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Vasodilator-stimulated phosphoprotein is a substrate for protein kinase C

K. Chitaley[a,*], L. Chen[a], A. Galler[b], U. Walter[b], G. Daum[a], A.W. Clowes[a]

[a] *Department of Surgery, University of Washington, Seattle, WA 98195, USA*
[b] *Institut fur Klinische Biochemie und Pathobiochemie, Universität Würzburg, Germany*

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Abstract Vasodilator-stimulated phosphoprotein (VASP), an actin binding protein localized to areas of focal contacts, is a substrate for the cyclic adenosine monophosphate/cyclic guanosine monophosphate (cAMP/cGMP)-dependent protein kinases (PKA, PKG). In this study, we show that serum stimulation of vascular smooth muscle cells (SMCs) induces VASP phosphorylation on Ser157, in a mechanism not dependent on PKA or PKG. We tested the possibility that protein kinase C (PKC), a regulator of cytoskeletal function, is involved. PKC inhibition or down-regulation prevented serum-induced phosphorylation of VASP at Ser157 in rat vascular SMCs. Additionally, recombinant PKCα directly phosphorylated Ser157 on VASP. In summary, our data support the hypothesis that PKC phosphorylates VASP and mediates serum-induced VASP regulation.
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Key words: Phosphorylation; Ser/Thr kinase; Focal adhesion

1. Introduction

Vasodilator-stimulated phosphoprotein (VASP), the first identified member of the Ena/Mena/VASP family, is an actin binding protein that is localized to focal adhesions and cell-to-cell contacts [1,2]. VASP is present in numerous cell types including platelets, endothelial cells, smooth muscle cells (SMCs) and fibroblasts [1,3]. Through highly conserved EVH1 and EVH2 binding domains, VASP interacts directly with F-actin, the Listeria surface protein, ActA, and the focal adhesion proteins, vinculin, zyxin, and profilin [2,4–7]. Various studies describe a role for VASP in the assembly and bundling of actin filaments, a dynamic process that mediates cellular functions such as adhesion, motility, cell cycle progression, and contraction [8–12].

Studies using VASP-deficient mice have demonstrated enhanced platelet aggregation and diminished cyclic nucleotide-mediated platelet inhibition, but no significant effect on SMC function such as contraction of isolated aorta and fundus [13,14]. This lack of effect may be explained by a potential redundant role of the VASP-related protein Mena, which is expressed in SMCs but absent in platelets. The role of VASP in cellular motility has generated much debate. Recent studies suggest that VASP may play a paradoxical role by increasing lamellipodial velocity, while decreasing whole cell speed, in part, through the inhibition of actin-capping proteins [12,15,16].

VASP is phosphorylated on three sites by the cyclic adenosine monophosphate/cyclic guanosine monophosphate (cAMP/cGMP)-dependent protein kinases, protein kinase A (PKA) and protein kinase G (PKG) [17]. The preferred phosphorylation site for PKA is Ser157, which leads to a retardation of VASP mobility during sodium dodecyl sulfate (SDS)–gel electrophoresis, resulting in an apparent shift in molecular weight of VASP from 46 to 50 kDa. Phosphorylation of Ser239 on VASP, the preferred site for PKG, as well as Thr278 (a less favored site for both PKA and PKG), does not alter VASP mobility during SDS–gel electrophoresis. To date, the exact role of each of the three phosphorylation sites is unclear. A positive correlation between the phosphorylation of Ser157 on VASP and the inhibition of integrin $\alpha_{IIb}\beta_3$ (fibrinogen receptor) activation in platelets is reported [18]. In endothelial cells, PKG-induced phosphorylation resulted in the detachment from focal adhesions of wild-type VASP, but not of a mutant VASP containing Ser/Thr to Ala substitutions at all three phosphorylation sites [19]. Phosphorylation of Ser157 on VASP correlated with the detachment and spreading of fibroblasts and epithelial cells [20]. Further, PKA-induced phosphorylation of VASP decreased actin nucleation and binding, but had no effect on the association of VASP with vinculin, zyxin or profilin [21]. Although the exact function of VASP phosphorylation is not defined, these studies suggest that discrete phosphorylation events may be critical for the regulation of VASP-mediated modulation of actin cytoskeletal and focal adhesion-related processes.

