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Multiple Kinase Involvement in the Regulation of Vascular Growth

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1. Introduction

The initial discovery of protein phosphorylation as a regulatory mechanism for the control of glycogen metabolism has led to intense interest of protein phosphorylation in regulating protein function (Cohen *et al.*, 2001). Kinases play a variety of roles in many physiological processes within cells and represent one of the largest families in the human genome with over 500 members comprising protein serine/threonine, tyrosine, and dual-specificity kinases (Manning *et al.*, 2002). Phosphorylation of proteins is one of the most significant signal transduction mechanisms which regulate intracellular processes such as transport, growth, metabolism, apoptosis, cystoskeletal arrangement and hormone responses (Bononi *et al.*, 2011; Heidenreich *et al.*, 1991; Manning *et al.*, 2002; Pawson *et al.*, 2000). As such, abnormal phosphorylation of proteins can be either a cause or a consequence of disease. Kinases are regulated by activator and inhibitor proteins, ligand binding, and phosphorylation by other proteins or via autophosphorylation (Hanks *et al.*, 1991; Hug *et al.*, 1993; Scott, 1991; Taylor *et al.*, 1990; Taylor *et al.*, 1992). Since kinases play key functions in many cellular processes, they represent an attractive target for therapeutic interventions in many disease states such as cancer, inflammation, diabetes and arthritis (Cohen *et al.*, 2010; Fry *et al.*, 1994; Karin, 2005; Mayers *et al.*, 2005). In particular, the serine/threonine family of kinases comprises approximately 125 of the 500 family of kinases and includes the cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA), the cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG), and protein kinase C (PKC). These kinases are implicated in the regulation of cell growth and are the focus of this current study.

Nitric oxide (NO) is the primary endogenous activator of soluble guanylate cyclase (sGC) and cGMP generation in the vasculature with PKG historically acknowledged as its primary downstream kinase (Ignarro, 1991; Ignarro *et al.*, 1985). However, this area of research is controversial since the effects of NO are tightly regulated and specific to certain sub-cellular microenvironments. The NO-sGC-cGMP signaling pathway is of major importance in the cardiovascular system where it modulates functions such as vascular smooth muscle cell (VSMC) relaxation and growth (Ignarro, 1991; Ignarro *et al.*, 1986; Ignarro *et al.*, 1985; Lucas *et al.*, 2000). Vascular remodeling is associated with injury and the pathogenesis of disorders

such as atherosclerosis and it is well established that the proliferation and migration of VSMCs play a major role in these processes (Gibbons *et al.*, 1994; Herity *et al.*, 1999; Shi *et al.*, 1996). The molecular mechanisms of cGMP signaling are not well understood, since studies suggest that NO can both promote and inhibit pathological vascular remodeling (Kawashima *et al.*, 2001; Ozaki *et al.*, 2002; Rudic *et al.*, 1998). Furthermore, recent studies provide sound evidence for NO-independent heme oxygenase (HO) and carbon monoxide (CO) as physiologically relevant signals capable of activating sGC and promoting cGMP signal transduction (Christodoulides *et al.*, 1995; Siow *et al.*, 1999; Tulis *et al.*, 2001a; Tulis *et al.*, 2001b; Tulis *et al.*, 2005). BAY 41-2272 (BAY) is a prototype of a novel class of sGC modulators that potentiate both NO- and CO-mediated signaling and that has potential for use in clinical trials for the treatment of cardiovascular disease, yet mechanisms of action for BAY and similar agents still remain to be determined (Boerrigter *et al.*, 2007; Mendelev *et al.*, 2009; Roger *et al.*, 2010). One study suggests that BAY may have phosphodiesterase (PDE) 5 inhibitory activity at high concentrations (Mullershausen *et al.*, 2004); however this is still controversial. BAY interacts with sGC via a different mechanism than that of NO; therefore, the amount of cGMP generated and subsequent targets that are activated may be distinct leading to different responses than that of traditional NO-based signaling. This is important and a perfect example of a drug-induced versus a cell-specific response.

Individual drugs interact with cells differently depending on the cellular microenvironments within the cell. Recent reports demonstrate the ability of cell- and agonist-specific responses following ligand stimulation due to intracellular localization of proteins including kinases which operate largely to ensure signal specificity (Di Benedetto *et al.*, 2008; Lissandron *et al.*, 2006; Xiang, 2011). An increase in cyclic nucleotide signaling can affect multiple signaling pathways because of activation of such kinases in particular intracellular domains. Hence, compartmentalization of proteins is very critical in cellular/biomolecular signaling in order to ensure a specific response to a particular stimulus in cells.

VASP, a cytoskeletal protein that belongs to the Ena/VASP family, was originally characterized as a substrate for cyclic nucleotide-dependent PKA and PKG (Krause *et al.*, 2002; Reinhard *et al.*, 2001). At least four distinct phosphorylation sites on VASP (Ser_{157}, Ser_{239}, Thr_{278}, Ser_{322}) have been identified (Butt *et al.*, 1994; Chitaley *et al.*, 2004; Thomson *et al.*, 2011). Despite the conventional thought that phosphorylation of VASP at Ser_{239} and Ser_{157} occurs by PKG and PKA, respectively (Butt *et al.*, 1994), more recent studies suggest that crosstalk exists and that Ser_{157}, Ser_{239} and Thr_{278} can be phosphorylated by both PKA and PKG. However, phosporylation of VASP by PKC and AMP kinase is more selective (Benz *et al.*, 2009; Blume *et al.*, 2007; Zhuang *et al.*, 2004). The phosphorylation pattern of VASP is complex and dynamic and occurs in site-specific order with different priorities (Abel *et al.*, 1995; Butt *et al.*, 1994; Zhuang *et al.*, 2004). Functionally, VASP plays an important role in regulating cytoskeletal dynamics and processes such as cell adhesion and migration (Kwiatkowski *et al.*, 2003; Mitchison *et al.*, 1996; Vasioukhin *et al.*, 2000), and phosphorylation of VASP has been shown to be important physiologically as it is involved in these processes. It was reported that VASP phosphorylation at Ser_{157} is required for the growth stimulatory effect and phosphorylation at Ser_{239} is required for the growth inhibitory effect of NO on SMCs (Chen *et al.*, 2004). Although VASP phosphorylation continues to be used as a biochemical marker for selective kinase activation, its precise cellular and molecular functions remain to be fully determined.

Previously, we demonstrated in both rat commercial (A7R5) VSMCs and rat primary VSMCs that BAY increases cyclic nucleotides and inhibits vascular growth through processes involving VASP phosphorylation (Joshi *et al.*, 2011; Mendelev *et al.*, 2009). In this report we demonstrate for the first time that BAY increases the activity of protein kinases and that this system has the ability to regulate growth of VSMCs. Novel results include demonstration that kinase activity from PKA, PKG and PKC has the capacity to increase phosphorylation of cellular proteins including VASP. Notably, the implication of multiple kinases in the regulation and activation of VASP provides sound evidence for potential crosstalk among otherwise distinct kinases within the cell. Functionally, strong evidence is presented linking cyclic nucleotide-mediated kinase activation and VASP phosphorylation with inhibition of vascular smooth muscle growth through modulation of proliferation and migration.

2. Methods

2.1 Rat primary VSMC culture

Following established procedures (Liu *et al.*, 2009; Tulis *et al.*, 2002), thoracic aorta VSMCs were obtained from male Sprague-Dawley rats (100-125 grams) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and Primocin (100mg/L) at 37^{o}C in 95% air/5% CO_2. Cells were split and propagated through passage 6 (unless otherwise specified). For select experiments higher passage cells were used between passage 7 and 10. All studies abided by the guidelines of the Institutional Animal Care & Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health, Publication No. 85-23, revised 1996).

