Activation of Mitogen-activated Protein Kinase Pathways by Cyclic GMP and Cyclic GMP-dependent Protein Kinase in Contractile Vascular Smooth Muscle Cells*

(Received for publication, June 7, 1999, and in revised form, September 1, 1999)

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Vascular smooth muscle cells (VSMC) exist in either a contractile or a synthetic phenotype in vitro and in vivo. The molecular mechanisms regulating phenotypic modulation are unknown. Previous studies have suggested that the serine/threonine protein kinase mediator of nitric oxide (NO) and cyclic GMP (cGMP) signaling, the cGMP-dependent protein kinase (PKG) promotes modulation to the contractile phenotype in cultured rat aortic smooth muscle cells (RASMC). Because of the potential importance of the mitogen-activated protein kinase (MAP kinase) pathways in VSMC proliferation and phenotypic modulation, the effects of PKG expression in PKG-deficient and PKG-expressing adult RASMC on MAP kinases were examined. In PKG-expressing adult RASMC, 8-para-chlorophenylthio-cGMP activated extracellular signal-regulated kinases (ERK1/2) and c-Jun N-terminal kinase (JNK). The major effect of PKG activation was increased activation by MAP kinase kinase (MEK). The cAMP analog, 8-Br-cAMP inhibited ERK1/2 activation in PKG-deficient and PKG-expressing RASMC but had no effect on JNK activity. The effects of PKG on ERK and JNK activity were additive with those of platelet-derived growth factor (PDGF), suggesting that PKG activates MEK through a pathway not used by PDGF. The stimulatory effects of cGMP on ERK and JNK activation were also observed in low-passaged, contractile RASMC still expressing endogenous PKG, suggesting that the effects of PKG expression were not artifacts of cell transfections. These results suggest that in contractile adult RASMC, NO-cGMP signaling increases MAP kinase activity. Increased activation of these MAP kinase pathways may be one mechanism by which cGMP and PKG activation mediate c-fos induction and increased proliferation of contractile adult RASMC.

Vascular smooth muscle cell (VSMC) proliferation and migration are associated with several vascular diseases such as atherosclerosis and restenosis following vascular injury (1–5). VSMC from several species when cultured in vitro undergo a change in phenotype from a contractile state to a synthetic state similar to the changes that occur with VSMC in vivo in response to vascular injury (6–8). Several lines of evidence suggest that nitric oxide (NO) inhibits VSMC proliferation (9–13), suggesting that signal transduction pathways regulated by NO may be important in VSMC phenotypic modulation. NO stimulates the production of cyclic GMP (14), which, in turn, regulates several functions of VSMC such as smooth muscle relaxation (15). The major receptor protein for cGMP in VSMC is cGMP-dependent protein kinase (PKG), a serine/threonine kinase that catalyzes the phosphorylation of important proteins that regulate intracellular Ca2+ levels and relaxation of vascular smooth muscle (16). Our laboratory has recently demonstrated that PKG also plays a major role in the regulation of the phenotype and morphology of VSMC (17, 18).

The expression of PKG is highly variable in VSMC. When adult rat aortic SMC are subcultured in vitro, PKG expression is reduced to nearly undetectable levels (16, 19). Coincident with the loss of expression of PKG, VSMC assume the more synthetic phenotype. Transfection of the PKG-deficient VSMC with cDNAs encoding either PKG Iα or the catalytic domain of type I PKG reverted the morphology from synthetic to the original contractile morphology (17, 18).

Mitogen-activated protein kinases are important mediators of signal transduction from the cell surface to the nucleus, thereby regulating several cellular responses such as growth and differentiation (20–22). The MAP kinase pathways are activated during proliferation (23–25) and differentiation (26, 27), and it has been proposed that it is the duration of the MAP kinase activation that determines whether a stimulus evokes proliferation or differentiation (28). Three distinct MAP kinase pathways have been identified in mammalian cells: extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase (JNK)/stress-activated protein kinase, and p38 kinase. MAP kinases are activated upon phosphorylation on both threonine and tyrosine residues by the dual function kinase MAP kinase kinase or MEK (mitogen-activated extracellular-regulated protein kinase kinase). The various MEKs are themselves activated by serine phosphorylation. In the case of the MEK that activates ERKs, phosphorylation on serine by Raf kinase is the first step in ERK activation. Using constitutively active and nonactive mutants of MEK, it was demonstrated that activation of MEK was necessary and sufficient for PC12 differentiation and transformation of NIH 3T3 cells (27).