To date, PKA and PKG are the only kinases known to phosphorylate VASP. However, many protein kinases are involved in the regulation of cytoskeletal and focal adhesion-mediated events, including protein kinase C (PKC) family members. For example, PKCδ and PKCα interact with focal adhesion proteins such as vinculin and talin, and PKC members mediate actin–membrane interactions as well as integrin-regulated signaling [22–25]. This study tested the hypothesis that serum modulates VASP phosphorylation in a PKC-dependent mechanism in vascular SMCs.

2. Materials and methods

2.1. Cell culture

Rat aortic SMCs were prepared as previously described [26]. Aortas from Fischer 344 rats (3 months of age, Simonsen Laboratories, Gilroy, CA, USA) were isolated and enzymatically digested. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (serum; Gibco Life Technologies, Rockville, MD, USA), and used at passages 5–9.

*Corresponding author. Fax: (1)-206-616 7495.
E-mail address: kanchanc@u.washington.edu (K. Chitaley).

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2.2. Cell treatment and Western blot analysis

SMCs were plated at ~150 000 cells/well using 6-well plates, and grown to 70% confluence in DMEM containing 10% serum. Serum-containing DMEM was then replaced with serum-free DMEM for 2 days in order to achieve quiescence. Cells were treated with inhibitors of PKA (Rp-8-Br-cAMPS, 50–100 μM, BioLog-Life Science-Institute and Calbiochem) and PKG (Rp-8-pCPT-cGMPS, 50 μM or Rp-8-Br-PET-cGMPS, 50 μM, BioLog-Life Science-Institute and Calbiochem) or PKC (GF109203X, 10 μM; Gö6976, 10 μM; Gö6983, 5 μM; rottlerin, 10 μM; all from Calbiochem) prior to and during stimulation with 10% serum or phorbol-12-myristate-13-acetate (PMA, 1 μM). In some experiments, quiescent cells were incubated with PMA (5 μM) or dimethyl sulfoxide (DMSO) vehicle for 24 h prior to serum treatment. Cells were lysed in Laemmli buffer and subjected to Western blot analysis as previously described [26]. Membranes were probed with rabbit polyclonal anti-VASP (1:2000 dilution, Alexis Co.), mouse anti-phospho-Ser157 (as previously described [27]), or mouse anti-phospho-Ser239 antibody (as previously described [28]). Antibody detection was visualized using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA) according to manufacturer's protocols.

2.3. In vitro kinase activity assay

Recombinant wild-type human VASP protein was prepared as previously described [28]. Wild-type VASP (0.1 μg/reaction) was diluted in ice-cold kinase buffer (10 mM magnesium chloride, 20 mM Tris–HCl, pH 7.5) with recombinant PKCα (human active, 0.008 U/reaction, Calbiochem). Calcium chloride (0.5 mM), a micelle suspension of 0.3 mg/ml phosphatidylserine and 30 μg/ml diacylglycerol, and adenosine triphosphate (ATP) (100 μM final concentration) with or without [γ^{32}P]ATP (1 μCi/reaction), was added to initiate the kinase reaction. Samples were incubated at 30°C for 5 min, and the kinase reaction stopped by the addition of four times Laemmli sample buffer. Reactions containing [γ^{32}P]ATP were run on 8% SDS–polyacrylamide gels, and then fixed in 10% trichloroacetic acid and 1% sodium pyrophosphate overnight. Gels were stained with Coomassie blue to

