Cyclic GMP-Dependent Protein Kinase Activation and Induction by Exisulind and CP461 in Colon Tumor Cells

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ABSTRACT

These studies report on the activation and induction of cGMP-dependent protein kinase (PKG) by exisulind and analogs and test the hypothesis that PKG is involved in the induction of apoptosis in colon tumor cells. Exisulind and analogs are pro-apoptotic drugs developed as inhibitors of cGMP phosphodiesterase gene families 5 and 2 that have been shown to sustain increased cGMP in SW480 and HT29 cells. At concentrations that induced apoptosis, both exisulind and CP461 increased PKG activity in SW480 cell supernatants. PKG activation was dose-dependent and sustained. Activation of PKG by exisulind and analogs was also seen in the colon tumor cell lines HT29, T84, and HCT116. The guanylyl cyclase activators YC-1 and guanylin increased PKG activity secondary to increased cellular cGMP and induced apoptosis in colon tumor cells. Exisulind and CP461 had no direct effect on purified PKG activity or on basal and stimulated PKG activity from cell supernatants. An additional effect of exisulind after 8 h of drug treatment was a dose-dependent increase of PKG β protein expression. β-Catenin, a potential new substrate for PKG, whose regulation influences apoptosis, was phosphorylated by PKG in vitro. 32P-labeled cells treated with exisulind showed increased phosphorylation of β-catenin. These data indicate that exisulind and analogs activate and induce PKG, resulting in increased phosphorylation of β-catenin and enhanced apoptosis to promote colon tumor cell death.

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Cyclic GMP levels are determined by soluble and particulate guanylyl cyclases (GCs: GC-A, GC-B, and GC-C), cGMP PDE (gene families 1, 2, 3, 5, 6, 9, 10, and 11), cGMP export pumps (Patel et al., 1995; Jedlitschky et al., 2000). In the intestine GC synthesis of cGMP from GTP can be activated by agonists such as guanylin, uroguanylin, bacterial toxins, nitric oxide, CO, and YC-1. Cyclic GMP PDE hydrolysis can control the intensity and duration of responses. In intestine and colon epithelium cells, GC-C is highly expressed and has been studied as a diagnostic marker for metastatic colorectal tumors in human extraintestinal tissues (Carrithers et al., 1996). PDE5 and PDE2 also show enhanced immunoreactivity in colon, lung, pancreatic, and bladder tumor tissues (Piazza et al., 2000, 2001a,b,c). Thus, cGMP metabolism in colon tumor cells may be a useful therapeutic target.

Exisulind (sulindac sulfone) is a proapoptotic drug that causes regression and prevents recurrence of polyps in patients with familial adenomatous polyposis (Stoner et al., 1999). Exisulind and its analogs CP461, CP78, and CP248 inhibit cell growth and induce apoptosis in SW480 colon tumor cells without cyclooxygenase (I or II) inhibition (Thompson et al., 2000b). The analogs of exisulind were developed as inhibitors of cGMP PDEs with a preference for PDE5, 2, and 1 gene families. It is apparently important to maintain cross-reactivity among these isoforms, because highly selective PDE5 inhibitors do not induce apoptosis in tumor cell lines (Thompson et al., 2000b). These drugs inhibit cGMP PDE5 and 2 expressed by SW480 cells and PDE5 expressed by HT29 colon tumor cells (Soh et al., 2000; Thompson et al., 2000b). Because these agents maintained similar rank orders of potency for apoptosis induction, growth inhibition, cGMP PDE5 and 2 inhibition, and also caused sustained intracellular cGMP increases in the colon tumor cells (Thompson et al., 2000b), we proposed a cGMP-
mediated mechanism underlying the actions of exisulind and analogs on apoptosis in neoplastic cells. Soh et al. (2000) found that cGMP mediated apoptosis in SW480 and HT29 cells by mechanisms involving activation of c-Jun NH2-terminal kinase 1 (JNK1). The studies reported here extend our initial finding that exisulind and analogs also activated PKG (Thompson et al., 2000b). Recent studies by Soh et al. (2001) have shown that PKG activates JNK1 via a novel PKG phosphorylation and activation of MEKK1.