The two isoforms of ERK, p42 and p44 (ERK2 and ERK1, respectively), are present in adult contractile mammalian VSMC at relatively high concentrations (24, 29–31). Additionally, it has been demonstrated that ERK1 and -2 are partially activated in fully differentiated contractile smooth muscle by
contractile agonists (29, 32–34). Several reports suggest that ERK activation may be involved in regulating contraction of differentiated smooth muscle (35), but others have found only small effects of ERK activation on contraction (34). Nevertheless, these findings suggest that the MAP kinase pathway may be involved in other functions besides the regulation of proliferation in fully contractile smooth muscle cells. Hence, it is possible that MAP kinase activation is regulatory in phenotypic modulation. Because PKG itself appears to be important for the expression of the VSMC contractile phenotype, it was of interest to examine the effects of PKG expression on MAP kinase pathways in VSMC compared with VSMC that do not express PKG. In addition, accumulating evidence indicates that PKG activation increases the expression of a number of protooncogene transcription factors in a variety of cell types (36–40). In particular, increased expression of c-fos and Jun-B in response to PKG activation may underlie the increases in VSMC proliferation observed by some investigators (41, 42) and endothelial cells (43). In the case of endothelial cells, Hoo and Granger (43) have shown that cGMP analogs activate Raf-1, an upstream regulator of ERK1/2. The results reported here are the first to examine the role of PKG on MAP kinase activation in contractile VSMC.

**EXPERIMENTAL PROCEDURES**

**Materials**—Collagenase (CLS III) and elastase were from Worthington. Fetal bovine and calf serum, Dulbecco’s modified Eagle’s medium (DMEM) and platelet-derived growth factor BB (PDGF) were purchased from Life Technologies, Inc. Transfectam reagent was from Promega (Madison, WI). 8-para-chlorophenylthio)-guanosine-3’,5’-monophosphate (8-CPT-cGMP) was from Biolog (La Jolla, CA). Geneticin was purchased from Sigma. Antibodies specific to the phosphorylated forms of ERK1/2, total ERK1/2, phospho-p38, total p38, phospho-MEK, and total MEK were purchased from New England Biolabs, Inc. (Beverly, MA). Anti-human JNK-1 antibodies were from PharMingen (San Diego, CA), and the anti-phospho JNK-1 antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Purified antibodies to PKG-I were produced in our own laboratory or were purchased from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada). All other reagents were purchased from either Sigma or Fisher.

**Culture of Rat Aortic Smooth Muscle Cells (RASMC)**—RASMC were isolated from the thoracic and abdominal aortas of adult Harlan Sprague-Dawley rats (150–200 g, Harlan) as described previously (16). Rats were sacrificed by CO₂ inhalation, and aorta were excised and placed in a wash medium of DMEM containing 20 mM HEPES, 1 mg/ml bovine serum albumin, 5 μg/ml amphotericin B, and 50 μg/ml gentamicin. The aortae were cleaned and placed in digestion medium (wash medium containing 1 mg/ml collagenase and 130 units/ml collagenase) for 8 min. The tunica adventitia were removed, and the medial layers were minced and further digested for 1–2 h in digestion medium containing 200 units/ml collagenase until a single-cell suspension was obtained. Cells were washed twice with the wash medium and plated in culture dishes. Cells were maintained in DMEM containing 10% fetal bovine serum and 50 μg/ml gentamicin and grown under 10% CO₂ and subcultured every 6 days.

**Transfection of RASMC**—The transfection of RASMC with the bovine PKG Iα cDNA was performed as described previously (17, 18). Cells at passage 3 were transfected with 5 μg of recombinant pcDNA1-neo/PKG Iα vector or empty (control) pcDNA1-neo vector using 10 μl of Transfectam reagent with precipitation of the DNA-licosome complex for 15 min at room temperature. The precipitate was added to the cell monolayer, and the cells were incubated for 6 h at 37 °C and 10% CO₂. The transfection was terminated by adding DMEM with 20% fetal bovine serum. Stably transfected cells were selected using 500 μg/ml Geneticin (G418). After isolating the colonies, the transfected cell lines were maintained in 250 μg/ml G418. The transfected cells from passage 6–12 were used for the experiments. For the experiments reported here, at least three different clones of cells expressing physiological levels of PKG and three different clones of cells transfected with empty vector and not expressing PKG were examined, as reported previously (17, 18).

**Cell Treatments and Preparation of Cell Lysates**—Cells plated in 60 mm culture dishes and grown to confluence were serum-deprived in DMEM containing 1 mg/ml bovine serum albumin for 24 h, treated with various agents in serum-free medium for different time intervals, quick-frozen in liquid nitrogen, and stored at −80 °C. Cells were harvested by scraping in 0.25–0.5 ml of ice-cold extraction buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and pepstatin A, 5 μg/ml aprotinin A, and 10 mM calcium (EGTA), rotated for 15 min at 4 °C, and centrifuged at 20,000 × g for 10 min. The supernatants were separated, and protein contents were estimated by using the Coomassie Plus protein assay reagent (Pierce) using bovine serum albumin as standard.