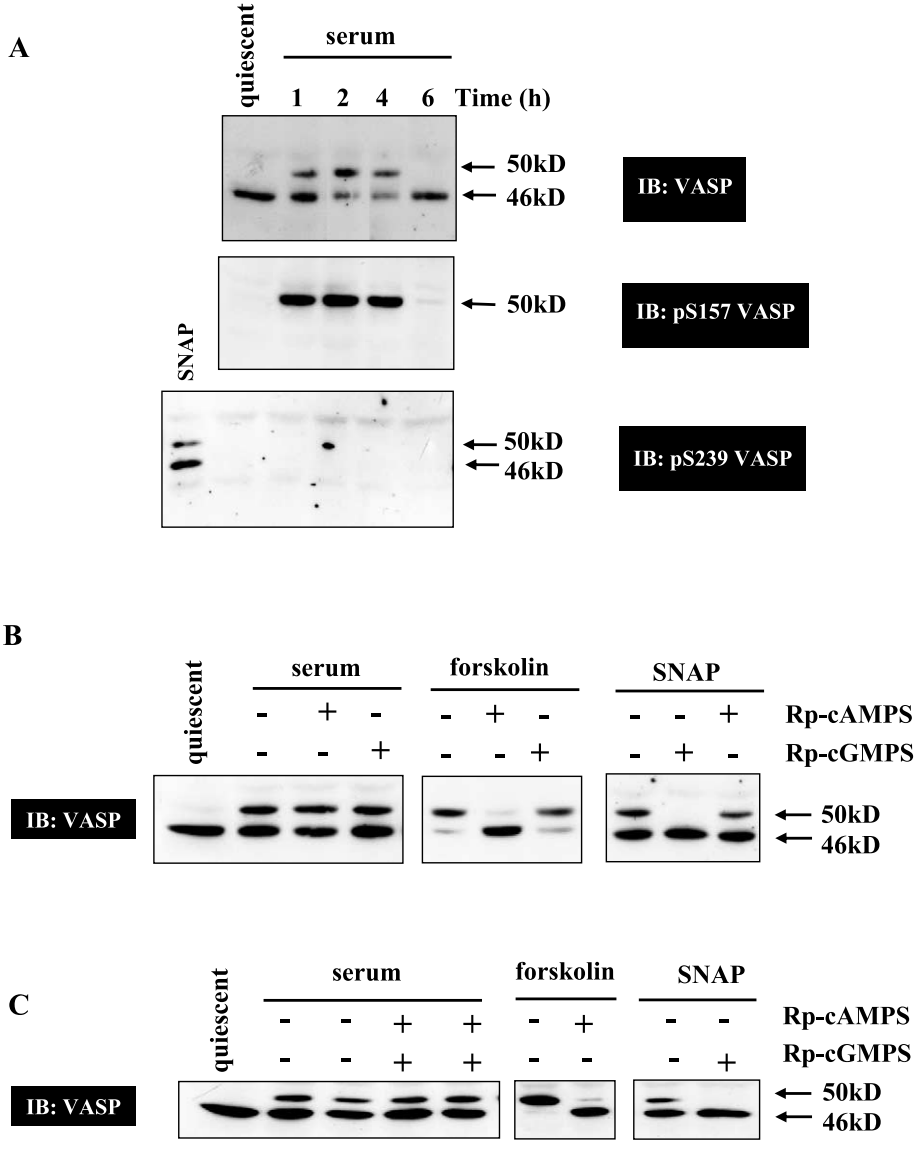

Fig. 1. Serum-induced phosphorylation of VASP at Ser157 is not dependent on PKA or PKG. A: Quiescent SMCs were treated for 1, 2, 4 and 6 h with serum (10%) and the phosphorylation of Ser157 and Ser239 on VASP examined by Western blot analysis. B: SMCs were treated with Rp-8-Br-cAMPS (50 μM) or Rp-8-pCPT-cGMPS (50 μM), inhibitors of PKA and PKG, respectively, and the phosphorylation of VASP on Ser157 in response to serum (10%) examined. C: Additionally, SMCs were treated with both Rp-8-Br-cAMPs (100 μM) and Rp-8-Br-PET-cGMPS (50 μM) and VASP phosphorylation in response to serum examined (representative blot depicts duplicate samples). Attenuation of VASP phosphorylation in response to the adenylate cyclase agonist, forskolin (1 μM), and the nitric oxide donor, SNAP (250 μM), by PKA and PKG blockade, respectively, served as positive controls. Blots shown are representative of four independent experiments.

identify the apparent molecular weight of VASP, dried, and radiolabeled phosphate incorporation visualized on autoradiograph film. Samples containing only non-radiolabeled ATP were loaded for SDS–polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membrane, and probed with polyclonal antibody to VASP.

