Vasodilator-stimulated Phosphoprotein (VASP) Phosphorylation Provides a Biomarker for the Action of Exisulind and Related Agents That Activate Protein Kinase G[1]

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Abstract

Recent studies provide evidence that exisulind and two potent derivatives, CP461 and CP248, induce apoptosis in colon cancer cells by inhibiting cyclic GMP (cGMP)-specific phosphodiesterases (phosphodiesterases 2 and 5). This causes an increase in intracellular levels of cGMP, thus activating the cGMP-dependent protein kinase G (PKG), which then activates pathways that lead to apoptosis. To further examine this mechanism and to provide a potential *in vivo* biomarker for activation of this pathway, we examined phosphorylation of the vasodilator-stimulated phosphoprotein (VASP), a ubiquitously expressed endogenous substrate for PKG. We found that VASP was phosphorylated after treating SW480 colon cancer cells with exisulind, CP461, or CP248. CP248-induced VASP phosphorylation was inhibited by a specific PKG inhibitor but not by a protein kinase A inhibitor. The drug 3-(5′-hydroxymethyl-2′-furyl)-benzylindazole and nitric oxide donors that activate cellular guanylyl cyclase and thus increase cellular levels of cGMP also caused VASP phosphorylation. With all of these agents, the phosphorylation of VASP was associated with increased intracellular levels of cGMP and the induction of apoptosis. We also demonstrated direct *in vivo* phosphorylation of VASP with constitutively activated mutants of PKG. These results suggest that VASP phosphorylation can provide a useful endogenous cellular biomarker for anticancer agents that cause cGMP-mediated apoptosis.

Introduction

cGMP[3] is an important second messenger that mediates the action of several hormones, neurotransmitters, and drugs (1). It thereby regulates various physiological functions including neurotransmission, platelet aggregation, and smooth muscle tone (2). There is also increasing evidence that it can play an important role in cellular proliferation, differentiation, and apoptosis (3–5). The intracellular level of cGMP is regulated through a dynamic balance between its rate of synthesis by GCs and its degradation by specific PDEs, especially PDEs 2 and 5 (6). cGMP has several intracellular targets. Thus, it can bind to specific PDEs, thereby activating or inhibiting their activities (7); it can bind to and activate cGMP-gated cation channels; it can bind to and activate PKG; and, under certain conditions, it can bind to and activate PKA (8).

The activation of PKG by cGMP has recently become of considerable interest as a novel molecular mechanism for the induction of apoptosis. Activation of the cGMP/PKG pathway has been implicated as a cause of apoptosis in cardiomyocytoes (3) and in pancreatic B cells (4). Recent studies indicate that exisulind, which is a metabolite of the nonsteroidal anti-inflammatory drug, and two potent derivatives of exisulind, CP248 and CP461, specifically inhibit the cGMP-specific PDEs 2 and 5. Evidence has also been obtained that the resulting increase in cellular levels of cGMP in human colon cancer cells leads to activation of PKG and thereby to the induction of apoptosis (9). The precise pathway by which PKG activation leads to apoptosis is not known, although it appears to involve both a decrease in cellular levels of β-catenin and the activation of JNK1 (9, 10). These novel effects of exisulind and related drugs may explain why these compounds exert anticancer effects in a variety of biological syntheses, even though, in contrast to conventional nonsteroidal anti-inflammatory drugs, they do not inhibit COX activity (11).

In view of the above findings, it is of interest that colon cancer cells have relatively high levels of PDE 5 when compared with normal colonic mucosa (12). In addition, the polypeptide GC activators guanylin and uroguanylin are expressed at reduced levels in colon adenoma and adenocarcinoma when compared with normal mucosa (13). Taken together, these findings suggest that cGMP-mediated path-

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[3] The abbreviations used are: cGMP, cyclic GMP; PDE, phosphodiesterase, PKG, protein kinase G; PKA, protein kinase A; YC-1; 3-(5′-hydroxymethyl-2′-furyl)-benzylindazole; JNK1, c-Jun NH_2-terminal kinase 1; VASP, vasodilator-stimulated phosphoprotein; GC, guanylyl cyclase; 4MQ, 4-{3′,4′-(methylenedioxy)-benzylamino}-6-methoxyquinazoline; HA, hemagglutinin; PE, phycoerythrin; GST, glutathione *S*-transferase; COX, cyclooxygenase; NO, nitric oxide.

ways are suppressed in colon cancer cells, presumably to inhibit the process of apoptosis. Colon cancer cells and other types of cancer cells may therefore be preferentially sensitive to drugs or other factors that increase cellular levels of cGMP and thereby activate PKG.