2.2 In-cell western

Phosphorylation of VASP was examined by use of an established cell-based immunocytochemical assay for monitoring kinase signaling pathways (Chen *et al.*, 2005). Rat VSMCs were seeded (~20,000 cells/well) in 96 well plates and once adhered, pretreated with either kinase and PDE inhibitors or vehicle for 30 minutes after which they were stimulated with either BAY for 60 minutes or cyclic nucleotide analogs (8Br-cAMP; 10μM or 8Br-cGMP; 10μM, Tocris) for 30 minutes. The inhibitors used for In-Cell Westerns were: adenosine 3',5'-cyclic monophosphate-dependent protein kinase inhibitor (5-24), (PKI; 10μM), a selective PKA inhibitor (Enzo); DT-2 trifluoroacetate salt, (DT2; 10μM), a selective PKG inhibitor (Sigma-Aldrich); calphostin C (CALC; 100nM), a selective inhibitor of PKC (Enzo); cilostazol (CILO; 10 μM), a selective PDE3 inhibitor (Sigma-Aldrich); rolipram (ROL; 10 μM), a selective PDE4 inhibitor (Tocris); zaprinast (ZAP; 10 μM; MP Biochemicals) and vardenifil (VAR; 50 nM; Toronto Research Chemicals), selective PDE5 inhibitors. The vehicle for CILO, ROL, CALC and ZAP was DMSO ($\leq$ 0.5%). After incubation, media was removed and cells were fixed with 4% formalin in PBS for 20 minutes. Cells were washed and permeabilized with PBS containing 0.1% Triton X-100, followed by 1X PBS + 0.1% Tween-20. Cells were blocked with IR blocking solution (Odyssey) for 3 hours and then incubated with primary antibodies directed against VASP at $pSer_{239}$ (1:500; Cell Signaling), VASP at $pSer_{157}$ (1:500; Cell Signaling) or α-tubulin (1:500; Sigma) overnight at 4^{o}C. Cells were washed with PBS + 0.1% Tween-20 followed by incubation with two secondary antibodies: IRDye 800CW

(1:500; Odyssey) and Alexa Fluor 680 for 1 hour. Cells were washed with PBS containing 0.1% Tween-20 followed by plain PBS. The plate was allowed to dry overnight and scanned using the appropriate channels for detection with the Odyssey Imager.

2.3 Western blot

Rat VSM cells were used between passages 4 and 6 for all experiments. Cells were lysed in buffer (50mM Tris, pH 6.8; 1% SDS; 0.1% Triton; protease inhibitor cocktail (Roche); phosphate inhibitor cocktail (Santa Cruz)) and sonicated on ice followed by centrifugation (14000g, 20 min). Protein concentration of whole cell lysates was determined by the BCA assay (Pierce). Proteins were separated on SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore) using Mini-Protean Electrophoresis and Mini Trans-Blot Cells (Bio-Rad). Blots were blocked with 5% non-fat milk in TTBS (25mM Tris/Tris-HCl; 0.0027M KCl; 0.13M NaCl; 0.1% Tween 20) or 5% BSA in TTBS for 1 hour and incubated with primary antibodies directed against protein kinase G (PKG) and protein kinase A (PKA) (1:1000; Abcam), phospho-PKA_{Ser338} (1:1000; Biosource), VASP, phospho-$VASP_{Ser157}$, and phospho-$VASP_{Ser239}$ (1:1000; Cell Signaling) and α-tubulin (1:1000; Sigma) overnight at 4 ^{o}C. Blots were washed with TTBS, incubated with peroxidase-linked secondary antibodies (1:5000) for 1 hour, visualized using Pierce ECL Western blotting kit, documented using an Alpha Imager 2200, and analyzed with ImageJ 1.40g software (NIH).

2.4 Hemocytometry assay

Rat VSMCs were seeded in 24 well plates (40,000 cells/well) and after adherence were quiesced overnight in 0.2% FBS. Cells were then pretreated with PKI, DT2 or vehicle for 30 mins, prior to stimulation with BAY (10μM) for 48 or 72 hrs. At the end of the time point, cells in each well were trypsinized and the cell count estimated using a hemocytometer at 10X magnification.

2.5 MTT assay

Rat VSMCs were seeded in 96 well plates and pretreated with PKI, DT2 or vehicle for 30 mins, prior to stimulation with BAY (10μM) for 24hrs. After treatment media was removed and MTT was added to each well and incubated for 4 hours at 37˚C. The MTT was removed, MTT solvent added and absorbance read after 15 mins.

2.6 BrdU assay

Rat VSMCs were seeded in 96 well plates and pretreated with PKI, DT2, CALC or vehicle for 30 mins, prior to stimulation with BAY (10μM) for 48hrs. After treatment media was removed and BrdU labeling solution was added to each well and incubated for 3 hours at 37˚C. Next the labeling solution was removed, cells were fixed and the anti-BrdU antibody added for 90 mins. Cells were washed, the substrate was then added, and absorbance was read after 30 mins.

2.7 Kinase assay

Rat VSMCs were seeded in 6 well plates and grown to confluence. The cells were then pretreated with PKI, DT2, CALC or vehicle for 30 mins, prior to stimulation with BAY

(10μM) for 1 hour. The cells were lysed, protein was isolated and protein concentration was determined via a BSA assay. Kinase activity was determined using an activity kit (Enzo Life Sciences). Briefly, samples were added to the wells, the reaction was initiated with ATP, and cells were incubated at 30˚C for 90 mins. A phosphospecific substrate antibody was added and incubated at RTP for 60 mins. Next the plate was washed and the HRP conjugate was added and incubated for 30 mins. Finally the plate was washed and substrate added for 30 mins and absorbance was read.

2.8 Scratch wound assay

Rat VSMCs were seeded in 24 well plates. Cells were injured with a 1 mm scrape and the images captured. Immediately cells were then treated with PKI, DT2, CALC or vehicle for 30 mins, prior to stimulation with BAY (10μM) for 6 or 16 hrs. Afterward the images were captured and distances migrated were measured.

2.9 Data analysis

Statistical significance between experiments was determined using an analysis of variance (ANOVA). In the event that the *F* ratio indicated a significant $P<0.05$ change occurred, a post hoc test was performed to identify individual differences. Results were reported as mean ± the standard error of the mean (SEM).

3. Results

3.1 The effect of kinase inhibitors on BAY-induced VASP phosphorylation

We recently demonstrated that BAY, a potent stimulator of sGC, increased phosphorylation of VASP in a site-specific fashion in rat A7R5 VSMCs (Mendelev *et al.*, 2009) and in rat primary VSMCs (Joshi *et al.*, 2011). In the current study we sought to identify kinases responsible for this selective phosphorylation of VASP induced by BAY. In primary VSMCs, BAY (10 μM) significantly increased VASP phosphorylation at both Ser_{157} and Ser_{239} (Figures 1A, 1B, respectively). Addition of PKI (10 μM) or CALC (100 nM) slightly (non-significantly) increased BAY-induced phosphorylation of VASP at Ser_{157} compared to effects of BAY alone (Fig. 1A), whereas CALC slightly (non-significantly) reduced VASP phosphorylation at Ser_{239} compared to BAY alone (Fig. 1B).

Due to the relative ineffectiveness of select kinase inhibition on site-specific VASP phosphorylation, the possibility of crosstalk between these kinases was examined. Intriguingly, the combination of PKI and DT2 increased the phosphorylation induced by BAY at Ser_{157} (Fig 2A), while the combination of PKI and CALC markedly increased the phosphorylation induced by BAY at Ser_{239} (Fig. 2B).