2.4. Statistical analysis

Western blots were quantified by densitometry (ImageQuant, Molecular Dynamics), and the ratio of phospho-Ser157 VASP to total VASP was compared between each condition and analyzed statistically using one-way analysis of variance (ANOVA) with Student's Newman–Keuls post hoc test.

3. Results

3.1. Serum-induced phosphorylation of VASP at Ser157 does not depend on PKA or PKG

The formation and regulation of focal adhesions and stress fibers is modulated by serum stimulation. We thus examined the effects of serum on the phosphorylation of VASP. Treatment of quiescent rat vascular SMCs with serum (10%, 1–6 h) resulted in VASP phosphorylation at Ser157, as determined by a shift in the apparent molecular weight of VASP from 46 to 50 kDa upon SDS–PAGE, and by detection with a phospho-specific Ser157 VASP antibody (Fig. 1A). The serum-induced phosphorylation peaked at 2 h, and was undetectable at 6 h after stimulation (Fig. 1A). Treatment with platelet-derived growth factor BB and basic fibroblast growth factor, also induced phosphorylation of Ser157 on VASP (data not shown). Treatment of SMCs with serum did not stimulate phosphorylation of Ser239 on VASP (Fig. 1A). The effect of growth factor treatment on the regulation of Thr278 on VASP, the third known phosphorylation site, was not similarly examined by Western blot analysis due to lack of available antibodies to date.

Because both PKA and PKG phosphorylate Ser157 on VASP, we investigated whether serum-induced phosphorylation of VASP is mediated by PKA or PKG. Pretreatment of cells with Rp-8-Br-cAMPS (50 μM) or Rp-8-pCPT-cGMPS (50 μM), inhibitors of PKA and PKG, respectively, had no effect on serum-induced phosphorylation of VASP on Ser157 (Fig. 1B). Treatment with Rp-8-Br-cAMPS or Rp-8-pCPT-cGMPS, however, prevented VASP phosphorylation in response to the adenylate cyclase agonist, forskolin, or an activator of soluble guanylate cyclase, the nitric oxide donor, *S*-nitroso-*n*-acetylpenicillamine (SNAP), respectively (Fig. 1B).

To examine the possibility of overlapping functions of PKA and PKG in mediating serum-induced VASP phosphorylation, quiescent SMCs were treated with both Rp-8-Br-cAMPS (100 μM) and Rp-8-Br-PET-cGMPS (50 mM) (to inhibit PKA and PKG, respectively), and the response to serum stimulation tested. Concurrent blockade of PKA and PKG had no effect on the phosphorylation of VASP by serum (Fig. 1C). Treatment with Rp-8-Br-cAMPS and Rp-8-Br-PET-cGMPS did prevent forskolin and SNAP-induced VASP phosphorylation, respectively (Fig. 1C).

3.2. A PMA-sensitive isoform of PKC mediates serum-induced VASP phosphorylation on Ser157

PKC regulates the formation of focal adhesions through phosphorylation of various cytoskeletal proteins. We tested whether PKC mediates serum-induced phosphorylation of VASP on Ser157. Quiescent SMCs were treated for 24 h with the phorbol ester, PMA (5 μM), in order to down-regulate PKC, or DMSO vehicle. Following treatment, cells were stimulated with serum (10%) or forskolin (1 μM). Serum induced phosphorylation of VASP at Ser157 in vehicle but not in PMA-treated cells (Fig. 2A). However, forskolin (and SNAP, 250 μM) induced VASP phosphorylation at Ser157 in both controls and PMA-treated cells (as shown for forskolin in Fig. 2A). These data suggest that a PMA-sensitive isoform(s) of PKC mediates VASP phosphorylation on Ser157 in response to serum.

We next sought to verify that activation of PKC induces phosphorylation of Ser157 on VASP in SMCs. PKC was stimulated by acute treatment of quiescent SMCs with PMA. Treatment with PMA (1 μM, 30 min) resulted in phosphorylation of Ser157 on VASP (Fig. 2B). The PMA-induced phosphorylation of VASP was completely blocked by inhibition of PKC with GF109203X (10 μM) (Fig. 2B). Phosphorylation of Ser239 on VASP was not detected in response to PMA stimulation (data not shown).