The possible involvement of cGMP and PKG in apoptosis is supported by studies in rat myocytes (Wu et al., 1997), pancreatic β-cells (Loweth et al., 1997), and endothelial cells (Suenobu et al., 1999), as well as data showing that transfection of PKG increases the sensitivity of vascular smooth muscle cells to apoptosis inducers (Chiche et al., 1998). Boerth et al. (1997) have shown that PKG also plays a major role in the phenotype and morphology of vascular smooth muscle cells with the involvement of extracellular signal receptor-activated kinase activation with increased proliferation (Komalavilas et al., 1999). To test our hypothesis and further explore cGMP-induced apoptosis, we have used a sensitive solid phase assay to determine PKG activity changes from cell supernatants treated with exisulind and a higher affinity analog, CP461, as well as the GC activators, YC-1 and guanylin. CP461, YC-1, and guanylin increased changes from cell supernatants treated with exisulind and a higher affinity analog, CP461, as well as the GC activators, YC-1 and guanylin. CP461, YC-1, and guanylin increased.

The initial finding that exisulind and analogs also activated PKG phosphorylation and processing, providing a mechanism to circumvent β-catenin accumulation from colon genetic mutations.

**Experimental Procedures**

**Materials.** Cyclic GMP was obtained from ICN (Costa Mesa, CA). 8-Br-cGMP and Rp-8-Br-cGMPs were obtained from BIOMOL (Plymouth Meeting, PA). 8-Br-cGMP was further purified with Sephadex G-25 (Amerham Pharmacia Biotech, Piscataway, NJ) chromatography (Corbin et al., 1988). [3-5'-Hydroxymethyl-2'-furyl]-benzylindazole (YC-1), human guanylin, and forskolin (FSK) were purchased from Alexis Biochemicals (San Diego, CA). E4021 was from Eisai Co., Ltd. (Tokyo, Japan). Exisulind (sulindac sulfone) and CP461 were synthesized by Cell Pathways, Inc. (Horsham, PA). Isopropyl-β-D-thiogalactoside and GSH-Sepharose 4B were from Amersham Pharmacia Biotech. Purified PKG Iα, catalytic subunit of cAMP-dependant protein kinase (PKA, mouse recombinant), GSK-3β (recombinant), and PKA inhibitor PKI (5–24) were obtained from Calbiochem-Novabiochem (San Diego, CA).

**Cell Culture.** SW480, HCT116, HT29, and T84 colon tumor cells were obtained from American Type Culture Collection (Rockville, MD). SW480, HCT116, and HT29 cells were grown in RPMI 1640 media supplemented with 5% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and 0.25 µg/ml amphotericin. T84 cells were grown in 47% Ham’s F-12 media (American Type Culture Collection) and 47% Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO) supplemented with 5% fetal bovine serum, 8.4 mM sodium bicarbonate, 100 units/ml penicillin, 100 units/ml streptomycin, and 0.25 µg/ml amphotericin, pH 7.25. Cells were harvested at 70 to 90% confluence with either trypsin/EDTA or pancreatin and used immediately. Exisulind and CP461 were solubilized in 100% DMSO and diluted with media to obtain a final DMSO concentration of 0.5% or less.

**Cloning and Expression of GST-PDE536–529 for PKG Activity Assay.** RT-PCR methods were used to obtain a domain of PDE536–529 (Val36-Glu529 relative to bovine PDE5) (McAllister-Lucas et al., 1993) from HT-29 cells as a PKG substrate. The forward primer (GTT-AGA-AAA-GGC-ACC-AGA-ATA-G) and the reverse primer (AGC-TCT-CTT-GTT-TCC-TCT-GCT) defined a 1484-base pair fragment containing phosphorylation and high- and low-affinity cGMP binding sites of PDE5 (PDE536–529). The PCR product was cloned into a pGEX-5X-3 GST fusion vector (Amerham Pharmacia Biotech) with EcoRI and XhoI cloning sites. The DNA construct was sequenced by Applied Biosystems (Foster City, CA) model 377 Prism DNA sequencers at the DNA Sequencing and Synthesis Facility at Iowa State University, Ames, IA.