3.2 Effect of PDE inhibition and kinase activity on VASP phosphorylation

In order to determine if kinase inhibition affects the ability of PDEs to regulate VASP, VSMCs were pretreated with inhibitors of kinases and PDEs prior to stimulation with BAY. In the presence of BAY and DT2, the PDE 3 inhibitor CILO and the PDE 5 inhibitors VAR or ZAP, significantly increased phosphorylation at Ser_{157} compared to BAY alone (Fig. 3A). PKI increased Ser_{157} phosphorylation induced by BAY (Fig. 3B); however, PDE inhibition

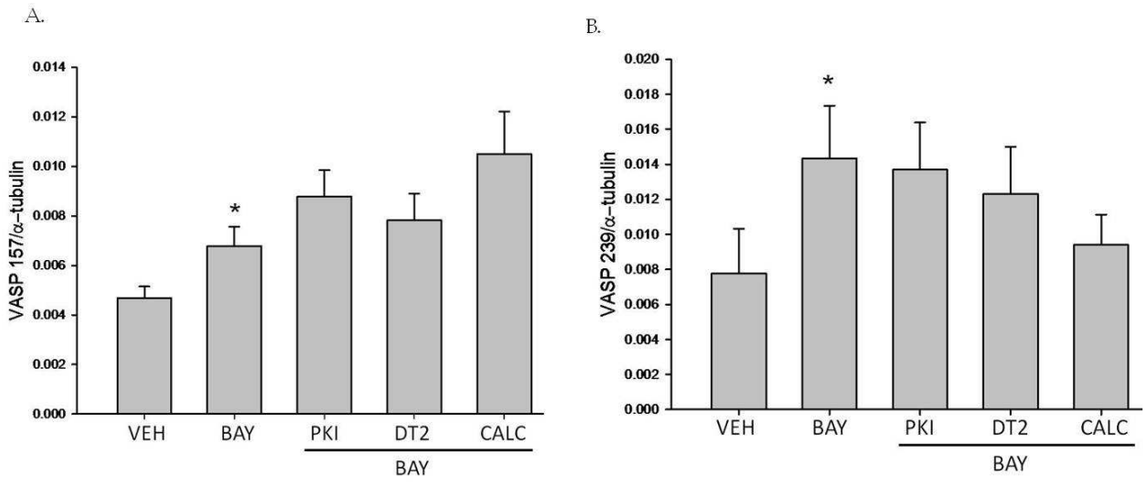


Fig. 1. (A) Effect of kinase inhibitors on BAY 41-2272 (BAY)-induced VASPSer$_{157}$ phosphorylation. VSMCs were pretreated with PKI, DT2, CALC or vehicle for 30 min prior to stimulation with BAY (10uM) for 60 minutes (n=15). (B) Effect of kinase inhibitors on BAY-induced VASPSer$_{239}$ phosphorylation. VSMCs were pretreated with PKI, DT2, CALC or vehicle for 30 min prior to stimulation with BAY (10uM) for 60 minutes (n=15). Values are the means ± SE. * = different from vehicle (P<0.05).

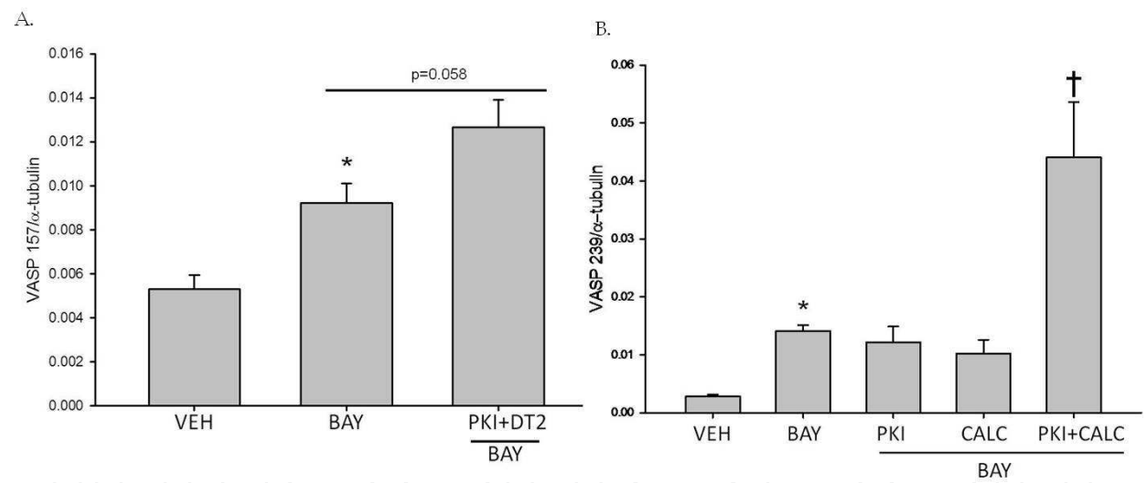


Fig. 2. (A) Effect of PKI and DT2 on BAY-induced VASPSer$_{157}$ phosphorylation. VSMCs were pretreated with PKI and DT2 or vehicle for 30 min prior to stimulation with BAY (10uM) for 60 minutes (n=5). (B) Effect of PKI and CALC on BAY-induced VASPSer$_{239}$ phosphorylation. VSMCs were pretreated with PKI or CALC individually, PKI+CALC or vehicle for 30 min prior to stimulation with BAY (10uM) for 60 minutes (n=5). Values are the means ± SE. * = different from vehicle (P<0.05); † = different from vehicle and BAY (P<0.05).

had no further effect on Ser$_{157}$ phosphorylation. BAY-induced Ser$_{239}$ phosphorylation was potentiated only in the presence of PKI and the PDE 5 inhibitor ZAP (Fig 3C). Neither an inhibitor of PKA nor PKG and PDEs had any effect on Ser$_{239}$ in any other instance (data not shown).

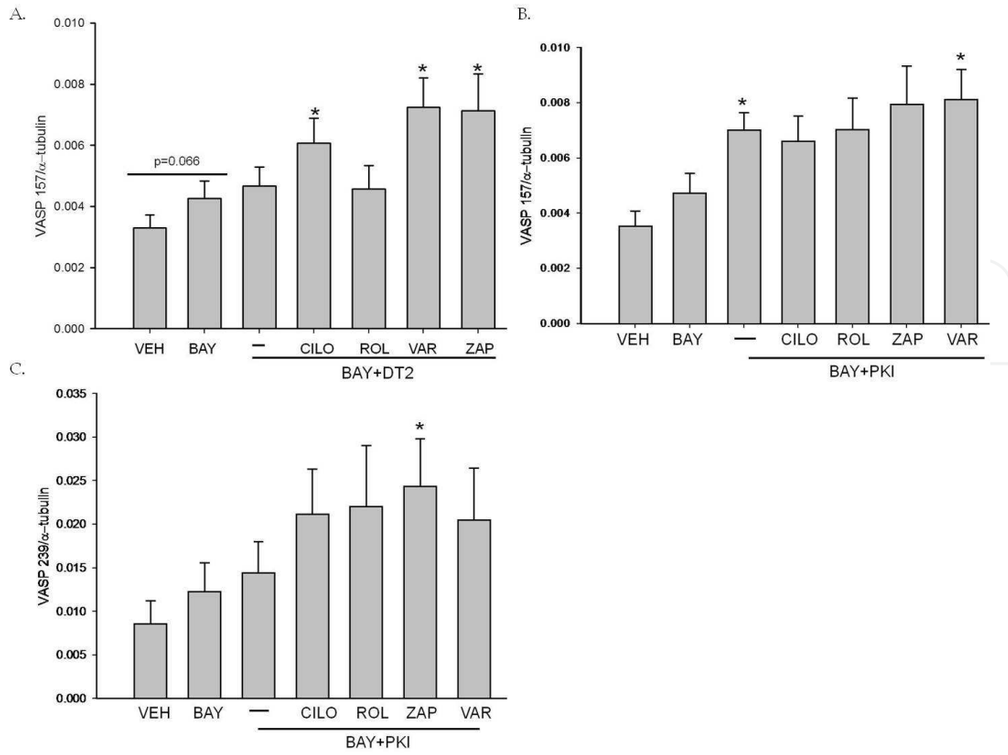


Fig. 3. (A) Effect of DT2 and PDE inhibitors on BAY-induced VASPSer$_{157}$ phosphorylation. VSMCs were pretreated with DT2 alone or CILO, ROL, VAR, or ZAP in the presence of DT2 or vehicle for 30 min prior to stimulation with BAY (10uM) for 60 minutes (n=13). (B) Effect of PKI and PDE inhibitors on BAY-induced VASPSer$_{157}$ phosphorylation. VSMCs were pretreated with PKI alone, CILO, ROL, VAR, or ZAP in the presence of PKI, or vehicle for 30 min prior to stimulation with BAY (10uM) for 60 minutes (n=8). (C) Effect of PKI and PDE inhibitors on BAY 41-2272 (BAY)-induced VASPSer$_{239}$ phosphorylation. VSMCs were pretreated with PKI alone, CILO, ROL, VAR, or ZAP in the presence of PKI, or vehicle for 30 min prior to stimulation with BAY (10uM) for 60 minutes (n=14). Values are the means ± SE. * = different from BAY (P<0.05).