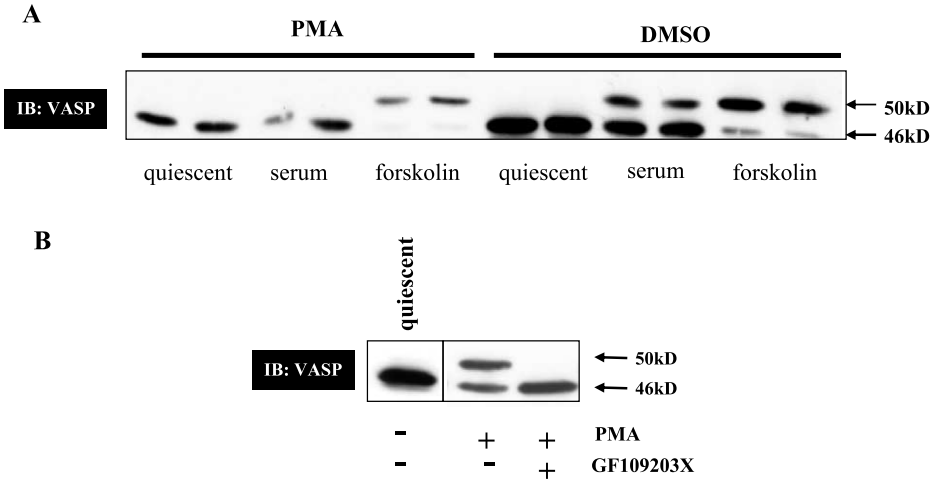

Fig. 2. A PMA-sensitive isoform of PKC is involved in phosphorylation of Ser157 on VASP. A: Quiescent SMCs were treated for 24 h with PMA (5 μM) or DMSO before exposure to serum (10%) or forskolin (1 μM). Phosphorylation of VASP on Ser157 was detected by Western blot analysis. B: Quiescent SMCs were treated with PMA (1 μM, 30 min) in the presence or absence of GF109203X (10 μM) and the phosphorylation of Ser157 on VASP examined. Blots shown are representative of four independent experiments.

3.3. Serum-induced stimulation of Ser157 on VASP is attenuated by inhibition of PKCα and β

Using inhibitors of various PKC isoforms, we sought to determine which PKC isoform(s) mediates serum-induced VASP phosphorylation. Treatment of rat vascular SMCs with various PKC inhibitors, GF109203X (10 μM, to inhibit PKCα, β, γ, δ, and ε), Gö6976 or Gö6983 (10 μM and 5 μM, respectively, to inhibit the classical isoforms) [29–32], attenuated serum-induced Ser157 phosphorylation on VASP 51 ± 5%, 63 ± 4% and 66 ± 10%, respectively (Fig. 3). However, treatment with the PKCδ inhibitor, rottlerin (10 μM), had no significant effect on serum-induced Ser157 VASP phosphorylation (Fig. 3). These data are consistent with the involvement of diacylglycerol-dependent PKC, and suggest that the α, β or γ isoform mediates serum-induced VASP phosphorylation in rat vascular SMCs.

3.4. Recombinant PKCα directly phosphorylates VASP at Ser157

We next examined whether PKCα directly phosphorylates VASP. PKCα has been previously shown to account for the majority of PKC activity in rat vascular SMCs, and may localize to areas of focal adhesions [22–26]. Kinase activity was first confirmed by the phosphorylation of myelin basic protein by recombinant PKCα (data not shown). Further analysis demonstrated human recombinant PKCα to phosphorylate recombinant human VASP, as detected by radio-