Two major forms of PKG have been identified in mammalian cells, PKG I and PKG II. In addition, there are two splice variants of PKG I, which are designated Iα and Iβ (2, 14). PKG I is expressed in many cell types and at especially high concentrations in platelets and endothelial cells. One of the major substrates for PKG I is the protein VASP. VASP was originally discovered as a substrate for both PKA and PKG in human platelets (15), but it is ubiquitously expressed in many types of cells, including vascular smooth muscle and endothelial cells. Furthermore, VASP binds to actin and is associated with microfilaments, highly dynamic membrane regions, and focal adhesions (16). In platelets, VASP phosphorylation correlates with vasodilator-induced inhibition of platelet activation and aggregation and with inhibition of fibrinogen receptor activation (17). In VASP knockout mice, cGMP-mediated inhibition of platelet aggregation is reduced, and agonist-induced platelet activation is enhanced (18). PKG I knockout mice also display defective cGMP-mediated inhibition of platelet aggregation (19). Three phosphorylation sites have been identified in VASP (serine 157, serine 239, and threonine 278). All three sites can be phosphorylated by PKA or PKG, although serine 239 is preferentially phosphorylated by PKG (20). Phosphorylation at serine 157 causes a marked shift in the apparent molecular mass of VASP from 46 to 50 kDa when analyzed by SDS-PAGE, thus providing a simple method for detecting the *in vivo* phosphorylation of VASP by PKG or PKA (21).

The purpose of the present study was to further explore the effects of several PDE inhibitors and GC activators in human colon cancer cells with respect to their effects on cellular levels of cGMP and the induction of apoptosis. An additional goal was to determine the effects of these compounds on the *in vivo* phosphorylation of the VASP protein in colon cancer cells because VASP phosphorylation might provide a convenient biomarker of PKG activation in tumor cells obtained from patients undergoing treatment with exisulind or related compounds. As part of this analysis, we also constructed constitutively activated mutants of PKG Iα and PKG Iβ to directly examine the effects of PKG on VASP phosphorylation in colon cancer cells.

Materials and Methods

Materials and Cell Culture. Exisulind, sulindac sulfide, CP248, and CP461 were supplied by Cell Pathways, Inc. YC-1 was purchased from Alexis Biochemicals (San Diego, CA). NOR3, NOC18, 4MQ, Rp-8-pCPT-cGMPs, PKA inhibitor 5-24, PKA inhibitor 14-22 amide (cell permeable, myristoylated, designated PKI), and an anti-PKG Iβ antibody were purchased from Calbiochem (La Jolla, CA). The anti-VASP antibody was purchased from BD Transduction Laboratories (San Diego, CA). The anti-phospho-Ser239-VASP antibody was purchased from nanoTools (Teningen, Germany). The anti-HA antibody was purchased from Covance (Richmond, CA).

SW480 and COS7 cells were cultured in DMEM with 10% fetal bovine serum.

Assays for Intracellular Levels of cGMP. SW480 cells were plated at 1×10^6 cells/10-cm dish, and on the following day, the test chemicals were added. After 1 h, the cells were lysed, and the cell extracts were assayed for cGMP using an enzyme immunoassay according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ). The results were expressed as fmol cGMP generated/mg cell protein.

Assays for Apoptosis Using Annexin V Staining. SW480 cells were treated with the indicated compounds for 48 h. The harvested cells were then stained with PE-conjugated annexin V (PharMingen, San Diego, CA) for 15 min in the dark, according to the manufacturer's protocol. The stained cells were then analyzed on a FACSCalibur instrument using CELLQuest software (Becton Dickinson, Mountain View, CA). The extent of apoptosis was expressed as a percentage by calculating the number of cells that were annexin-V-PE positive divided by the total number of cells examined.