3.3 Effect of kinase inhibitors on cyclic nucleotide analog-induced VASP phosphorylation

Since BAY increases cAMP and cGMP (Joshi *et al.*, 2011; Mendelev *et al.*, 2009; Stasch *et al.*, 2001; Stasch *et al.*, 2009) as well as the activity of their respective kinases (Joshi *et al.*, 2011), and considering that both kinases can phosphorylate VASP at either kinase-preferred site (Butt *et al.*, 1994), cyclic nucleotide analogs were used to determine the kinase(s) specifically involved in VASP phosphorylation. VSMCs were incubated with selective kinase inhibitors prior to addition of a cAMP or cGMP analog. Results show that 8-Br-cAMP alone failed to significantly increase phosphorylation at Ser$_{157}$ or Ser$_{239}$, but PKI significantly increased phosphorylation at both sites induced by the analogs (Figs. 4A, 4B). Neither DT2 nor CALC had an effect on VASP phosphorylation in the presence of 8Br-cAMP. Additionally, when using 8Br-cAMP multiple kinase inhibition had no effect greater than that of PKI alone at either site (data not shown).

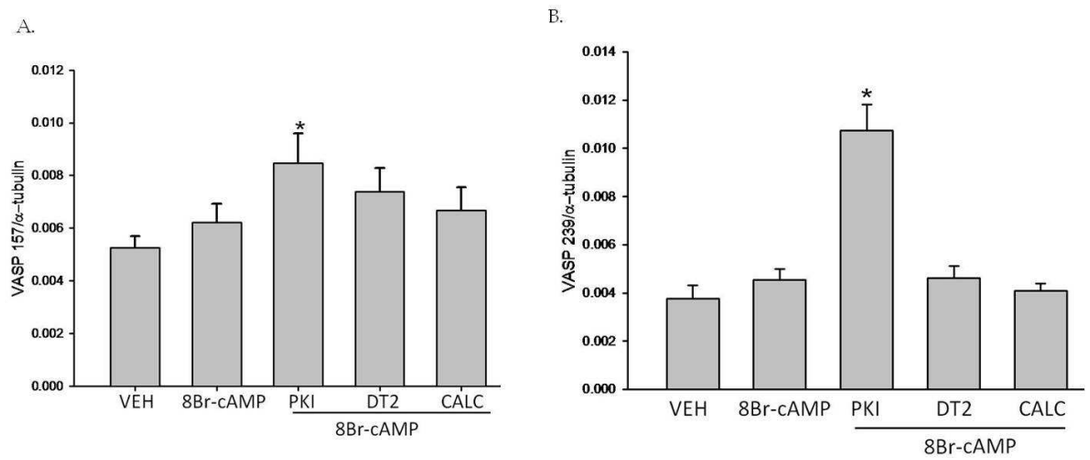


Fig. 4. (A) Effect of kinase inhibitors and 8Br-cAMP on VASPSer$_{157}$ phosphorylation. VSMCs were pretreated with PKI, DT2, CALC or vehicle for 30 min prior to stimulation with 8Br-cAMP (10uM) for 30 minutes (n=10). (B) Effect of kinase inhibitors and 8Br-cAMP on VASPSer$_{239}$ phosphorylation. VSMCs were pretreated with PKI, DT2, CALC or vehicle for 30 min prior to stimulation with 8Br-cAMP (10uM) for 30 minutes (n=10). Values are the means ± SE. * = different from vehicle (P<0.05).

In comparison, in these cells 8Br-cGMP alone failed to increase Ser$_{157}$ phosphorylation; however, co-treatment with PKI or CALC increased Ser$_{157}$ phosphorylation (Fig. 5A). On the other hand, 8Br-cGMP alone significantly increased phosphorylation at Ser$_{239}$ and a trend for potentiation by PKI was observed (p = 0.059; Fig. 5B).

Co-treatment with an inhibitor of PKG and PKC blunted the increase induced by 8-Br-cGMP at Ser$_{239}$ (Fig. 6).

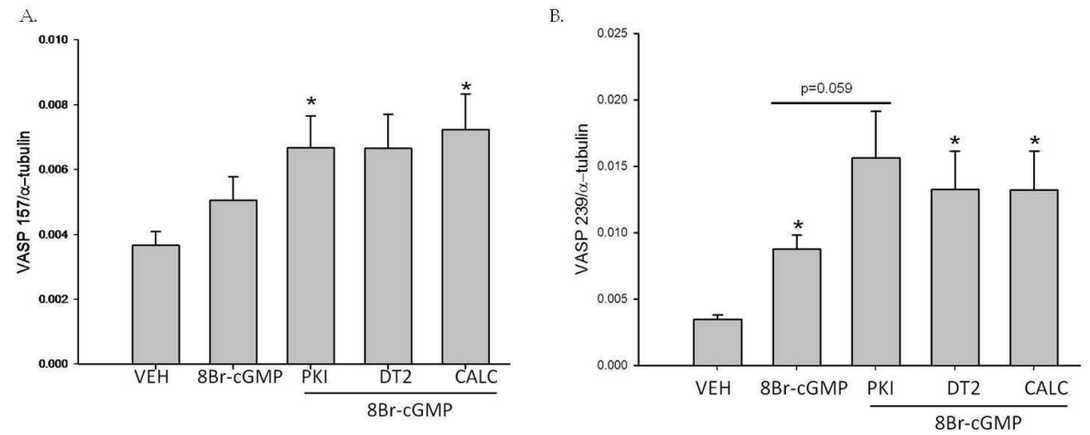


Fig. 5. (A) Effect of kinase inhibitors and 8Br-cGMP on VASPSer$_{157}$ phosphorylation. VSMCs were pretreated with PKI, DT2, CALC or vehicle for 30 min prior to stimulation with 8Br-cGMP (10uM) for 30 minutes (n=10). (B) Effect of kinase inhibitors and 8Br-cGMP on VASPSer$_{239}$ phosphorylation. VSMCs were pretreated with PKI, DT2, CALC or vehicle for 30 min prior to stimulation with 8Br-cGMP (10uM) for 30 minutes (n=10). Values are the means ± SE. * = different from vehicle (P<0.05).

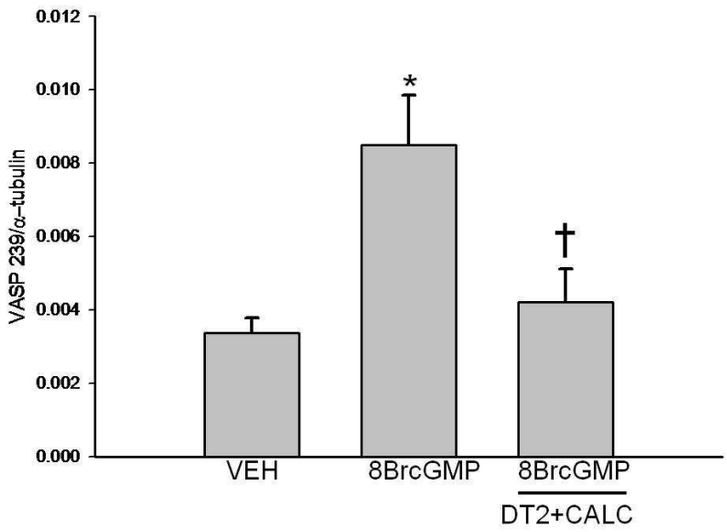


Fig. 6. Effect of CALC and DT2 on 8Br-cGMP-induced VASPSer$_{239}$ phosphorylation. VSMCs were pretreated with CALC and DT2 or vehicle for 30 min prior to stimulation with 8Br-cGMP for 30 minutes (n=8). Values are the means ± SE. * = different from vehicle (P<0.05); † = different from 8Br-cGMP (P<0.05).

