediated activation of nuclear transcription factors such as Sp-1 has a critical role in VEGF gene transcription (66, 67). The importance of PKC activity in VEGF mRNA regulation was further demonstrated, as GFX and Ro-31-8220 were found to rapidly down-regulate VEGF mRNA in PC12 cells maintained in complete medium. This suggests that VEGF mRNA is maintained at a high constitutive level in PC12 cells most likely because of high basal PKC activity resulting from growth factors present in the serum-supplemented medium. Indeed, VEGF mRNA and protein expression are markedly lower in serum-starved PC12 cells relative to cells maintained in complete medium (32).

The findings with the PKC inhibitors suggested that down-regulation of VEGF mRNA in response to CGS21680 and PMA over a prolonged period may occur as a result of a decrease in PKC activity, and several approaches were taken to examine this possibility. These studies demonstrated the differential regulation and roles of distinct PKC isoforms in the CGS21680- and PMA-induced responses. Treatment of PC12 cells with PMA for 6 h significantly decreased PKCα levels, and PKCe expression was nearly abolished. It is well documented that...
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long term (≥24 h) treatment with PMA down-regulates conventional and novel PKC (59), but it was surprising to observe such a dramatic decrease at 6 h. In cells depleted of conventional and novel PKC isoforms by prolonged exposure to PMA, CGS21680, but not PMA, maintained the ability to inhibit the cobalt chloride-induced up-regulation of VEGF mRNA. This finding strongly implies a role for conventional/novel PKC isoforms in the PMA-induced VEGF down-regulation whereas PKC\textsubscript{\alpha} may mediate the response observed with A\textsubscript{2A}AR activation.

The proteasome inhibitor MG-132 was employed to determine whether PKC degradation was central to the mechanism by which PMA down-regulates VEGF mRNA. MG-132 blocked PMA-induced down-regulation of VEGF mRNA, but had no effect on the CGS21680-induced response. PMA, a phorbol ester reported not to promote the degradation of PKC\textsubscript{\alpha} (62), was administered to determine which PKC isoform may mediate the PMA-induced down-regulation of VEGF mRNA. PMA promoted the degradation of specifically PKC\textsubscript{\alpha}, and in a consistent temporal fashion down-regulated VEGF mRNA. These findings indicate that PMA-induced down-regulation of VEGF mRNA occurs because of the degradation of specifically PKC\textsubscript{\alpha} that follows the activation of this PKC isoform.

To further explore the putative role of PKC\textsubscript{\alpha} in the A\textsubscript{2A}AR-mediated down-regulation of VEGF mRNA, replication-deficient adenoviruses containing either wild-type PKC\textsubscript{\alpha} or a dominant negative PKC\textsubscript{\alpha} construct were employed. In cells overexpressing wild-type PKC\textsubscript{\alpha} and treated for 24 h with PMA, CGS21680 did not down-regulate VEGF mRNA. One possibility for this lack of responsiveness is that the mechanism by which prolonged stimulation of the A\textsubscript{2A}AR abrogates PKC\textsubscript{\alpha} signaling is overwhelmed in the presence of excess PKC\textsubscript{\alpha} levels. We did not observe up-regulation of VEGF mRNA levels upon overexpression of PKC\textsubscript{\alpha}, and this was similarly reported for retinal capillary pericytes (47). Conversely, in other cell lines, an up-regulation of VEGF mRNA occurs upon overexpression of PKC\textsubscript{\alpha} (25, 46). It is possible that, in PC12 cells, a relatively high basal activity of endogenous PKC\textsubscript{\alpha} prevents any activity of experimentally introduced PKC\textsubscript{\alpha} to be apparent. When a dominant negative PKC\textsubscript{\alpha} isoform is expressed, VEGF mRNA levels were reduced relative to those in β-galactosidase-expressing cells, an observation in agreement with the hypothesis regarding high constitutive activity of endogenous PKC\textsubscript{\alpha} in PC12 cells. In these cells, CGS21680 did not down-regulate VEGF mRNA. Thus, the reduction in PKC\textsubscript{\alpha} function induced via expression of the dominant negative construct may result in the loss of the signaling activity that is typically targeted for inhibition upon prolonged A\textsubscript{2A}AR stimulation.

Our findings implicating PKC\textsubscript{\alpha} and PKC\textsubscript{\alpha} in VEGF regulation in PC12 cells are consistent with observations in other cell lines. For example, Kawata et al. (48) found that the specific translocation of PKC\textsubscript{\alpha} to the nucleus of cardiomyocytes 10 min after an ischemia/reperfusion protocol in rats up-regulated VEGF mRNA at 3 h. A PKC inhibitor blocked both the translocation of PKC\textsubscript{\alpha} and the up-regulation of VEGF mRNA. PKC\textsubscript{\alpha} has also been shown to regulate VEGF mRNA expression in several cell types. In both human glioblastoma (46) and fibrosarcoma (25) cells, overexpression of PKC\textsubscript{\alpha} resulted in constitutive up-regulation of VEGF mRNA. Similarly, expression of a dominant negative PKC\textsubscript{\alpha} has been shown to abrogate VEGF up-regulation induced by various stimuli (25, 47). Particularly intriguing is the present finding that the reduction, and not the direct activation, of PKC signaling by a physiologically relevant stimulus, adenosine, underlies the VEGF mRNA down-regulation. The cellular effects occurring in response to acute PKC activation have been extensively explored. However, there have been reports of PKC deactivation/degradation upon prolonged agonist exposure as the mechanism underlying cellular response. For example, in an examination of the tumor-promoting effects of PMA in rat fibroblasts overexpressing c-Src, Lu and co-workers (68) found that phenotypic transformation of these cells corresponded temporally with phorbol ester-induced depletion of PKC\textsubscript{\alpha}. Similar to results we describe, inhibitors of PKC and expression of a dominant negative PKC\textsubscript{\alpha} also promoted phenotypic changes similar to those observed with prolonged PMA exposure (68). Additionally, Shizukuda et al. (69) reported that the VEGF-induced migration and proliferation of endothelial cells was mediated by a decrease in PKC\textsubscript{\alpha} activity although PKC\textsubscript{\alpha} expression remained unchanged. Overexpression of wild-type PKC\textsubscript{\alpha} blocked the ability of VEGF to induce cell response (69), and this finding is similar to our observation that CGS21680 did not regulate VEGF in cells overexpressing PKC\textsubscript{\alpha}. It is apparent that deactivation of PKCs, either through reduction in kinase activity or protein expression, can be as important or more important than their activation.

Our findings also raise questions regarding the mechanisms of differential feedback regulation of PKC isoforms. It is clear that PMA ultimately promotes the proteasomal degradation of PKC\textsubscript{\alpha}, but it is not known how A\textsubscript{2A}AR activation apparently decreases PKC\textsubscript{\alpha} activity without modifying protein levels. Indeed, relatively little is known about the regulation of atypical PKC isoforms although it has been reported that PKC\textsubscript{\eta} decreases PKC\textsubscript{\alpha} activity without modifying protein levels. In PC12 cells expressing the Par-4 gene product (data not shown), and it is currently being examined whether A\textsubscript{2A}AR activation modulates Par-4 expression or promotes its complex formation with PKC\textsubscript{\alpha}.

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