Western Blot Analysis. SW480 cells were harvested and then sonicated in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 25% glycerol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM NaF, 0.1 mM Na_3VO_4, 10 mM β-glycerophosphate, and 1 mM phenylmethylsulfonyl fluoride]. The lysates were electrophoresed on a 10% polyacrylamide gel and then electophoretically transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% dry milk and incubated with the indicated antibody. After washing, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech). Protein bands were visualized with the enhanced chemiluminescence Western blotting system (Amersham Pharmacia Biotech).

Construction of PKG Mutants. Wild-type cDNAs for PKG Iα and PKG Iβ were kindly provided by Dr. Sharron H. Francis, and constitutively active and dominant negative mutants of both of these forms of PKG were constructed, following procedures similar to those previously used by Collins *et al.* (22) and Gudi *et al.* (23). The related structures are shown in Fig. 3. Appropriate PCR primers were used to generate the point mutants PKGIαS65D, IαK390R, IβS80D, and IβK405R and the NH_2-terminal truncated mutants PKGI$\alpha\Delta$ and PKGI$\beta\Delta$. These mutant forms of PKG Iα and PKG Iβ were then subcloned into the expression vector pHACE, which has a COOH-terminal HA tag and a cytomegalovirus promoter, as described previously (24).

PKG Kinase Assays. COS7 cells were plated at 1×10^6 cells/10-cm plate, and 24 h later, they were transfected with 10 μg of DNA of the indicated PKG expression vector using Lipofectin (Invitrogen, Carlsbad, CA). After 18 h, the cells were harvested and sonicated in KPEM buffer [10 mM potassium phosphate (pH 6.8), 1 mM EDTA, 25 mM 2-mercaptoethanol, 150 mM NaCl, 12% sucrose, 1 μg/ml pepstatin A, 10 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride]. The supernatant fraction obtained after

centrifugation at 10,000 × *g* for 10 min was used as the enzyme source. Then, 20 μg of this protein extract were assayed for PKG activity using as the substrate a GST fusion protein fragment of human VASP in PKG assay buffer [20 mM Tris-HCl (pH 7.5), 20 mM MgAc, 10 mM DTT, 10 mM NaF, 500 μM 3-isobutyl-1-methylxanthine, 200 μM ATP, 20 μM PKA inhibitor 5-24, and 11 nM [γ-^{32}P]ATP]. The VASP fragment contains residues 158 to 277, which includes the PKG-preferred phosphorylation site, *i.e.,* Ser-239. Assay mixtures were incubated for 10 min at 30°C, the assays were terminated by the addition of SDS sample buffer, and the mixtures were boiled for 5 min. The reaction products were then analyzed by SDS-PAGE and autoradiography.

Results

Effects of cGMP-PDE Inhibitors and GC Activators on Intracellular cGMP Levels and Induction of Apoptosis in SW480 Cells. We first examined the effects of several compounds on intracellular levels of cGMP in the SW480 human colon cancer cell line, using cGMP enzyme immunoassays (see "Materials and Methods"). Sulindac sulfide is known to have at least two types of cellular targets, the cGMP PDEs 2 and 5 and the COXs COX1 and COX2 (25).[4] Sulindac sulfone (exisulind) and two potent exisulind derivatives, CP248 and CP461, also inhibit PDEs 2 and 5 but do not inhibit COX1 and COX2 (9). As shown in Fig. 1*A,* sulindac sulfide, exisulind, CP248, CP461, and the PDE inhibitor 4MQ (26) caused, within 1 h, a dose-dependent increase in intracellular levels of cGMP. We also examined the effects of the GC activator YC-1 (27) and NO donors NOR3 and NOC18 because NO is a known activator of GC (28). These three compounds also showed a dose-dependent increase in intracellular levels of cGMP.

We then examined whether concentrations of each of the above-mentioned compounds that increased intracellular levels of cGMP also induced apoptosis in SW480 cells. The cells were treated with these concentrations of each compound (see the Fig. 1*B* legend) for 48 h and then examined for apoptosis by annexin V-PE staining. Indeed, we found that under these conditions, sulindac sulfide, exisulind, CP248, CP461, 4MQ, YC-1, NOR3, and NOC18 caused about a 4- to 6-fold increase in apoptosis (Fig. 1*B*).