3.4 Effect of BAY on Kinase activity

To determine if BAY increases kinase activity, VSMCs were pretreated with inhibitors of kinases prior to stimulation with BAY and selective kinase activity assays were performed. BAY increased the activity of PKA ~86% from (0.0278 ± 0.0223 to 0.0519 ± 0.0415). PKI decreased the activity back to control levels (0.0214 ± 0.0160). Similarly, BAY increased the activity of PKG ~55% from (0.0108 ± 0.0042 to 0.0167 ± 0.007) and DT2 decreased the activity back to control levels (0.0116 ± 0.003).

3.5 Effect of kinase inhibitors and BAY on Proliferation

Previously we concluded that BAY inhibits proliferation of both rat A7R5 (Mendelev *et al.*, 2009) and rat primary VSMCs (Joshi *et al.*, 2011). To determine the influence of kinases on BAY-induced growth suppression, three complementary assays were performed. In the MTT assay, a mitochondria reductase-dependent approach, cells were pretreated with kinase inhibitors prior to stimulation with BAY. BAY (non-significantly) decreased proliferation (~25%) after 24 hrs, and inhibition of either PKA or PKG significantly potentiated this effect (Fig. 7A). Using the BrdU assay, BAY significantly inhibited DNA replication after 48 hours, yet inhibition of any individual kinase had no further effect at this time point (Fig. 7B). Using hemocytometry, BAY reduced cell numbers after both 48 (Fig. 7C) and 72 hrs (Fig. 7D). Inhibition of PKA at 48 hrs (Fig. 7C) and either PKA or PKG at 72 hrs (Fig. 7D) potentiated this effect. On the other hand, a lower concentration of DT2 (1μM) had no effect at 72 hrs (Fig. 7E). The Neutral red and MTT assays done at 16 hrs demonstrate that BAY and all inhibitor combinations were not cytotoxic (data not shown).

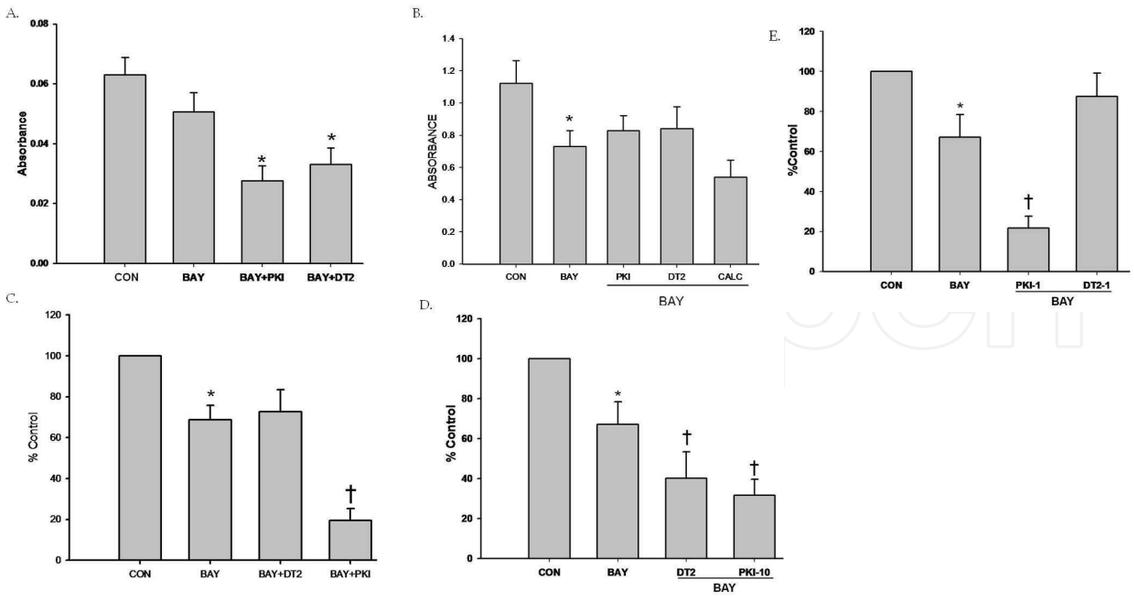


Fig. 7. (A) MTT assay to test the effect of PKI, DT2 and BAY on proliferation. VSMCs were pretreated with PKI, DT2 or vehicle for 30 min prior to stimulation with BAY (10uM) for 24 hours (n=9). (B) BrdU assay to test the effect of PKI, DT2, CALC and BAY on proliferation. VSMCs were pretreated with PKI, DT2, CALC or vehicle for 30 min prior to stimulation with BAY (10uM) for 48 hours (n=10). (C) Hemocytometry assay to test the effect of PKI, DT2 and BAY on proliferation. VSMCs were pretreated with PKI, DT2 or vehicle for 30 min prior to stimulation with BAY (10uM) for 48 hours (n=7). (D) Hemocytometry assay to test the effect of PKI, DT2 and BAY on proliferation. VSMCs were pretreated with PKI, DT2 or vehicle for 30 min prior to stimulation with BAY (10uM) after 72 hours (n=16). Values are the means ± SE. * = different from vehicle (P<0.05); † = different from vehicle and BAY (P<0.01). (E) Hemocytometry assay to test the effect of PKI, DT2 and BAY on proliferation. VSMCs were pretreated with PKI, DT2 or vehicle for 30 min prior to stimulation with BAY (10uM) for 72 hours (n=4). Values are the means ± SE. * = different from vehicle (P<0.05); † = different from vehicle and BAY (P<0.05).

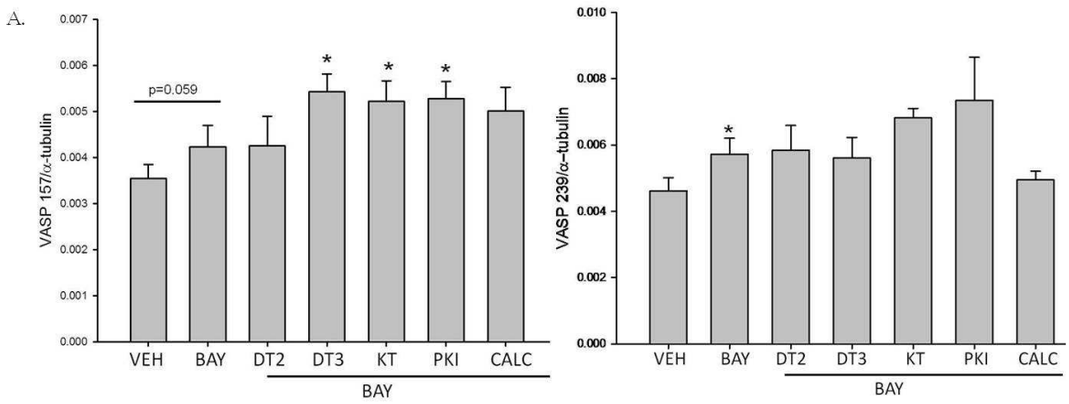
3.6 Effect of kinase inhibitors and BAY on Migration

Previously we observed that BAY inhibits cell migration in rat primary VSMCs (Joshi *et al.*, 2011). In order to determine the role of kinases on this anti-migratory effect of BAY, VSMCs were pretreated with inhibitors of PKG, PKA or PKC prior to stimulation with BAY and migration was assessed using the wounding assay. BAY inhibited migration ~40% after 6 hours and co-incubation of BAY with DT-2 or CALC inhibited migration ~13% or ~17%, respectively (data not shown).