**JNK Activation Assay**—The activity of JNK was measured by an immunocomplex kinase assay using an antibody specific for JNK-1 (PharMingen, clone G 151–333) and c-Jun (amino acids 5–89) fused to glutathione S-transferase (GST-c-Jun) as the substrate as described previously (44). 200 μg of cell lysate protein was incubated with anti-JNK-1 antibodies for 1 h at 4 °C, followed by an additional 1-h incubation with Protein G-agarose beads. The immunocomplexes were washed four times with the extraction buffer followed by two washes with JNK assay buffer (20 mM HEPES, pH 7.6, 20 mM MgCl₂, 20 mM β-glycerophosphate, 20 mM p-nitrophenyl phosphate, 0.1 mM sodium orthovanadate, 2 mM dithiothreitol). The immunocomplexes were further incubated in 20 μl of JNK assay buffer containing GST-c-Jun (5 μg/sample), 50 μCi of [γ-32P]ATP for 8 min at 30 °C. The reaction was terminated by the addition of 5 μl of a 5X electrophoresis stop buffer (312.5 mM Tris hydroxymethylaminomethane (Bio-Rad), 0.5X sucrose, 15% sodium dodecyl sulfate (SDS), 0.8 M 2-mercaptoethanol, and 0.002% bromphenol blue). The samples were heated for 10 min at 100 °C, and the denatured proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and subjected to autoradiography. The radioactivity incorporated into the phosphorylated GST-c-Jun protein bands was quantitated by scintillation counting and by densitometry of the autoradiogram.

**Western Blot Analysis for the Activation of ERK1/2, MEK1/2, JNK1, and p38 Kinases**—Cell lysates (10–50 μg of protein) from the treated samples were resolved by 10% SDS-PAGE, protein transferred to nitrocellulose membranes (Bio-Rad), and probed with antibodies specific to the phosphorylated forms of ERK1/2, p38, and MEK1/2. As a control, the amount of total ERK1/2 protein, p38 protein, JNK-1 protein, and MEK1/2 protein was determined by using antibodies that are specific to total ERK1/2, p38, JNK-1, and MEK1/2, respectively. PKG was detected using goat anti-bovine type I PKG prepared in our laboratory or purchased from StressGen. Proteins were visualized using the enhanced chemiluminescence (ECL) detection system (Pierce). The intensity of the bands was quantitated by densitometry.

**Statistical Analysis**—Experiments were performed a minimum of three times, and the data are presented as the mean ± S.E. and analyzed by Student’s t test. A p value of less than 0.05 was used as the criterion of significance.

**RESULTS**

**Expression of PKG Iα in Transfected RASMC**—It was demonstrated earlier that the level of PKG decreases with each passage when adult RASMC are cultured in vitro (16, 19). Similar results have been reported by other laboratories for adult rat aortic and pulmonary arterial smooth muscle cells (13, 45). On the other hand, RASMC isolated from juvenile animals have been reported to maintain PKG expression under certain plating and growth conditions (46). Therefore, in order to ensure that PKG expression was suppressed for the studies described below, cells were isolated from adult animals and passaged several times. The lack of PKG expression was also examined by Western blot analysis, Northern blot analysis, and enzymatic assay. Any non-transfected cell lines still expressing PKG were excluded from these studies. Transfection of RASMC with either the cDNA encoding PKG Iα or the catalytic domain of PKG-I in passages 3–5 reversed the morphology of the cells to a more contractile phenotype (17, 18). Fig. 1 shows the morphology of RASMC stably expressing physiological levels of PKG Iα compared with control transfected not expressing PKG. The RASMC transfected with the empty vector had a synthetic morphology as demonstrated by the

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*2 C. M. Brophy, unpublished observations.*
flattened appearance and the irregular growth pattern (panel A), whereas the RASMC transfected with pcDNA1-neo/PKG Iα had a contractile morphology as demonstrated by the spindle-shaped appearance and “hill and valley” growth pattern (panel B). Similar results were obtained by transfection of the catalytic domain (data not shown). To determine whether the phenotypic changes were associated with alterations in MAP kinase expression, RASMC deficient in PKG and those expressing PKG Iα were used to study the activation of ERK1/2, JNK, and p38. Fig. 2 demonstrates the levels of protein expression for these MAP kinases along with PKG expression in transfected RASMC. It is important to note the absence of detectable PKG expression in RASMC transfected with the empty vector. No PKG was detected by Western blot analysis when up to 50 μg of soluble protein were probed using either a polyclonal, immunopurified anti-PKG generated in our own laboratory or an antibody purchased from StressGen. It is also important to note that the loss of PKG expression in passaged adult rat VSMC has been found by at least three other laboratories independent of our own (13, 45).2 RASMC expressing PKG Iα (Ia) have somewhat higher endogenous levels of ERK1 and -2, especially with respect to the 42-kDa protein (2.9 ± 0.87-fold) compared with RASMC deficient in PKG expression. There were no significant differences in the levels of JNK and p38 kinases in these two cell types.