To introduce a Ser92 to Ala mutation, a linker primer, 5′-CTG AAT TTG ACC GGC CTC-3′ was used to connect the fragment domains beside Ser92. Primers (forward, 5′-GAA TTC TCT TAG AAA AGC CAC CAG AGA AAT G-3′; reverse, 5′-AGG CCG GTC AAA TTC AGA GGC AGC GAT TTT CTT G-3′) were used for amplification of the N-terminal domain and primers (forward, 5′-GCC TCT GAA TTA GCG CCG CTT C-3′; reverse, 5′-CTG GAG CTC TCT TGG TCT TTC TCT GTC TG-3′) were used for amplification of the C-terminal domain with pCR2.1 plasmid, encoding the wild-type PDE536–529 domain, as the template. PCR was carried out for 30 cycles and both PCR products were gel-purified. Another PCR was carried out for seven cycles without primers followed by 25 cycles with primers (forward, 5′-GAA TTC TCT TAG AAA AGC CAC CAG AGA AAT G-3′; reverse, 5′-CTG GAC CTC TCT TGG TCT TTC TCT GTC TG-3′). The amplified fragment of 1484 base pairs with the S92A mutation was cloned into pGEX-5X-3 and DNA was purified from midi scale plasmid preparation by using QIAGEN plasmid kit (Valencia, CA) according to the manufacturer’s protocol. The DNA constructs were verified by sequencing performed at the DNA Sequencing and Synthesis Facility at Iowa State University.

The cloned pGEX-5X-3-PDE536–529 (WT) and pGEX-5X-3-PDE536–529 (S92A) plasmids were transfected into BL21 (DE3) bacterial cells. GST-PDE536–529 (WT) and GST-PDE536–529 (S92A) fusion proteins were expressed using 100 µM isopropyl-β-D-thiogalactoside induction at 20°C for 18 h. Cells were sonicated and induced GST-fusion proteins were purified from the supernatant of the bacterial cell extract by binding to a GSH-Sepharose 4B affinity column and eluting with 10 mM reduced GSH in 50 mM Tris-HCl, pH 8.0, according to the manufacturer’s instructions (Amersham Pharmacia Biotech).

**Apparent Km Determination of GST-PDE536–529 Phosphorylation.** The protein phosphorylation assay was performed in 10 mM potassium phosphate buffer, pH 6.8, containing 190 µM [γ32P]ATP (3000 Ci/mmol; PerkinElmer Life Science Products, Boston, MA) and 4.5 mM MgCl2. The phosphorylation reaction was initiated by adding PKG Iα to affinity-purified GST-PDE536–529 in phosphorylation buffer with added cGMP (20 µM) unless indicated otherwise. Incubation was at 30°C and the reaction was terminated by spotting 50 µl of reaction mixture onto Whatman P-81 phosphocellulose paper (Roskoski, 1983). After four washes with 75 mM phosphoric acid, the paper was air-dried and counted on a Beckman Coulter LS 5800 liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA). The phosphate incorporation of GST-PDE536–529 was also analyzed by SDS-PAGE followed by phosphor imaging (Cyclone; Packard, Meriden, CT).

**Cell PKG Activity Assay.** SW480, HCT116, HT29, and T84 colon tumor cells were treated with compounds or DMSO (0.5%) by using culture conditions as described above. Cells (~1 × 105) were washed with cold PBS and lysed with cold modified RIPA buffer (400 µl).
containing 50 mM Tris-HCl, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, 500 μM 3-isobutyl-1-methylxanthine, and Complete protease inhibitor cocktail (Roche Molecular Biochemicals, Palo Alto, CA). Cell supernatants were obtained by centrifugation of the cell lysates at 14,000 rpm with an Eppendorf model 5417C for 15 min at 4°C. Protein was quantitated by Bio-Rad DC protein assay (Heracles, CA). PKG activities from cell supernatants were measured using, as a phosphate acceptor, the fusion protein fragment of PDE5 (GST-PDE536–529) bound to GSH-Sepharose affinity beads. Cell supernatant (100 μg), substrate (20 μg of bound protein), 0.5 μM PKI, 4.5 mM Mg2+, and [γ-32P]ATP (10 μCi, 190 μM) were mixed with or without added cGMP and incubated at 30°C for 30 min. The phosphorylated PDE5 fragment on beads (∼85 kDa) was resolved on 7.5% SDS-PAGE and [32P] incorporation quantitated (digital light units) by using a phosphor imaging system (Cyclone; Packard). Exposure times were optimized to maintain a linear range. In addition digitized images in the figures are normalized to no drug treatment (fold versus no drug).