Effects of cGMP Inhibitors and GC Activators on Cellular Levels of PKG Iβ and VASP Phosphorylation. As discussed in the "Introduction," an increase in intracellular levels of cGMP can lead to the activation of PKG, and PKG activation has been implicated in the induction of apoptosis in human colon cancer cells. Thompson *et al.* (9) have reported that when SW480 cells were treated with exisulind, there was not only activation of PKG but also an increase in the amount of the PKG Iβ protein. Fig. 2*A* indicates that treatment of SW480 cells with the PDE inhibitors CP248, CP461, exisulind, and 4MQ for 24 h caused about a 2- to 3-fold increase in cellular levels of PKG Iβ. An increase was also seen with the GC activators YC-1, NOR3, and NOC18,

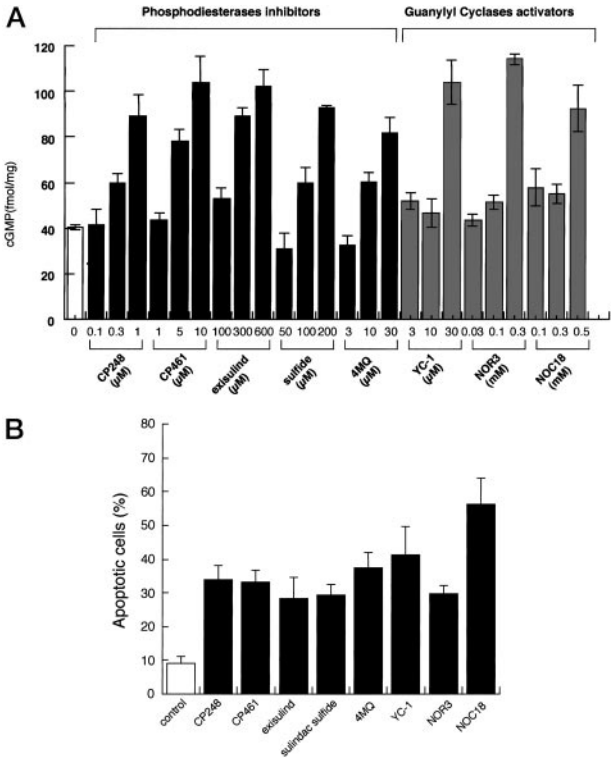

Fig. 1. Effects of PDE inhibitors and GC activators on cellular levels of cGMP and apoptosis in SW480 cells. *A,* intracellular levels of cGMP. Cells (1×10^6) were treated with the indicated compounds for 1 h. cGMP levels were then determined by enzyme immunoassays, as described in "Materials and Methods." *B,* induction of apoptosis. Cells (1×10^6) were treated with 1 μM CP248, 10 μM CP461, 600 μM exisulind, 30 μM 4MQ, 30 μM YC-1, 0.3 mM NOR3, and 0.5 mM NOC18 for 48 h, and the percentage of apoptotic cells was determined by annexin V-PE staining and flow cytometry, as described in "Materials and Methods."

but this increase was less than 2-fold. The reason for this smaller effect with the GC activators is not apparent because the latter compounds were very effective in increasing cellular levels of cGMP (Fig. 1*A*) and causing VASP phosphorylation (Fig. 2*A*). A time course study with CP248 (Fig. 2*B*) confirmed the increase in PKG Iβ and also indicated that this occurred at early time points (15 min and 30 min), with a secondary increase at 24 h.

The VASP protein, which is ubiquitously expressed in various cell types, is known to be a major substrate for PKG in certain cell types (15, 21). Therefore, it was of interest to determine whether the concentrations of the compounds described in Fig. 1*A* that induced increased cellular levels of cGMP and induced apoptosis (Fig. 1*B*) also caused the phosphorylation of VASP in SW480 cells. *In vivo* phosphorylation of VASP can be readily detected because when serine 157 of the VASP protein is phosphorylated, the protein displays a mobility shift from 46 to 50 kDa when analyzed by SDS-PAGE (21). Fig. 2*A* demonstrates that when SW480 cells were treated with CP248 (1 μM), CP461 (10 μM), exisulind (600 μM), 4MQ (30 μM), YC-1 (30 μM), NOR3 (0.3 mM), or NOC18 (0.5 mM) for 24 h, there was a marked increase in VASP phosphorylation.