3.7 The effect of kinase inhibitors on BAY-induced VASP phosphorylation in high passaged rat primary VSMCs

In comparison to studies performed at low passage, in higher passage VSMCs (passage > 6) either PKI or two chemically dissimilar inhibitors of PKG, DT3 and KT5823, significantly increased phosphorylation of VASP at Ser_{157} but had no effect at Ser_{239} (Suppl. Figs. 1A and

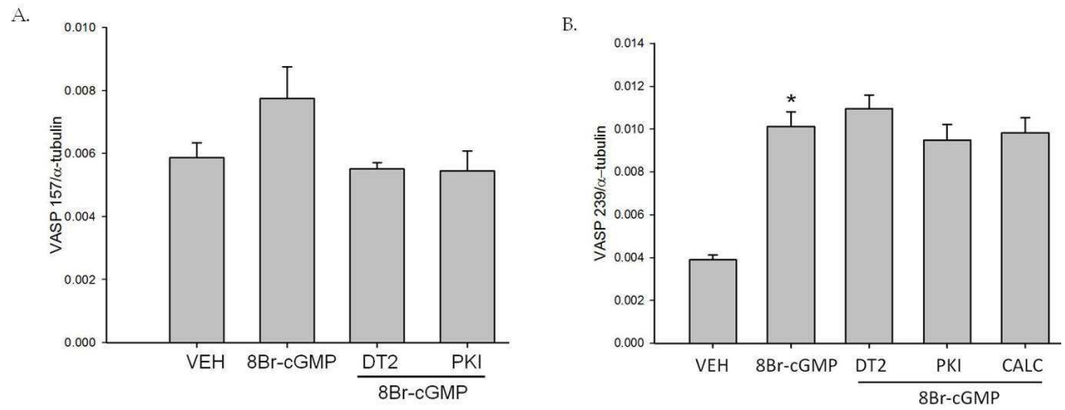
1B, resp.). Notably, in these higher passage cells inhibition of PKC by CALC did not affect phosphorylation at either site versus that of BAY alone. In these cells, inhibition of multiple kinases had no effect on Ser_{157} but any combination of kinase inhibitors inhibited VASP phosphorylation at Ser_{239} (data not shown).



Suppl. Fig. 1. (A) Effect of kinase inhibitors on BAY 41-2272 (BAY)-induced $VASPSer_{157}$ phosphorylation. Higher passaged (p > 6) VSMCs were pretreated with PKI, DT2, DT3, KT, CALC or vehicle for 30 min prior to stimulation with BAY (10uM) for 60 minutes (n=8). Values are the means ± SE. * = different from BAY (P<0.05). (B) Effect of kinase inhibitors on BAY-induced $VASPSer_{239}$ phosphorylation. Higher passaged (p > 6) VSMCs were pretreated with PKI, DT2, DT3, KT, CALC or vehicle for 30 min prior to stimulation with BAY (10uM) for 60 minutes (n=9). Values are the means ± SE. * = different from vehicle (P<0.05).

3.8 Effect of kinase inhibitors on cyclic nucleotide analog-induced VASP phosphorylation

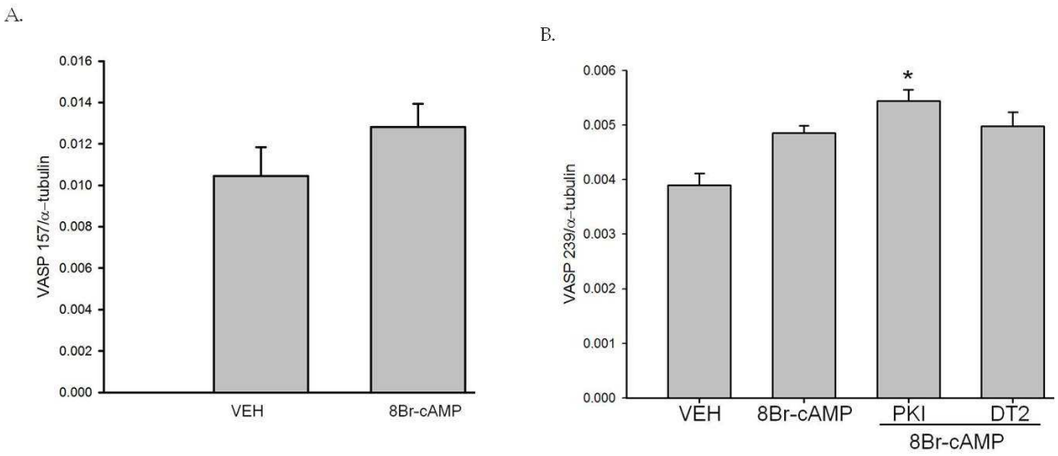
In higher passage VSMCs (p > 6), 8-Br-cGMP alone increased phosphorylation at Ser_{239} only (Suppl. Figs. 2A, 2B), and kinase inhibition was without effect at either site.



Suppl. Fig. 2. (A) Effect of kinase inhibitors and 8Br-cGMP on $VASPSer_{157}$ phosphorylation. VSMCs were pretreated with PKI, DT2 or vehicle for 30 min prior to stimulation with 8Br-cGMP (10uM) for 30 minutes (n=3). (B) Effect of kinase inhibitors and 8Br-cGMP on $VASPSer_{239}$ phosphorylation. VSMCs were pretreated with PKI, DT2, CALC or vehicle for

30 min prior to stimulation with 8Br-cGMP (10uM) for 30 minutes (n=21). Values are the means ± SE. * = different from vehicle (P<0.05).

8-Br-cAMP had no effect on site-specific VASP phosphorylation (Suppl. Fig 3A, 3B.). However, PKI further increased 8-Br-cAMP-induced phosphorylation at Ser_{239} (Suppl. Fig. 3B). In VSMCs p > 6 any combination of kinase inhibitors did not affect cAMP or cGMP-selective VASP phosphorylation (data not shown).



Suppl. Fig. 3. (A) Effect of 8Br-cAMP on $VASPSer_{157}$ phosphorylation. VSMCs were stimulated with 8Br-cAMP (10uM) for 30 minutes (n=15). (B) Effect of kinase inhibitors and 8Br-cAMP on $VASPSer_{239}$ phosphorylation. VSMCs were pretreated with PKI, DT2 or vehicle for 30 min prior to stimulation with 8Br-cAMP (10uM) for 30 minutes (n=20). Values are the means ± SE. * = different from vehicle (P<0.05).

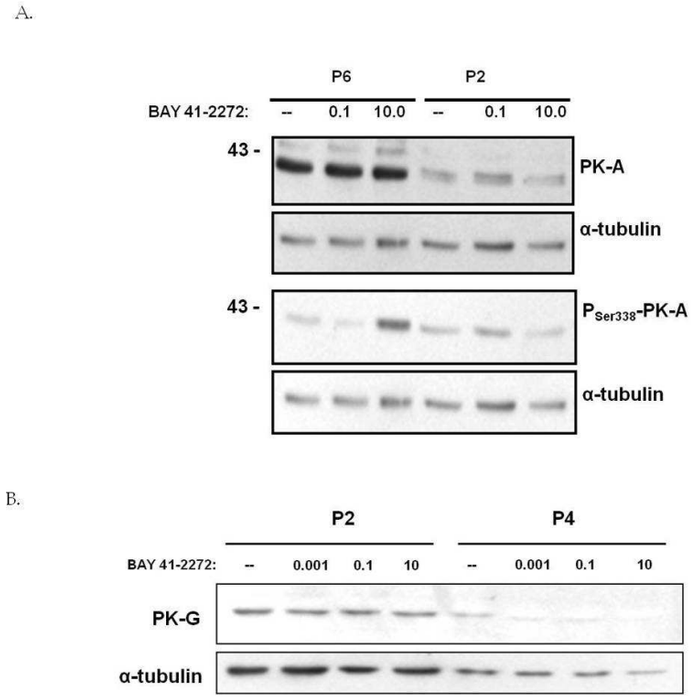
3.9 Effect of BAY on Kinase expression

In untreated VSMCs, Western analysis shows that there is an increase in total PKA in passage 6 cells versus passage 2 cells, yet concentration-dependent stimulation with BAY did not alter the amount of total PKA in either cohort (Suppl. Fig. 4A). Interestingly, BAY (at 10 μM) largely increased the amount of phosphorylated PKA (at Ser_{338}) in p6 VSMCs but not in p2 cells (Suppl. Fig. 4A). In comparison, the amount of basal untreated PKG decreased with increasing passage, and stimulation with BAY had no additive effect (Suppl. Fig. 4B).