Western Blot. Total protein per sample (50 μg) of cell supernatant prepared as described above was loaded onto 10% NuPAGE Bis-Tris gels (Novex, San Diego, CA) and transferred. Western blots were probed with affinity-purified rabbit polyclonal antibody (PKO02; Stressgen Biotechnologies, Victoria, BC, Canada) specific for human PKG Iβ and detecting an 80-kDa band. Another affinity-purified peptide antibody (PKO05; Stressgen Biotechnologies) detecting both human PKG Iα (75-kDa) and Iβ (80-kDa) isoforms has been studied. Bound antibody was identified with the corresponding horseradish peroxidase-conjugated anti-IgG secondary antibodies by using BM blue substrate (Roche Molecular Biochemicals). The results were analyzed using an AlphaImager 2000 and software (Alpha Innotech Corporation, San Leandro, CA).

Radioimmunoassay for cGMP and cAMP. Approximately 5 × 10^6 cells were used for each assay. Cells were plated on 100-mm dishes and drugs were added after 2 days of growth at specified times and doses. This was followed by a rapid wash, 0.2 N HCl/50% methanol extraction (1 ml), and drying. The dried samples were reconstituted in water and acetylated before radioimmunoassay with anti-cGMP and anti-cAMP antibodies (Brooker et al., 1979).

Cell Growth Inhibition and DNA Fragmentation. Cell growth inhibition was determined by plating cells at 1900 cells/well in 96-well plates. Cells were dosed after 24 h and incubated for six more days. Cells were fixed with 50% trichloracetic acid at 4°C for 1 h, rinsed five times with deionized H2O, and incubated for 10 min with 0.4% sulforhodamine B in 1% acetic acid. Plates were rinsed four times with 1% acetic acid, dried 30 min, and solubilized in 10 mM Tris. Absorbance was determined at 540 nM by using a Molecular Devices Spectra Max 340 plate reader. For apoptosis assay, cells were seeded at 10,000 cells/well in 96-well plates. After 24 h, cells were dosed and grown for an additional 48 h. DNA fragmentation was measured using a double antibody ELISA kit (Roche Molecular Biochemicals) that detects histone protein and fragmented DNA.

β-Catenin Phosphorylation in Vivo and in Intact Cells. SW480 cells were lysed using modified RIPA buffer with protease inhibitors and cell supernatants were obtained as described above. From 500 μg of supernatant, β-catenin was immunoprecipitated using 4 μg of rabbit anti-β-catenin IgG for 2 h at 4°C followed by an additional overnight incubation with 100 μl of protein A-agarose beads. The immunoprecipitates were washed three times with cell lysis buffer and one time with kinase assay buffer. Human β-catenin (2–698) (Hulsken et al., 1994) was expressed as a GST fusion protein in Escherichia coli (BL21) by using β-catenin cDNA cloned by RT-PCR from SW480 cells and purified by GSH-Sepharose 4B affinity chromatography.

To label β-catenin in vitro either β-catenin immunoprecipitates (20 μl) or recombinant GST-β-catenin (1 μg) was phosphorylated with PKG Iα in buffer containing 4.5 mM Mg2+ and [γ-32P]ATP (10 μCi, 190 μM) at 30°C for 30 min. Phosphorylated β-catenin was resolved on 7.5% SDS-PAGE and quantitated by phosphor imaging (Cyclone; Packard).

To label β-catenin in intact cells SW480 cells were plated for 24 h in phosphate-free media and treated with 0.2% DMSO or exisulind (500 μM) in phosphate-free media containing [32P]orthophosphate (1 mCi/10 ml, 9000 Ci/mmol; PerkinElmer Life Science Products) for 18 h. Labeled cells were washed three times with cold PBS, lysed using modified RIPA buffer, and immunoprecipitated with anti-β-catenin IgG-coated protein A-agarose beads. Western blots using anti-β-catenin IgG and second antibody detection were used to calibrate the amount of β-catenin in each lane and to ensure the same amount of β-catenin was loaded from both DMSO control and exisulind-treated cells. [32P]β-catenin was resolved on 7.5% SDS-PAGE and quantitated by phosphor imaging.