[4] W. J. Thompson *et al*., unpublished observations.

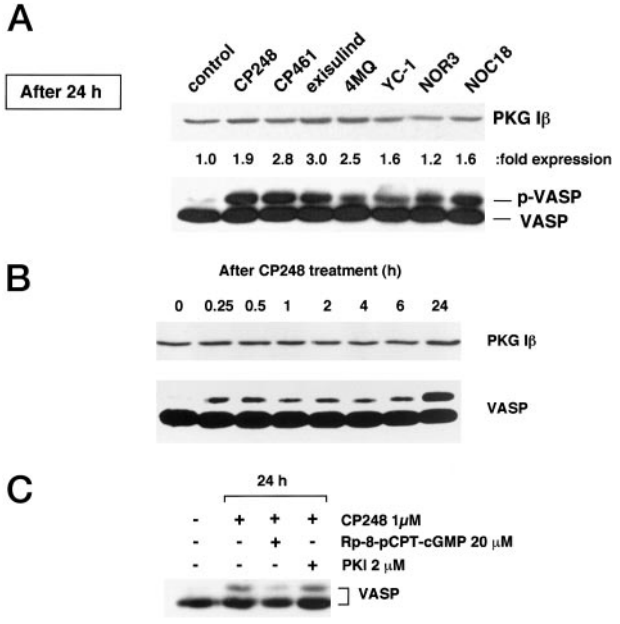

Fig. 2. Effects of PDE inhibitors and GC activators on VASP and PKG Iβ. *A,* SW480 cells were treated with 1 μM CP248, 10 μM CP461, 600 μM exisulind, 30 μM 4MQ, 30 μM YC-1, 0.3 mM NOR3, and 0.5 mM NOC18. After 24 h, the cells were examined by Western blot analysis with an anti-PKG Iβ or anti-VASP antibody. Fold expression was analyzed with NIH Image. *B,* time course study of the effects of CP248 on PKG Iβ and VASP. Cells were treated with 1 μM CP248 for the indicated times, and extracts were examined by Western blot analysis using the respective antibodies. Fold expression and fold phosphorylation were analyzed with NIH Image. *C,* as indicated, cells were pretreated with the specific PKG inhibitor Rp-8pCPT-cGMPs (20 μM) or the cell-permeable PKA inhibitor PKI (2 μM) for 4 h and then treated with CP248 (1 μM) for 24 h. The cells were lysed and examined by Western blot analysis using the anti-VASP antibody.

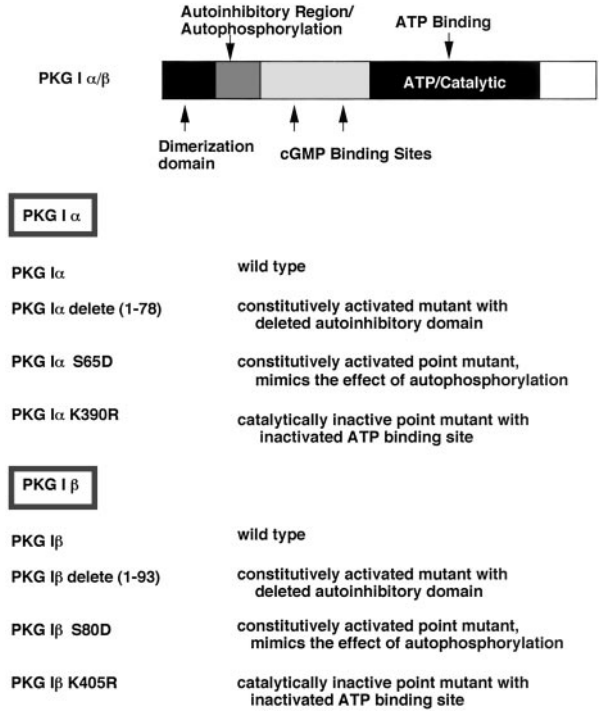

Fig. 3. Structures of PKG mutants. The *top* of this figure displays the full-length open reading frame of wild-type PKG Iα and Iβ, which contain a dimerization domain, an auto-inhibitory region, an autophosphorylation region, two cGMP-binding domains, and an ATP-binding domain. Also displayed are two types of constitutively activated mutants, truncated mutants (PKG I$\alpha\Delta$78 and PKG I$\beta\Delta$93) and point mutants (PKG IαS65D and PKG IβS80D). Also shown are the dominant negative mutants (PKG Iα K390R and PKG Iβ K405R). For additional details, see "Materials and Methods."