4. Discussion

The sGC signal transduction system plays an important regulatory role in cardiovascular disease due to the production of cyclic nucleotides and their impact on downstream effectors. VSMCs primarily regulate vascular tone and contraction and are important in the medial layer of arteries. In animal models of vascular injury, intimal and medial thickening is attributed to phenotypic switching of resident VSMCs and their proliferation and migration from the adventitia and media to the intimal space (Janssens *et al.*, 1998; Kawashima *et al.*, 2001; von der Thusen *et al.*, 2004). Therefore, augmentation of neointimal formation in iatrogenic response to therapeutic intervention compromises flow and

increases the risk of a cardiac event. For that reason understanding the cellular mechanisms involved in vascular growth is of critical importance.



Suppl. Fig. 4. (A) Western analysis for Protein Kinase A (PKA) and phosphorylated-PKASer_{338}. VSMCs cytosolic preparations were incubated with antibodies generated against PKA and phospho-PKA. (B) Western analysis for Protein Kinase G (PKG). VSMCs cytosolic preparations were incubated with an antibody generated against PKG.

In this study we provide sound evidence that BAY, a synthetic stimulator of sGC, increases the activity of protein kinases and downstream effectors and that this system has the ability to control growth of VSM. Novel results show that activity from PKA, PKG and PKC has the capacity to increase phosphorylation of cellular proteins including VASP, a multi-functional cytoskeletal protein capable of regulating VSM cell proliferation and migration. Notably, the implication of multiple kinases in the regulation and activation of VASP provides evidence for potential crosstalk among otherwise distinct kinases within the cell. Functionally, strong evidence is presented linking cyclic nucleotide-mediated kinase activation and VASP phosphorylation with inhibition of VSM growth through modulation of proliferation and migration. BAY-induced site-specific VASP phosphorylation is regulated by kinases even though cross-talk exists among these (and perhaps other) kinases in VSM. These data demonstrate the importance of understanding cyclic nucleotide-directed kinases in regulating signaling proteins and their roles for inducing specific cellular responses.

VSMC proliferation, a critical mechanism in vessel remodeling, has been reported to be positively controlled by phosphorylation of VASP at Ser_{157} in VSMCs (Chen *et al.*, 2004). $VASP_{Ser157}$ is the site preferentially phosphorylated by PKA, but evidence suggests that both PKG and PKC can also phosphorylate Ser_{157} (Butt *et al.*, 1994; Chitaley *et al.*, 2004). In this

study we report that inhibition of PKA alone has no effect on Ser_{157} phosphorylation of VASP induced by BAY (Fig 1A); however, in the presence of a PKG inhibitor phosphorylation at this site is increased (Fig 2A). On the other hand, VASPSer_{239} is the site reported to be preferentially phosphorylated by PKG (Butt *et al.*, 1994), but PKA can also phosphorylate this site (Butt *et al.*, 1994). In this study, inhibition of PKG alone had no effect on VASP phosphorylation at Ser_{239} induced by BAY (Fig. 1B); however, in the presence of an inhibitor of PKC, BAY-induced phosphorylation was increased. These intriguing results demonstrate that there is crosstalk among these kinases in response to VASP phosphorylation induced by BAY: with respect to Ser_{157}, PKA and PKG are primarily involved, whereas at Ser_{239} the major kinases are PKA and PKC.

Cyclic nucleotide activity and localization in cells is regulated by PDEs which modulate the levels of cyclic nucleotide levels and maintains them at a steady state. Moreover, kinases have been shown to regulate PDE activity (Bender *et al.*, 2006). It has been reported that PKA phosphorylates and increases the activities of PDE3 and 4 (Bender *et al.*, 2006; Degerman *et al.*, 1997; Houslay *et al.*, 2007), while an increase in cGMP binding and phosphorylation by PKG increases the activity of PDE5 (Bender *et al.*, 2006; Rybalkin *et al.*, 2002). BAY has been shown to increase cyclic nucleotide content in VSMCs (Joshi *et al.*, 2011; Mendelev *et al.*, 2009); therefore, it is logical that PDE and kinase activity could potentially be modulated by BAY treatment. Preliminary data suggest that with respect to Ser_{157}, inhibition of PDE5 increased its phosphorylation while inhibition of PDE3 and PDE4 decreased its phosphorylation in response to BAY. Here we demonstrate that inhibition of PDE3 or PDE5 in the presence of an inhibitor of PKG increases the phosphorylation at this site while inhibition of PDE4 had no effect (Fig 3A). In the presence of PKA inhibition, Ser_{157} phosphorylation was increased and PDE inhibition had no additional effect (Fig 3B). Considering that PDE3 is a cGMP-inhibited PDE, following concomitant inhibition of both PDE5 and PKG, the resulting increase in cAMP and PKA is expected as an enhancement of BAY-induced signaling. The same observation is seen following simultaneous inhibition of PKG and PDE5, as there is an increase in cGMP which could then inhibit PDE3 and increase cAMP leading to additive phosphorylation at Ser_{157}. On the other hand, in the presence of an inhibitor of PKA and individual PDE inhibitors, no further phosphorylation is shown at Ser_{157} suggesting that this previously observed inhibitory effect is rescued.

At $VASP_{Ser239}$, preliminary data suggest that inhibition of PDE activity decreased phosphorylation at this site in response to BAY. Here we demonstrate that in the presence of an inhibitor of PKA or PKG, inhibition of PDEs no longer decreases Ser_{239} phosphorylation and in fact, inhibition of PDE5 with ZAP in the presence of PKI increased Ser_{239} phosphorylation (Fig 3C). These results support involvement of kinases in the regulation of PDEs. In this study we are inhibiting activation of PDEs via kinases as well as inhibiting the actual PDE, thereby increasing the cyclic nucleotide content of the cell and as a result increasing phosphorylation of VASP. This supports the argument that an increase in both cyclic nucleotides in a cell at the same time is inhibitory because two kinases are active simultaneously thereby activating opposing pathways. In addition, these findings confirm the theory of intracellular compartmentalization of second messengers and their effectors as described elsewhere (Di Benedetto *et al.*, 2008; Houslay *et al.*, 2007; Zaccolo *et al.*, 2002).

Activation of kinases in other pathways may negatively regulate phosphorylation of VASP. It is reported that PKG is the primary kinase responsible for phosphorylation of Ser_{239} (Butt

et al., 1994); in turn, PKG inhibition would hinder Ser_{239} phosphorylation and PKA would then become the primary kinase responsible for phosphorylation at this site. To further add to the complexity of these findings and to demonstrate the importance of kinase regulation and localization of proteins we demonstrate that inhibition of PDE5 in the presence of an inhibitor of PKA potentates the phosphorylation at Ser_{239}. This demonstrates that cGMP does indeed directly phosphorylate VASP at this site independent of cAMP/PKA signals (Fig. 3C).