Results

Solid Phase PKG Activity Assay. The solid phase substrate used to determine PKG activity was GST-PDE536–529 bound as a GSH conjugate to Sepharose 4B beads (Fig. 1A). PKI, a specific inhibitor of PKA, was added to the assay mixture to block phosphorylation by PKA in cell lysates. As shown in Fig. 1B, 0.5 μM PKI completely blocked PKA phosphorylation of GST-PDE536–529 without influencing PKG phosphorylation. Phosphorylation of GST-PDE536–529 by SW480 cell supernatants was time- and cGMP-dependent (Fig. 1, C and D). The assay was linear for up to 60 min and 100 μM cGMP activated PKG maximally. Rp-8-Br-cGMP, a PKG-specific inhibitor, blocked the phosphorylation of GST-PDE536–529 by SW480 cell supernatants (Fig. 1E). The apparent K_m for PKG Iα phosphorylation of GST-PDE536–529 was 3 μM (Fig. 2A), indicating a higher affinity than BP-DEtide at 68 μM (Colbran et al., 1992) and suggesting that GST-PDE536–529 is an improved substrate over BPDEtide for PKG. PKG phosphorylation showed 0.9 moles of phosphate incorporated per mole of GST-PDE536–529 protein after 1 h (Fig. 2B), data consistent with the phosphorylation of bovine PDE5 at Ser92 (Thomas et al., 1990, Colbran et al., 1992). Moreover, mutation of Ser92 to Ala (S92A) in GST-PDE5 at Ser92 (Thomas et al., 1990; Colbran et al., 1992).

Exisulind Increases PKG Activity in Tumor Cells. SW480 cells were treated with various concentrations of exisulind for 40 min and PKG activities determined using the solid phase assay. Treatment with exisulind showed a concentration-dependent increase in PKG activity both in the absence or presence of cGMP in the assay (Fig. 3A). E4021, a drug that did not sustain cGMP increases, inhibit cell growth, or induce apoptosis (Thompson et al., 2000b), had no effect on PKG activity in SW480 cells. The doses of exisulind that activate PKG (200–600 μM) are consistent with concentrations needed to induce apoptosis or inhibit cell growth (Thompson et al., 2000b).

PKG activation could be detected as early as 5 min after 400 μM exisulind exposure to SW480 cells (Fig. 3B). Supernatants from treated cells showed increased PKG enzyme activity throughout the duration of the 24-h drug treatment. At 24 h the increased basal activity showed a much higher fold increase with slightly less fold in vitro stimulation by
cGMP in the assay, suggesting increased enzyme protein in the assay and substrate limitation. At 48 h of exisulind treatment, PKG activity continued to increase (Fig. 3C), but the lower dose of drug was more effective compared with the no-drug control than seen at 40-min activation, also suggesting increased enzyme protein.

Exisulind-Induced PKG Protein Expression. PKG activity increases were studied further in SW480 cells treated with exisulind by using antibody specific for the human Iβ/H9252 isoform (Fig. 4). Induction of PKG Iβ/H9252 protein was detectable at 8 h after exisulind (600 μM) treatment. Exisulind continued to increase PKG expression in the cells attached to the plates for up to 72 h (Fig. 4A). PKG Iβ/H9252 protein induction by exisulind was dose-dependent (Fig. 4B). Consistent with a lack of effect on cGMP accumulation, the kinase was not induced by treatment with E4021 treatment. A different affinity-purified peptide antibody detecting both human PKG Iα/H9251 and Iβ/H9252 isoforms showed Iβ 80-kDa immunoreactivity but no band at PKG Iα 75 kDa by Western blot (data not shown).

RT-PCR with published primers (Selvaraj et al., 2000) detected PKG II mRNA in these colon tumor cells (data not shown). PKG II isoforms have not been studied further due to lack of commercially available antibody.