A detailed time course study was carried out with 1 μM CP248 (Fig. 2*B*). Increased phosphorylation was detected within 15 min of the addition of CP248 to SW480 cells, and this persisted for at least 24 h. It is curious that between 6 and 24 h, there was a further and marked increase in VASP phosphorylation. This may reflect the effects of CP248 on the actual abundance of PKG (Fig. 2*B*), as discussed below. The serine 157 residue of VASP is a substrate for both PKG and PKA, and indeed it is a preferred site for PKA (20). Thus, it was important to demonstrate that the VASP phosphorylation seen with CP248 and related compounds was due to activation of PKG and not PKA. Therefore, SW480 cells were pretreated with the PKG-specific inhibitor Rp-8-pCPT-cGMPs (29) or the PKA-specific cell-permeable inhibitor PKI (30) for 4 h and then treated for 24 h with 1 μM CP248 (Fig. 2*C*). It is apparent that the PKG inhibitor markedly inhibited the VASP phosphorylation induced by CP248, but this was not the case with the PKA inhibitor.

Effects of Mutant Forms of PKG on VASP Phosphorylation. To obtain direct evidence for the role of PKG on VASP phosphorylation, we constructed two different types of constitutively activated mutants and a dominant negative mutant of both PKG Iα and PKG Iβ (Fig. 3) using an approach similar to that used by other investigators (22, 23). To generate a monomeric enzyme that is cGMP independent and constitutively active, we deleted the dimerization and auto-inhibitory domains by NH_2-terminal truncation of the molecule at glutamine 79 and valine 94, for PKG Iα and PKG Iβ, respectively. To develop another type of constitutively activated mutant, we substituted aspartic acid for serine 65 of PKG Iα and aspartic acid for serine 80 of PKG Iβ, because this mimics the autophosphorylation of PKG that is known to activate PKG kinase activity (22). To obtain dominant negative mutants, we substituted arginine for lysine 390 of PKG Iα and arginine for lysine 405 of PKG Iβ to inactivate the ATP-binding domain of both proteins. These PKG variants and the wild-type forms of PKG Iα and PKG Iβ were recloned into the expression vector pHACE, which has a cytomegalovirus promoter, a neomycin resistance gene for selection, and a convenient HA epitope tag that can be used to readily detect the respective proteins.

To examine the expression and activities of these PKG mutants in intact cells, the respective vector constructs were transfected into COS7 cells. After 24 h, extracts were prepared and examined by Western blot analysis with an anti-HA and an anti-VASP antibody (Fig. 4*A*). The anti-HA antibody revealed that all of the respective proteins were expressed at relatively high levels and were of the expected sizes. The anti-VASP antibody revealed that transfection with