Time Course of Activation of ERK1/2, JNK, and p38 Kinases by 8-CPT-cGMP—Empty vector- and PKG Iα-transfected cells were treated with an analog of cGMP, 8-CPT-cGMP, for different time intervals. Fig. 3 is the time course of activation of ERK1/2 by cGMP in vector only (lanes 1–4) and PKG Iα-transfected RASMC (lanes 5–8). Compared with empty vector-transfected cells, the untreated control (time 0) from the PKG-Iα RASMC had an increased activity of JNK. 8-CPT-cGMP increased JNK activation at 15 min in the PKG-expressing cells but had no significant effects on JNK activation in the PKG-deficient cells. The total level of JNK protein in these two cell lines was not significantly different (panel F), and the statistical analysis of the data for JNK activation by 8-CPT-cGMP is shown in panel G. PKG transfection and 8-CPT-cGMP had no significant stimulatory effects on p38 kinase in the PKG-expressing cells compared with the PKG-deficient cells (data not shown) and was not studied further. Panel C in Fig. 3 illustrates the level of PKG in the vector alone-transfected cells (lanes 1–4) and the PKG Iα-transfected cells (lanes 5–8). Again, it is important to note the lack of detectable expression of PKG-I in vector alone-transfected adult RASMC.

Sorbitol is an osmotically stressfull agent to cultured cells that is known to activate MAP kinase pathways. Sorbitol significantly activated both ERK1/2 and JNK in vector alone-transfected RASMC (panels A and C and the statistical analysis in panel D in Fig. 4). In PKG-expressing RASMC, sorbitol increased JNK activation more potently than did 8-CPT-cGMP. In these same cells, however, sorbitol was not more potent in increasing ERK1/2 than was 8-CPT-cGMP. The total increase in JNK activation by sorbitol in PKG-deficient and PKG-expressing cells was similar.

Activation of ERK1/2 and JNK by PDGF—Growth factors such as PDGF activate MAP kinases through both Ras-dependent pathways and G-protein coupled pathways in smooth muscle cells (47). To gain further insight into possible mechanisms by which PKG activation increases MAP kinase activity in RASMC, the effects of PDGF on ERK and JNK activation were monitored in PKG-expressing and PKG-deficient cells. Maximal concentrations of PDGF (10 ng/ml) increased ERK1/2 activity in both PKG-deficient and PKG-expressing cells (Fig. 5, panels A and C). ERK1/2 activity at 10 min induced by PDGF was significantly greater in the PKG-expressing cells compared with the PKG-deficient cells. Because total ERK1/2 was not significantly different between RASMC deficient in PKG and
The cells were grown as described under “Experimental Procedures,” and the confluent cells were serum-deprived for 24 h and treated with 100 μM 8-CPT-cGMP. At the various times indicated, vector alone-transfected RASMC (lanes 1–4) and PKG-Ia-transfected RASMC (lanes 5–8) were analyzed for activated (phospho-)ERK1/2 (panel A) and total ERK1/2 (panel B), PKG Iα levels (panel C), activity of JNK (GST-c-Jun, panel E), and total JNK (panel F). Panels D and G represent averaged data quantified by densitometry of immunoblots (D) or autoradiographs (G), expressed as fold increases in activation in which the activation observed in unstimulated vector-alone-transfected cells was defined as 1.0. The fold activations (the means ± S.E. for three independent experiments, *, p < 0.05 for increase in fold activation with 8-CPT-cGMP at 15 min compared to basal activation in PKG-expressing cells) for the various different cell lines examined are presented as bar graphs for ERK1/2 (panel A), total ERK; panel C, GST-c-Jun phosphorylation. Panels D represent averaged data quantified by densitometry of immunoblots (ERK1/2) or autoradiographs (GST-c-Jun), expressed as relative intensity of the bands (means ± S.E. for three independent experiments) for the various different cell lines. The open bars are vector alone-transfected cells, and the filled bars are PKG Iα-transfected cells. *, p < 0.05 for increase in fold activation with 8-CPT-cGMP compared with activation in vector-transfected cells.

FIG. 3. Time course of activation of MAP kinases by 8-CPT-cGMP in transfected RASMC. The cells were grown as described under “Experimental Procedures,” and the confluent cells were serum-deprived for 24 h and treated with 100 μM 8-CPT-cGMP. At the various times indicated, vector alone-transfected RASMC (lanes 1–4) and PKG-Ia-transfected RASMC (lanes 5–8) were analyzed for activated (phospho-)ERK1/2 (panel A) and total ERK1/2 (panel B), PKG Iα levels (panel C), activity of JNK (GST-c-Jun, panel E), and total JNK (panel F). Panels D and G represent averaged data quantified by densitometry of immunoblots (D) or autoradiographs (G), expressed as fold increases in activation in which the activation observed in unstimulated vector-alone-transfected cells was defined as 1.0. The fold activations (the means ± S.E. for three independent experiments, *, p < 0.05 for increase in fold activation with 8-CPT-cGMP at 15 min compared to basal activation in PKG-expressing cells) for the various different cell lines examined are presented as bar graphs for ERK1/2 (panel A), total ERK; panel C, GST-c-Jun phosphorylation. Panels D represent averaged data quantified by densitometry of immunoblots (ERK1/2) or autoradiographs (GST-c-Jun), expressed as relative intensity of the bands (means ± S.E. for three independent experiments) for the various different cell lines. The open bars are vector alone-transfected cells, and the filled bars are PKG Iα-transfected cells. *, p < 0.05 for increase in fold activation with 8-CPT-cGMP compared with activation in vector-transfected cells.