Lack of Direct Effect of Exisulind or CP461 on PKG Activity. To determine whether exisulind or CP461 had a direct effect on PKG to activate the enzyme and therefore, with exisulind by using antibody specific for the human Iβ isoform (Fig. 4). Induction of PKG Iβ protein was detectable at 8 h after exisulind (600 μM) treatment. Exisulind continued to increase PKG expression in the cells attached to the plates for up to 72 h (Fig. 4A). PKG Iβ protein induction by exisulind was dose-dependent (Fig. 4B). Consistent with a lack of effect on cGMP accumulation, the kinase was not induced by treatment with E4021 treatment. A different affinity-purified peptide antibody detecting both human PKG Iα and Iβ isoforms showed Iβ 80-kDa immunoreactivity but no band at PKG Iα 75 kDa by Western blot (data not shown).

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Lack of Direct Effect of Exisulind or CP461 on PKG Activity. To determine whether exisulind or CP461 had a direct effect on PKG to activate the enzyme and therefore,
would not require an intact cell for activation, we studied PKG activation in vitro. No direct activating effects were seen when exisulind or CP461 was added to the phosphorylation reaction mixtures (Fig. 5). Neither exisulind nor CP461 was effective with recombinant PKG I/H9251 added to solid phase substrate (Fig. 5A). PKG in untreated SW480 cell supernatants was not directly activated by either drug after the cells were lysed (Fig. 5B). Furthermore, PKG in supernatants from cells treated with exisulind for 48 h was not activated by adding exisulind or CP461 directly to the assay (Fig. 5C). To ensure enzyme integrity cGMP (10 M) and 8-Br-cGMP (10 M) were added to activate PKG in the same phosphorylation reaction mixtures (Fig. 5, A and C).

### PKG Activation and Induction by Exisulind in Multiple Colon Tumor Cell Lines
Exisulind also increased PKG activity in HT29, HCT116, and T84 colon tumor cells (Fig. 6A). Although the basal activity varied from cell line to cell line, PKG activity was increased at 1-h drug treatment. After 48-h drug treatment, all four colon tumor cell lines showed PKG Iβ induction by Western blot analysis (Fig. 6B).

### CP461 and YC-1 Activation of PKG in SW480
CP461 inhibits cGMP PDE isoforms with more selectivity than exisulind and shows higher affinity for PDE5 and PDE2 than exisulind (IC50 = 3 and 14 μM for CP461, respectively, and 114 and 335 μM for exisulind, respectively). Because YC-1 and CP461 have been shown to increase cGMP, inhibit cell growth, and induce apoptosis in SW480 cells (Thompson et al., 2000b), we studied PKG activation by these agents.
CP461 and YC-1 increased PKG activity in SW480 cells after 40-min and 24-h treatments (Fig. 7, A and B) and induced more PKG I/\(\beta\) protein after 24-h drug treatments (Fig. 7C). FSK (10 \(\mu\)M) increased cellular cAMP 32-fold in SW480 cells, but did not increase cellular cGMP, inhibit cell growth, induce apoptosis, or activate PKG after 24-h treatment (Fig. 7B). Exisulind-, CP461-, and YC-1-induced PKG activity remained sensitive to activation by 100 \(\mu\)M cGMP in the assay.

**Guanylin Increased cGMP and Induced Apoptosis in T84 Cells.** T84 colon tumor cells express membrane-bound GC-C (Singh et al., 1991). Guanylin is a 15-amino acid peptide homolog of bacterial heat-stable enterotoxin and an endogenous activator of GC-C. When T84 colon tumor cells were treated with 200 nM guanylin for 40 min, cellular cGMP was increased 3.6-fold (Fig. 8A). Exisulind (400 \(\mu\)M) increased cGMP in T84 cells at 40 min and an additive accumulation of cGMP was detected in T84 cells treated with guanylin in combination with exisulind (Fig. 8A). Consistent with cGMP increases, guanylin and exisulind also induced apoptosis in T84 cells and showed an additive effect on apoptosis in combination at 48-h treatment (Fig. 8B). Guanylin (200 nM) also increased PKG activity 2-fold in T84 cells after 1-h incubation (data not shown).