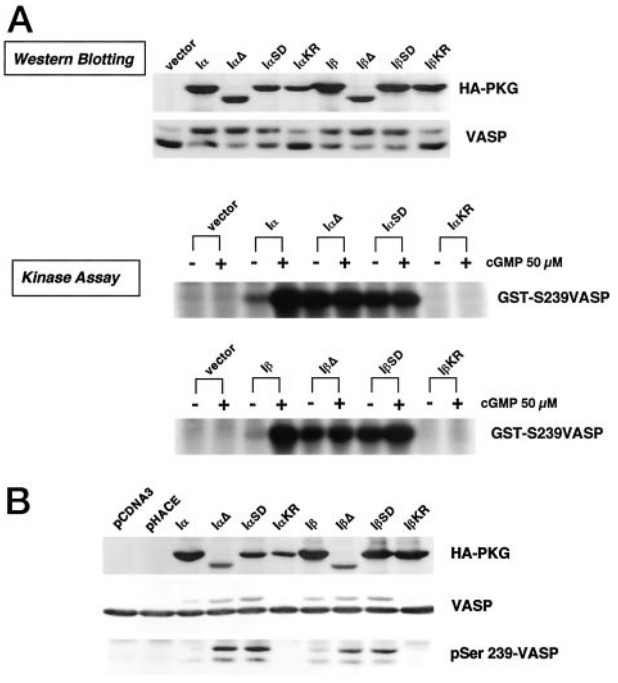

Fig. 4. Expression of PKG mutants in intact cells. *A,* expression and activities of PKG mutants in COS7 cells. Wild-type PKG Iα and Iβ and the PKG mutants shown in Fig. 3 were transfected into COS7 cells. After 24 h, extracts were examined by Western blot analysis with an anti-HA or anti-VASP antibody. In addition, the extracts were analyzed for *in vitro* kinase activities in the absence or presence of cGMP, using a GST-Ser239-VASP peptide as the substrate. For additional details, see "Materials and Methods." *B,* expression of PKG mutants and VASP phosphorylation in SW480 cells. Wild-type PKG Iα and Iβ and the PKG mutants shown in Fig. 3 were transfected into SW480 cells. After 24 h, extracts were examined by Western blot analysis using the respective antibodies.

the wild-type forms of PKG Iα and PKG Iβ and their respective constitutively activated forms caused phosphorylation of VASP, as evidenced by its shift in mobility. On the other hand, the dominant negative mutants (KR) of PKG Iα and PKG Iβ did not cause significant phosphorylation of VASP (Fig. 4*A*). The phosphorylation of VASP obtained with wild-type PKG Iα and PKG Iβ, even in the absence of agents that increase cellular levels of cGMP, may reflect the high level of expression of these proteins in COS7 cells and/or the presence of significant levels of cGMP in these cells.

We also prepared extracts from the same COS7 transfected cells and assayed them for *in vitro* PKG kinase activity using a GST-serine 239 peptide (see "Materials and Methods") as the substrate (Fig. 4*A*). These assays were done in the absence and presence of added cGMP (50 μM). Wild-type PKG Iα had very low kinase activity in the absence of added cGMP but had high activity in the presence of cGMP. On the other hand, both of the constitutively activated mutants had high kinase activity even in the absence of added cGMP. As expected, the dominant negative mutant (KR) had no detectable activity in either the absence or presence of cGMP. Similar results were obtained with respective wild-type and mutants forms of PKG Iβ (Fig. 4*A*).

Furthermore, to confirm the activities of these mutants in SW480 cells, we transfected the wild-type and mutant forms of PKG Iα and PKG Iβ into SW480 cells. As shown in Fig. 4*B,* the anti-HA antibody revealed that, as in COS7 cells, all of the respective proteins were expressed at relatively high levels and were of the expected sizes. All of the constitutively activated mutants caused phosphorylation of VASP, as evidenced by its shift in mobility. We detected only a faint shift in mobility of VASP in the cells transfected with the wild-type PKG Iα and PKG Iβ constructs. As expected, the dominant negative mutants (KR) of PKG Iα and PKG Iβ did not cause significant phosphorylation of VASP (Fig. 4*B*). To obtain further evidence of PKG-mediated VASP phosphorylation, we also performed Western blot analysis with an anti-phospho-Ser239-VASP antibody. Only the constitutively activated forms of PKG caused VASP phosphorylation at Ser-239.

Discussion

The present studies (Fig. 1*B*) extend previous evidence (9) indicating that agents that increase cellular levels of cGMP in human colon carcinoma cells lead to the induction of apoptosis. These agents include the compounds exisulind, sulindac sulfide, CP248, CP461, and 4MQ, which increase cellular levels of cGMP by inhibiting specific PDEs, thus inhibiting the degradation of cGMP, as well as the compounds YC-1, NOR3, and NOC18, which increase cGMP levels by stimulating cellular GC activity, thus increasing the *de novo* synthesis of cGMP. The compounds NOR3 and NOC18 act indirectly by stimulating the synthesis of NO by cellular NO synthase, and NO is a known and physiologically important activator of GC (28). Our findings with NOR3 and NOC18 are of interest because a low concentration of NO can enhance cell survival, whereas higher concentrations of NO can induce apoptosis (31).