FIG. 4. Effects of sorbitol and 8-CPT-cGMP on the activation of MAP kinases in transfected RASMC. The cells were grown as described under “Experimental Procedures,” and the confluent cells were serum-deprived for 24 h and treated with 100 μM 8-CPT-cGMP or sorbitol for 30 min. Panel A, Western blot for ERK1/2 activation; panel B, total ERK; panel C, GST-c-Jun phosphorylation. Panels D represent averaged data quantified by densitometry of immunoblots (ERK1/2) or autoradiographs (GST-c-Jun), expressed as relative intensity of the bands (means ± S.E. for three independent experiments) for the various different cell lines. The open bars are vector alone-transfected cells, and the filled bars are PKG Iα-transfected cells. *, p < 0.05 for increase in fold activation with 8-CPT-cGMP compared with activation in vector-transfected cells.

those expressing PKG (Fig. 5, panel B), these results suggest that PKG expression appeared to augment ERK activation by PDGF. Similarly, PDGF activated JNK in both PKG-deficient and PKG-expressing cells (Fig. 5, panels D and F). PKG-transfected cells showed increased basal activity of JNK, which was further stimulated by PDGF treatment for 10 min when compared with vector alone-transfected cells. Thus, JNK activation was augmented by the presence of PKG. Basal or PDGF-stimulated activation of p38 kinase was not significantly different in the PKG-deficient and PKG Iα-expressing RASMC (data not shown).

Activation of MAP Kinase Pathways by TNF-α—Inflammatory cytokines such as TNF-α activate MAP kinase pathways in smooth muscle cells predominantly through STAT protein phosphorylation (48). To gain some insight into whether the activation of MAP kinases by PKGs shares a similar pathway, we examined the effects of PKG expression on ERK1/2 and JNK activation in TNF-α-treated cells. As shown in Fig. 6 (panels A and C), the time course of activation of ERK1/2 by TNF-α was different in the two RASMC cell types with the PKG Iα-expressing RASMC having a more prolonged activation of ERK1/2 compared with PKG-deficient cells. However, the peak level of ERK1/2 activation by TNF-α was similar. Likewise, panels D and F in Fig. 6 show that TNF-α activated JNK to a similar extent at 15 min in both the PKG-deficient and PKG-expressing cells. There were no differences in TNF-α-stimulated p38 kinase activation in the PKG-expressing and PKG-deficient cells (data not shown).

Activation of MEK by 8-CPT-cGMP—Since MEK is the upstream kinase that catalyzes the phosphorylation and activation of ERK1/2, the effects of cGMP on the activity of MEK was studied in the PKG-expressing and PKG-deficient cells. As shown in Fig. 7, treatment of cells with 8-CPT-cGMP for 10 min only stimulated the activity of MEK in the PKG Iα-expressing cells as determined by immunoblotting for phospho-MEK1/2. There were no significant differences in the level of total MEK in the PKG-expressing and PKG-deficient RASMC.

Activation of ERK1/2, JNK, and MEK in Primary Culture of RASMC—We have monitored the activation of ERK, JNK, and MEK in several colonies of cells expressing PKG through stable transfection and compared these results with colonies of cells transfected with empty vector and deficient in the expression of this kinase. All colonies expressing PKG demonstrated activation of MAP kinase pathways (i.e. ERK1/2, JNK, MEK) com-
Activation of MAP Kinases by PKG

The cells were grown as described under “Experimental Procedures,” and the confluent cells were serum-deprived for 24 h and treated with PDGF (10 ng/ml) for the times indicated. The cell lysates were prepared, and activation of ERK and JNK was measured as described under “Experimental Procedures.” Lanes 1–4 are protein extracts prepared from vector alone-transfected RASMC, and lanes 5–8 are protein extracts prepared from PKG-Iα-transfected RASMC. Panel A is a immunoblot for ERK1/2 activation; panel B, total ERK protein; panel D, GST-c-Jun phosphorylation; panel E, total JNK enzyme. Panels C and F represent averaged data quantified by densitometry of immunoblots (C) or autoradiographs (F), expressed as -fold increases in activation in which the activation observed in unstimulated vector alone-transfected cells was defined as 1.0. The -fold activation (the means ± S.E. for three independent experiments; *, p < 0.05 for increase in -fold activation with PDGF compared with basal activation in PKG-deficient and PKG-expressing cells) for the various different cell lines examined are presented as bar graphs for ERK1/2 (panel C) and JNK (panel F). The open bars are vector alone-transfected cells, and the filled bars are PKG Iα-transfected cells.

Fig. 5. Time course of activation of MAP kinases by PDGF in transfected RASMC. The cells were serum-deprived for 24 h and treated with 50 ng/ml TNF-α for the times indicated as described in Fig. 5 legend. Lanes 1–4 are protein extracts prepared from vector alone-transfected RASMC, and lanes 5–8 are extracts prepared from PKG Iα-transfected RASMC. Panel A is a Western blot for ERK1/2 activation, panel B is total ERK, panel D is the autoradiograph for GST-c-Jun phosphorylation, and panel E is the immunoblot for total JNK. Panels C and F represent averaged data quantified by densitometry of immunoblots (C) or autoradiographs (F), expressed as -fold increases in activation in which the activation observed in unstimulated vector alone-transfected cells was defined as 1.0. The -fold activation (the means ± S.E. for three independent experiments; *, p < 0.05 for increase in -fold activation with TNF-α compared with basal activation in PKG-deficient or PKG-expressing cells) for the various different cell lines examined are presented as bar graphs for ERK1/2 (panel C) and JNK (panel F). The open bars are vector alone-transfected cells, and the filled bars are PKG Iα-transfected cells.