**\(\beta\)-Catenin Phosphorylation by PKG.** Because we have postulated that \(\beta\)-catenin is a downstream target of PKG activated by exisulind or CP461 that could mediate regulation of apoptosis pathways (Thompson et al., 2000b), \(\beta\)-catenin phosphorylation was investigated further. PKG I\(\beta\) phosphorylated \(\beta\)-catenin immunoprecipitated from SW480 cells (Fig. 9A) or purified GST-\(\beta\)-catenin (Fig. 9B). GSK-3\(\beta\), known to require \(\beta\)-catenin complexed to APC and axin proteins to phosphorylate \(\beta\)-catenin, was not effective in the immunoprecipitates (Fig. 9A). PKG from SW480 cells activated by exisulind for 48 h increased GST-\(\beta\)-catenin phosphorylation (Fig. 9C), indicating the induced PKG also showed higher specific activity with \(\beta\)-catenin substrate.

SW480 cells incubated with \(^{32}\)P in the media showed increased phosphorylation of \(\beta\)-catenin when treated with 500 \(\mu\)M exisulind (Fig. 10). \(\beta\)-Catenins from the immunoprecipitates of control and treated cells were identified by Western blot with anti-\(\beta\)-catenin antibody. Phosphorylated \(\beta\)-catenins were quantitated by phosphor imaging. A 3-fold increase in \(\beta\)-catenin phosphorylation was determined in the immunoprecipitates from drug-treated cells by using equivalent proteins.

**Summary Correlations of Exisulind and CP461 on Colon Cell cGMP and Apoptosis.** Table 1 shows a summary of data published previously and the current study on.
colon tumor cell lines with exisulind and CP461 effects on cGMP-mediated apoptosis. Two of the cell lines show major cGMP PDE isoform expressions of PDE5 and PDE2 and two of the lines express only PDE5. Inhibition constants for fractionated isoforms correlate with growth inhibition and apoptosis determined by DNA fragmentation ELISA assays. Data shown are representative of three separate experiments. *P < 0.05 compared with control group, **P < 0.05 compared with either guanylin or exisulind treatment alone; two-tailed, unpaired Student’s t test.

Discussion

These studies support the proposed involvement of cGMP and PKG in exisulind- and CP461-mediated apoptosis in colon tumor cells (Thompson et al., 2000b; Soh et al., 2000, 2001). Although cGMP is a mediator of signal transduction with intracellular targets, including several cGMP PDEs, cGMP-gated cation channels, and PKG, Wu et al. (1997) have suggested a possible role for PKG and cGMP in smooth muscle cell apoptosis in addition to the more well studied platelet aggregation and smooth muscle relaxation events. Exisulind and CP461 caused PKG activation in SW480, HT-29, HCT116, and T84 colon tumor cell lines at concentrations that correlated with cGMP PDE inhibition constants, cGMP increases, and apoptosis induction and cell growth inhibition. Exisulind administration to patients with familial adenomatous polyposis induced apoptosis in dysplastic epithelial but not normal-appearing colonic mucosa, thus leading to the
ration of this manuscript, Pitari et al. (2001) showed that cancer inhibited the formation of polyps. During the preparation within 5 to 10%.

**Summary of exisulind and CP461 in colon tumor cells**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>cG-PDE</th>
<th>cG-PDE IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>CGMP 1, 24, 72 h</th>
<th>PKG Activity</th>
<th>Cell Growth IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Apoptosis EC&lt;sub&gt;50&lt;/sub&gt;</th>
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<tr>
<td>SW480</td>
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<td>1.1</td>
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<tr>
<td>T84</td>
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<td>*</td>
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<tr>
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<td>PDE5</td>
<td>113</td>
<td>3.5</td>
<td>↑</td>
<td>165</td>
<td>0.7</td>
</tr>
<tr>
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<td>198,133</td>
<td>8.13</td>
<td>↑</td>
<td>170</td>
<td>2</td>
</tr>
</tbody>
</table>

Exi, exisulind; N.D., not determined.