BAY has been shown to increase the content of both cAMP and cGMP in VSMCs (Joshi *et al.*, 2011; Mendelev *et al.*, 2009); therefore, to directly assess its mechanisms of action and what kinases may be involved in site specific phosphorylation of VASP, cyclic nucleotide analogs which directly activate PKA and PKG were used. 8Br-cAMP is a direct activator of PKA, the kinase which directly phosphorylates Ser_{157} (Butt *et al.*, 1994). As one would expect addition of this analog should increase Ser_{157} phosphorylation of VASP due to the increase in PKA activity. We did not observe an increase with the analog alone (Fig. 4A); however, inhibition of PKA in the presence of the analog significantly increased VASP phosphorylation at this site (Fig. 4A). Interestingly, inhibition of PKG or PKC in the presence of 8Br-cAMP had no effect at Ser_{157}. Although PKG is reported to be the primary kinase responsible for phosphorylation of Ser_{239} there could potentially be crosstalk involving other kinases at this site. This observation was evidenced with Ser_{239} phosphorylation whereby inhibition of PKA increased phosphorylation in the presence of the cyclic nucleotide analog (Fig. 4B). These data could provide an argument against the selectivity of the analogs in activating kinases because 8Br-cAMP may be activating PKG or some other protein that in reality is phosphorylating VASP. 8Br-cGMP is a direct activator of PKG and alone has no effect on Ser_{157} but markedly increases Ser_{239} as expected (Fig 5A, 5B). However, inhibition of PKA and PKC increased phosphorylation at Ser_{157} in the presence of 8Br-cGMP (Fig 5A). Inhibition of PKA slightly increased Ser_{239} phosphorylation above that of 8Br-cGMP alone (Fig 5B), while inhibition of either PKG or PKC slightly but not significantly increases phosphorylation. We could infer here that PKA and PKG are competing for the same site; therefore inhibition of PKA increases the phosphorylation at any particular site. Alternatively, we could conclude that PKA negatively regulates phosphorylation of VASP because inhibition of PKA increases phosphorylation of both sites. In any event, these results demonstrate that cyclic nucleotide analogs and BAY use different mechanisms to phosphorylate VASP. This is demonstrated in the results shown here whereby inhibition of both PKG and PKC (Fig. 6) inhibit phosphorylation at Ser_{239}. We can conclude that both PKG and PKC are actively involved in phosphorylation of Ser_{239} in response to 8Br-cGMP. In comparison, multiple kinase inhibition had no effect on 8Br-cAMP in its phosphorylation of VASP. These data clearly demonstrate that phosphorylation and thus regulation of VASP at these sites is complex in that all three major protein kinases (PKA/G/C) are involved. Furthermore, BAY, originally characterized as a direct activator of sGC, equally increases both PKA and PKG activity in these cells (Fig. 7). Thus, it appears that PKA may be the more active kinase involved in the phosphorylative control of VASP in VSM.

Direct involvement of PKA in BAY-mediated reduction in proliferation is seen in both the MTT and hemocytometry results in this study, which suggest that PKA activity is necessary for proliferation of VSMCs (Fig 8). The involvement of PKA in proliferation was previously suggested by Chen and colleagues (Chen *et al.*, 2004) where they found that phosphorylation of VASP by PKA stimulates proliferation. The difference between our study and that of

Chen and associates is that our study uses rat primary VSMCs and phosphorylation of VASP was in response to a pharmacologic stimulus, whereas theirs was done in VASP knockout (KO) mice where wild type or mutant VASP was transduced and phosphorylation induced by NO. These results suggest that the cellular mechanism of inhibition of cell growth induced by BAY is precise and unique to the drug. Interestingly, in our study the effects of PKG on the ability of BAY to inhibit proliferation in the MTT and BrdU assays (Figs. 8A, 8B) is similar to that of PKA; however, according to hemocytometric analysis these effects are not apparent until a later timepoint of 72 hrs (Fig 8C).

In the study by Chen and others (Chen *et al.*, 2004), the investigators show that genetic KO of VASPSer_{239} promotes migration. In our previous study (Joshi *et al.*, 2011), we showed that BAY phosphorylates Ser_{239} and in turn inhibits migration, which appears specific as a mechanism of action since inhibition of PKG fully reversed this effect. In the current study, data support these previous findings. Interestingly, the effect that PKG has on migration is similar to what is seen with PKC. Considering the influence of 8Br-cGMP on $VASP_{Ser239}$ (Fig. 6), these data support the involvement of PKG and PKC with VASP phosphorylation at Ser_{239} and their regulatory role in migration of VSMCs.

To summarize these functional data, with respect to proliferation VASPSer_{157} and PKA appear to be more involved and for migration VASPSer_{239} and PKG may be more involved in VSMCs. In an instance where one kinase is reduced or rendered dysfunctional, other kinases may compensate providing multiple kinase regulation of any particular functional event. Additionally, these data demonstrate that PDEs help to maintain tight regulation of cyclic nucleotides in these cells. Inhibition of PDEs increases the activity of proteins involved in phosphorylation of VASP, and inhibition or feedback regulation occurs because both cyclic nucleotides are high at the same time demonstrating the importance of compartmentalization of proteins. It is also possible that BAY increases the activity of some alternate pathways that may be involved with VASP phosphorylation in these cells.

In separate experiments, higher passaged cells were used to evaluate the influence of phenotypic switching in the response to cyclic nucleotide and kinase signaling. In these cells ($p>6$) treated with BAY, inhibition of PKA increased phosphorylation of VASP at Ser_{157} but had no effect on Ser_{239}, while inhibition of PKG increased phosphorylation of Ser_{157} but had no effect on Ser_{239} (Suppl. Fig. 1). On the other hand, inhibition of any kinase in the presence of a cyclic nucleotide analog had no effect (Suppl. Figs 2-3), except in the presence of 8Br-cAMP at Ser_{239} (suppl. Fig. 3B). We also show that the amounts of total PKA and phosphorylated PKA increase and that the amount of PKG protein decreases with passage (suppl. Figs. 5A, 5B). Thus, these signaling events and in particular kinase expression must be considered with elevated passage as a model of phenotypic changes associated with injury or disease.

5. Conclusion

In conclusion, in this study we demonstrate that: 1) BAY increases phosophorylation of VASP at both Ser_{157} and Ser_{239}; 2) BAY increases both PKA and PKG activity; 3) BAY reduces proliferation and migration in VSMCs; 4) inhibition of PKA in response to BAY augments inhibition of proliferation of VSMCs yet has no effect on migration; 5) multiple kinases appear to be involved in regulation of VASP phosphorylation; 6) PKA appears to be the kinase that is most active in these cells in response to BAY, and 7) kinases are involved in regulation of PDEs

that regulate VASP phosphorylation in VSMCs. These findings provide valuable details about the biochemical cell signaling associated with cyclic nucleotide second messengers in VSM and highlight BAY as a potential anti-growth modulator in VSM.

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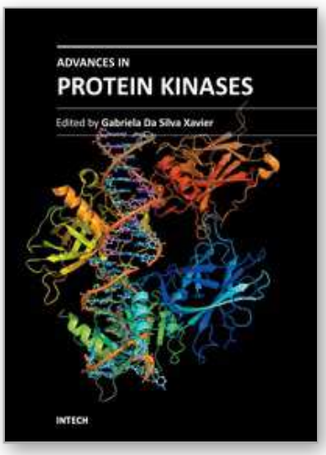
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Proteins are the work horses of the cell. As regulators of protein function, protein kinases are involved in the control of cellular functions via intricate signalling pathways, allowing for fine tuning of physiological functions. This book is a collaborative effort, with contribution from experts in their respective fields, reflecting the spirit of collaboration - across disciplines and borders - that exists in modern science. Here, we review the existing literature and, on occasions, provide novel data on the function of protein kinases in various systems. We also discuss the implications of these findings in the context of disease, treatment, and drug development.

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