Effects of 8-Br-cAMP on ERK Activity in RASMC—It is well established that PKG and the cAMP-dependent protein kinase (PKA) share several signaling functions in smooth muscle such as the mediation of relaxation. Furthermore, it has been suggested that cAMP is involved in phenotypic modulation of VSMC (49), and it is well known that cAMP is a physiological inhibitor of VSMC proliferation (50–53). Therefore, it was of interest to examine the effects of cAMP on MAP kinase activities in adult RASMC transfected with PKG. This was made possible from the standpoint that PKA expression, unlike that of PKG, is not reduced during culture of RASMC (54). Vector alone- and PKG Iα-transfected RASMC were treated with 100 μM 8-Br-cAMP for different time intervals and the activation of ERK and JNK was monitored. In contrast to the effects of 8-CPT-cGMP, 8-Br-cAMP markedly inhibited the activation of ERK1/2 in a time-dependent manner in both PKG-deficient

Fig. 6. Time course of activation of MAP kinases by TNF-α in transfected RASMC. The cells were serum-deprived for 24 h and treated with 50 ng/ml TNF-α in transfected RASMC. Panel A is a Western blot for ERK1/2 activation, panel B is total ERK, panel D is the autoradiograph for GST-c-Jun phosphorylation, and panel E is the immunoblot for total JNK. Panels C and F represent averaged data quantified by densitometry of immunoblots (C) or autoradiographs (F), expressed as -fold increases in activation in which the activation observed in unstimulated vector alone-transfected cells was defined as 1.0. The -fold activation (the means ± S.E. for three independent experiments; *, p < 0.05 for increase in -fold activation with TNF-α compared with basal activation in PKG-deficient or PKG-expressing cells) for the various different cell lines examined are presented as bar graphs for ERK1/2 (panel C) and JNK (panel F). The open bars are vector alone-transfected cells, and the filled bars are PKG Iα-transfected cells.

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Western blots for activation of MEK from PKG I into RASMC, while lanes 3 and 4 are Western blots for activation of MEK from PKG I-transfected RASMC. Panel B is the immunoblot for total MEK protein. Panel C represents averaged data quantified by densitometry of immunoblots, expressed as fold-increases in activation in which the activation observed in unstimulated vector alone-transfected cells was defined as 1.0. The -fold activation (the means ± S.E. for three independent experiments; *, p < 0.05 for increase in -fold activation with 8-CPT-cGMP compared with basal activation in PKG deficient cells) for the various different cell lines examined are presented as bar graphs for MEK. The open bars are vector alone-transfected cells and the filled bars PKG I-transfected cells.

Inhibition of ERK Activation by Selective Kinase Inhibitors—Previous studies have examined the effects of protein kinase inhibitors on MAP kinase activation in various cell types (12, 57). Although it is evident that compounds previously believed to be selective for inhibiting PKA and PKG are not selective when used as described in intact cells (58, 59), we were interested in determining whether or not there were any effects of these compounds on MAP kinase activity in the PKG-deficient and PKG-expressing adult RASM. Incubation of PKG I-transfected RASMC with 1 μM KT 5823 (a compound reported to be a selective inhibitor of PKG) for 30 min partially inhibited the stimulation of ERK activity (13.6 ± 2.1%, p < 0.0067) produced by 8-CPT-cGMP in PKG-transfected cells. Incubation with 1 μM KT 5720 (reportedly a selective inhibitor of PKA) did not significantly inhibit the stimulation of ERK activity (0.99 ± 1.2%, p > 0.474) in these same cells treated with 8-CPT-cGMP (data not shown). Neither KT 5823 nor KT 5720 inhibited the basal activity of ERK in either vector alone- or PKG-transfected RASM. In primary cultures of RASM expressing PKG, incubation of the cells for 30 min with 1 μM KT 5823 partially inhibited ERK activation produced by 8-CPT-cGMP by 27.5 ± 7.5%. In contrast, the selective MEK inhibitor, PD 98059, potently inhibited the effects of 8-CPT-cGMP on ERK1/2 activation in both PKG I-transfected RASMC and primary cultures of RASM, as well as in vector alone-transfected RASM (Fig. 10).