We also found that the same concentrations of the above-mentioned compounds that cause an increase in cellular levels of cGMP in SW480 human colon carcinoma cells (Fig. 1*A*) and induce apoptosis in these cells (Fig. 1*B*) also lead to phosphorylation of the VASP protein on serine 157, as evidenced by a characteristic shift in mobility of this protein on gel electrophoresis, from 46 to 50 kDa (Fig. 2*A*). It is known that this serine residue can be phosphorylated by either PKA or PKG (15). Furthermore, high concentrations of cGMP can activate PKA (8). However, we found that the phosphorylation of VASP induced by treating cells with CP248 was inhibited by the PKG-specific inhibitor Rp-pCPT-cGMPs but not by the cell-permeable PKA-specific inhibitor PKI (Fig. 2*C*). Similar results with these inhibitors were obtained when VASP phosphorylation was induced with the compound exisulind (data not shown). Therefore, the phosphorylation of VASP that we found with various compounds that increase cellular levels of cGMP is most likely due to activation of PKG.

To confirm that PKG activation is sufficient to cause VASP phosphorylation in intact cells, we constructed two types of constitutively active mutants of PKG Iα and PKG Iβ and also constructed dominant negative mutants of PKG Iα and PKG Iβ (Fig. 3). All of the constitutively active mutants increased VASP phosphorylation when transiently expressed into COS7 cells. Furthermore, when extracts prepared from cells that expressed the constitutively activated mutants of PKG

were assayed for *in vitro* kinase activity, there was phosphorylation of a VASP-derived peptide, even in the absence of added cGMP. On the other hand, the two dominant negative mutants, PKG Iα KR and PKG Iβ KR, did not display VASP kinase activity, either *in vivo* or *in vitro* (Fig. 4*A*). Similar results were obtained when these mutant forms of PKG Iα and PKG Iβ were transfected into SW480 cells (Fig. 4*B*). Our findings are consistent with previous studies by Collins *et al.* (22) indicating that constitutive activation of PKG causes phosphorylation of VASP in HEK293 cells. Because there is considerable interest in the use of exisulind and related compounds in the chemoprevention and treatment of colon, prostate, and other types of cancer (32), assays that detect increased phosphorylation of VASP in tumor biopsies after the administration of these drugs might provide a biomarker for indicating that the administration of these drugs caused the activation of PKG in the tumor tissue. However, in some cases, control studies might be required to exclude other causes of VASP phosphorylation.

At the present time, the mechanisms by which activation of PKG by exisulind, CP461, CP248, and the other cGMP-elevating agents examined in the present study causes the induction of apoptosis are not known with certainty. Because Thompson *et al.* (9) found that treatment of colon cancer cells with exisulind causes apoptosis and a decrease in cellular levels of β-catenin, the decline in cellular levels of β-catenin could play a critical role. They have also obtained evidence that activated PKG can directly phosphorylate β-catenin (9, 33). In recent studies, we demonstrated that activation of PKG in SW480 cells leads to rapid activation of the mitogen-activated protein kinase kinase kinase 1 (MEKK1)-stress-activated protein kinase/extracellular signal-regulated kinase kinase (SEK1)-JNK1 pathway, apparently through the direct phosphorylation and activation of mitogen-activated protein kinase kinase kinase 1 by PKG (10, 34). Furthermore, we obtained evidence that this activation of JNK1 plays a critical role in the induction of apoptosis by CP248 (10). Other investigators have provided evidence that activation of PKG inhibits the Ras/mitogen-activated protein kinase pathway, presumably through phosphorylation and inactivation of Raf-1 (35). As discussed in the "Introduction," the VASP protein is associated with cellular microfilaments and focal adhesions (16). Smolenski *et al.* (36) have found that in endothelial cells, phosphorylation of VASP by PKG inhibits the attachment of focal adhesions and thus inhibits migration, and in recent unpublished studies,[5] we found that overexpression of PKG Iα or PKG Iβ in SW480 colon cancer cells also inhibits migration. Therefore, the phosphorylation of VASP seen with exisulind, CP461, CP248, and other agents examined in the present studies might contribute to the growth-inhibitory and antitumor effects of these compounds, but this aspect requires further study.

[5] Unpublished studies.

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