Exisulind and CP461 appear to increase cellular cGMP, and not cAMP, by inhibiting cGMP PDEs for which they show a PDE5 preference, but not specificity, because in vitro both drugs inhibit PDE2 and 1 gene family isoforms in addition to PDE5. The colon tumor cell lines tested all prominently expressed PDE5 with two of the lines showing PDE2. Analogs of exisulind are proapoptotic drugs developed from screening for inhibitors of PDE5 and 2 while maintaining apoptosis and growth-inhibiting activity. It is not clear what isoforms of PDE are effected by exisulind and analogs when cGMP is increased in colon tumor cells; however, inhibitors lacking the chemical structure to inhibit both isoforms in vitro lose apoptosis-inducing properties. Thus, as shown previously, very selective PDE5 inhibitors in vitro, such as E4021, are not proapoptotic drugs and these agents do not sustain cGMP increases in intact cells or activate PKG in colon tumor cells. We have not found PDE 1, 9, 10, or 11 isoforms in these cell lines. PDE5, and to a lesser extent PDE2, show enhanced immunoreactivity in biopsies of human colon adenomas and adenocarcinomas, suggesting a role for these enzymes in colon cell survival (Piazza et al., 2000). As discussed elsewhere (Thompson et al., 2000b), the less selective PDE5 inhibitors, such as SAANDs, may be more effective as antineoplastic agents because of the multiple inductive effects of PDE inhibitors on alternative isoforms that could circumvent selective inhibitor actions.

Although the mechanism of PKG activation in intact colon tumor cells is under investigation, in vitro activation models show that activity of PKG Iα and β isoforms is increased by cGMP binding and autophosphorylation (Hofmann et al., 1985; Francis et al., 1996; Smith et al., 1996; Chu et al., 1998). These studies showed that cGMP binding stimulated basal kinase activity and increased the sensitivity of the enzyme to cGMP. Autophosphorylation as a result of increased cGMP has been shown to prolong increased kinase...
activity after cellular cGMP declines. Because exisulind and CP461 cause sustained cGMP increases, cGMP and auto-
phosphorylation could both provide mechanisms to sustain an increased activation of the enzyme by the drugs until
protein induction at approximately 8 h of drug treatment.

Our homogenization conditions apparently preserve the active state of PKG Iβ induced by exisulind or CP461. No
evidence for PKG Iα induction by exisulind treatment was seen in SW480 cells. SAANs are the first class of drugs
shown to induce the synthesis of PKG. Because most cultured cells appear to adapt to little or no PKG expression (Cornwell
et al., 1994), regulation of PKG protein synthesis needs further study to determine the role of cGMP and PKG in the
induction mechanism, if any. The concentrations of exisulind and CP461 required for PKG induction were consistent with
cGMP PDE inhibition and PKG activating concentrations. E4021, did not induce PKG consistent with its lack of PKG
activation, further suggesting that the sustained effects of exisulind and CP461 are important for apoptosis induction.

Exisulind and analogs decrease β-catenin levels in SW480 cells, suggesting a mechanism to regulate apoptosis (Thom-
son et al., 2000b). Cytosolic and nuclear β-catenin accumulations occur in a variety of tumors, including SW480,
HCT116, HT29, and T84 colon tumor cells due to mutations in the protein and defective phosphorylation from APC
mutations (Morin et al., 1997; Efstatichou et al., 1998). Based on in vitro phosphorylation data, we have proposed previously that β-catenin might be a downstream target of PKG (Thompson et al., 2000b). Ubiquitination, and thus turnover of β-catenin via proteosomal processing, requires phosphorylation of the protein. The results of the current studies show that purified PKG phosphorylated immunoprecipitated β-catenin from SW480 cells, as well as recombinant β-catenin
expressed as a GST fusion protein. Furthermore, cell supernatants activated by exisulind treatment of SW480 cells
for 48 h showed increased phosphorylation of β-catenin compared with untreated cell supernatant phosphorylation.

In intact cells prelabeled with 32P, exisulind treatment resulted in a 3-fold increase in β-catenin phosphorylation. These data indicate that PKG activation by exisulind results in β-catenin phosphorylation to initiate degradation by the ubiquitin-proteosomal system. The PKG phosphorylation site(s) on β-catenin, a protein that contains multiple potential sites, is under investigation. Reduced β-catenin in SW480 cells would provide a mechanism leading to enhanced apoptosis by exisulind and other SAANs. Activation of the proapoptotic Jun kinase by exisulind and analogs through PKG activation and phosphorylation of MEKK1 (Soh et al., 2000, 2001) would be expected to complement β-catenin re-
duction to provide antiapoptotic cell killing by SAANs.

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