DISCUSSION

One of the hallmarks of cGMP and PKG action in a variety of cultured cells is activation of c-fos gene expression (36–40). In turn, c-fos expression promotes increased cell proliferation. Currently there is much debate and controversy surrounding the role of cGMP and PKG in the regulation of cell proliferation. There are several studies suggesting that cGMP inhibits the proliferation of VSMC. Most of these studies were performed using adult VSMC that had been passaged several times and that exist in the synthetic phenotype. Except for neonatal or juvenile VSMC (or cell lines derived from embryonic VSMC such as the A7r5), synthetic phenotype VSMC do not express appreciable levels PKG. Thus, the role of the NO-cGMP-PKG signal transduction pathway in regulating adult VSMC proliferation is still unknown. In one report, Hassid and colleagues (41) demonstrated that NO and cGMP do not inhibit the proliferation of freshly isolated adult RASM, which express PKG and exhibit the contractile phenotype. In contrast,
neonatal RASMC, which express PKG but do not exhibit the adult contractile phenotype, are exquisitely sensitive to growth inhibition by NO. Similar to the studies of Hassid et al., we have found that cGMP and PKG activation do not inhibit RASMC proliferation provided that the cells are contractile (17). With regard to adult RASMC that do not express PKG, we have suggested that NO and cGMP are capable of inhibiting proliferation due to the ability of cGMP to “cross-activate” PKA (60). Using adenovirus-mediated transfer of PKG Iα, Chiche et al. (13) demonstrated that activation of PKG inhibited rat pulmonary artery VSMC proliferation. Since proliferation was monitored 48 h after infection, these cells would have been in a synthetic phenotype, not contractile. We have found that when sufficient time for phenotypic modulation was allowed after adenoviral-induced PKG Iα expression, cGMP did not inhibit the proliferation of RASMC (17). On the other hand, if proliferation was measured before PKG-induced the expression of contractile proteins and a contractile phenotype, then cGMP attenuated serum-induced proliferation. Based on these concepts, the control of VSMC proliferation by the NO-cGMP-PKG pathway may be quite complex and dependent upon the phenotype in which the cells exist. Clearly, a molecular approach toward understanding the effects of PKG on VSMC proliferation is important.

The MAP kinase family has been implicated in a variety of changes in VSMC function including increased proliferation and migration (23–25), two hallmarks of modulation of contractile VSMC to the synthetic phenotype. In VSMC, the majority of studies conducted on MAP kinases have been performed in cultured VSMC that already exist in a stable synthetic phenotype. Activation of the MAP kinase pathway, and especially the ERK pathway, is necessary for protooncogene expression (including c-fos) and cell proliferation. Fewer

**FIG. 9.** Effects of 8-Br-cAMP on MAP kinase activation in transfected RASMC. Cells were grown as described under “Experimental Procedures,” and the confluent cells were serum-deprived for 24 h. The RASMC were treated with 100 μM 8-Br-cAMP for the times indicated, and cell lysates were prepared as described in Fig. 5. Panel A is a Western blot for the activation of ERK1/2, panel C is a Western blot for the activation of MEK1/2, and panel E is an autoradiogram for GST-c-Jun phosphorylation. Lanes 1–5 are extracts from vector alone-transfected RASMC whereas lanes 6–10 are extracts from PKG Iα-transfected RASMC. Panels B, D, and F represent averaged data quantified by densitometry of immunoblots (B and D) or autoradiographs (F), expressed as -fold increases in activation which the activation observed in unstimulated vector alone-transfected cells was defined as

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**FIG. 10.** Effects of PD98059 on the activation of ERK1/2 in primary and transfected RASMC. Cells were grown as described under “Experimental Procedures,” and the confluent cells were serum-deprived for 24 h. The RASMC were treated with 50 μM PD98059 for 30 min followed by 100 μM 8-CPT-cGMP for 10 min; cell lysates were prepared; and the activations of ERK, MEK, and JNK were performed. Averaged data were quantified by densitometry of immunoblots and expressed as percentage of activation of ERK1/2. The percentage of activation (the means ± S.E. for three independent experiments; *, p < 0.05 for decrease in percentage of activation with PD98059 compared with basal activation in PKG-deficient or PKG-expressing cells) of ERK1/2 from primary or transfected RASMC is shown in which the activation observed in the unstimulated cells from the vector-transfected (open bar), PKG-transfected (closed bar), or primary (hatched bar) RASMC was defined as 100%.
studies have been performed on fully contractile smooth muscle cells, and those studies that have been done, a role for the MAP kinases in regulating contractile behavior has been suggested (32–35). Therefore, it was of great interest to us to examine the role of PKG on MAP kinase activity in RASMC whose contractile phenotype had been restored.

In this study, we demonstrated that restoration of PKG expression to PKG-deficient adult RASMC mediates cGMP activation of MEK, ERK1/2, and JNK. These effects were observed in all clonally derived cell lines expressing PKG. The cellular phenotype is clearly contractile in the PKG-expressing cell lines as judged by morphological, biochemical, and functional criteria. In PKG-deficient cell lines that are synthetic in phenotype, 8-CPT-cGMP has little or no effect on MAP kinase activities. In addition to the findings using stably transfected cells, we also observed that cGMP activates the same MAP kinases in freshly isolated, primary RASMC that still express PKG and exist in the contractile phenotype (Fig. 8). Therefore, the stimulatory effects of cGMP and PKG on MAP kinase activities are not due to prolonged culturing of the cells or due to artifacts associated with transfection. These results are similar to those of Hood and Granger (43) for the effects of PKG in cultured endothelial cells. To our knowledge, this is the first report demonstrating the PKG-mediated activation of MAP kinases in contractile smooth muscle cells. Hence, the finding that PKG activates MAP kinase pathways provides a plausible mechanism underlying NO- and cGMP-induced increases in c-fos expression and proliferation of adult, contractile VSMC and other cell types.

As mentioned above, every clonally derived RASMC line expressing PKG demonstrated higher levels of ERK and JNK activity compared with clonally derived RASMC lines transfected with empty vector and deficient in PKG expression. We observed significant variability in the basal ERK and JNK activity in PKG-expressing cells in these experiments. The variable levels of phospho-ERK and JNK were likely due to the rapidly fluctuating levels of intracellular cGMP, a phenomenon that is known to occur in cultured cells (61). During the growth phase of RASMC in the presence of serum, the intracellular levels of cGMP are some 10–100-fold greater when compared with cells approaching confluence (62). These high levels of cGMP activate PKG I during growth, thus accounting for the higher basal activity of ERK and JNK in and the stably transfected cell lines.

At first, we were somewhat surprised by these effects of PKG given previous reports that NO and cGMP inhibit VSMC proliferation (9, 10, 13, 24, 57). Yu et al. (12) reported that cGMP-elevating agents suppress proliferation of VSMC, presumably through the activation of PKG and the phosphorylation of Raf-1. The major evidence presented that suggested the involvement of PKG was the sensitivity of the effects to the PKG inhibitor, KT 5823. In another study using BHK cells, Suhasini et al. (57) reported that overexpression of PKG inhibits ERK activation via the phosphorylation of Raf-1 kinase on serine 43. In neither of these studies were the effects of cAMP and PKA activation on MAP kinase activity and Raf-1 phosphorylation determined. A well known effect of cAMP and PKA activation is the phosphorylation of Raf-1 on serine 43, leading to the inability of ras to activate the pathway (55, 56). Consequently, activation of the PKA pathway could have been responsible for the effects of cGMP elevating agents, insomuch as high concentrations of cGMP are known to activate PKA (60). It is also clear that PKA and PKG share overlapping substrate specificities in vitro and in the intact cell (15). Hence, overexpression of PKG in cells may result in the phosphorylation of substrate proteins normally selective for PKA. In either case, our results clearly demonstrate a specific effect of PKG to activate MEK and the ERK pathway. Indeed, the exact opposite effects of 8-Br-cAMP were observed in the current study where there was a clear inhibition by 8-Br-cAMP of ERK activation in both PKG-expressing and -deficient RASMC. Thus, it is possible that the inhibitory effects of cGMP on MAP kinase activity observed by other investigators using PKG-deficient VSMC is due to PKA-dependent phosphorylation of Raf-1 on serine-43.

Our finding that MAP kinases are also activated in differentiated contractile smooth muscle is consistent with these results and those obtained earlier by several investigators who study contractile function of differentiated VSMC. For example, the role of MAP kinase in the contractile response of smooth muscle may involve the phosphorylation of caldesmon (35). Although these results are somewhat controversial (34), the function of MAP kinase in the contractile phenotype of vascular smooth muscle is likely to be very different from its role in the synthetic phenotype.

Because JNK is also activated in the PKG Ia-transfected RASMC, the possibility exists that NO and cGMP may regulate stress pathway outcomes such as apoptosis. Induction of apoptosis by PKG in VSMC has been documented by Pollman et al. (63) and Chiche et al. (13). JNK has been implicated in the apoptotic pathway in at least some cell types (22). Thus, JNK activation in the PKG-expressing contractile VSMC may explain the increased sensitivity of these cells for apoptosis upon growth factor withdrawal, compared with the synthetic PKG-deficient VSMC.

The mechanism by which PKG leads to MAP kinase activation in the contractile phenotype is unclear. The results shown in Fig. 5 indicate that PKG-mediated activation of ERK is at least partially additive with PDGF, suggesting an alternative mechanism to the pathways utilized by these biological modulators. Since MEK is also activated in these cells, PKG may be acting upstream to MEK. Potential candidates for regulation by PKG include p21 Ras-GTPase-activating protein since Ras-GTPase-activating protein is phosphorylated in vitro using PKG. Other possible candidates include the MAP kinase phosphatase 1, since it has been demonstrated that PKG regulates the expression of at least one protein-tyrosine phosphatase in VSMC (64–67). More recently, phenotypic modulation of vascular and non-vascular smooth muscle cells has been linked to the balance between phosphoinositide 3-kinase and Akt with MAP kinases (68). Perhaps an upstream point of divergence between PKG-regulated MAP kinases and the PDGF and TNF-α signaling pathways is at the level of phosphoinositide 3-kinase. On the other hand, the results in Fig. 6 demonstrating that PKG and TNF-α may utilize similar pathways in the activation of ERK and JNK which suggest a common target for these two signaling pathways. Clearly, more studies are needed to identify the steps regulated by PKG in VSMC.

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