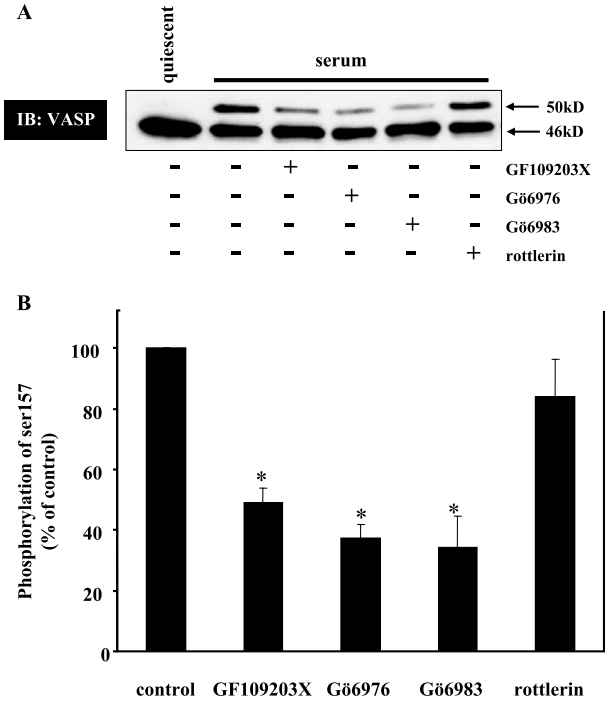

Fig. 3. Serum-induced phosphorylation of VASP at Ser157 is mediated by PKCα or β. Quiescent SMCs were treated for 30 min with inhibitors of PKC: GF109203X (10 μM), Gö6983 (5 μM), Gö6976 (10 μM), or rottlerin (10 μM), prior to and during stimulation with serum (10%) and the phosphorylation of Ser157 on VASP examined by Western blot analysis. A: Representative Western blot. B: Phosphorylated Ser157 on VASP as a percent of the serum-stimulated control response. The percent of VASP that was phosphorylated Ser157 was calculated from arbitrary densitometry units as a ratio of phosphorylated Ser157 (50 kDa band) divided by total VASP (50 kDa band+46 kDa band). Graph of results from four to six independent experiments; $*P < 0.05$, ANOVA.

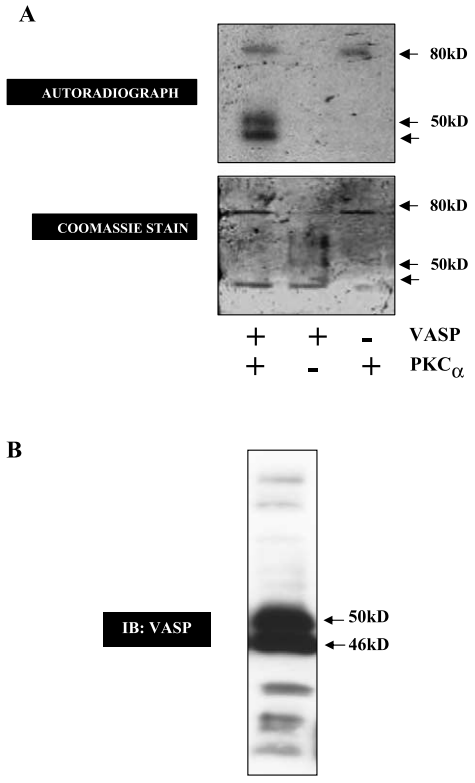

Fig. 4. Recombinant PKCα directly phosphorylates recombinant human VASP. Kinase activity of recombinant PKCα was initiated by the addition of phospholipids and ATP, and the phosphorylation of recombinant VASP (46–50 kDa bands) measured through analysis of radiolabeled phosphate incorporation or by immunoblotting with antibody against VASP (kinase reaction run at 30°C, 5 min). A: Autoradiograph of [^{32}P]phosphate incorporation and corresponding Coomassie-stained gel. Radiolabeled phosphate incorporation was not detected in the 46–50 kDa molecular weight bands in reactions lacking substrate or enzyme. Incorporation of radiolabeled phosphate corresponding to ~80 kDa may be indicative of autophosphorylation of PKCα, and is absent in reactions in which no enzyme was added. B: Western blot probed with polyclonal antibody against VASP.

labeled phosphate incorporation corresponding to the apparent molecular weights of 46 and 50 kDa (Fig. 4A). The higher molecular weight band corresponded to the electrophoretic mobility-shifted phosphorylated form of Ser157 on VASP. No radiolabeled phosphate incorporation corresponding to 46–50 kDa by SDS–PAGE was detected in reactions lacking either recombinant VASP or PKCα (Fig. 4A). Immunoblot analysis with an antibody against total VASP demonstrated activation of PKCα to result in a shift in wild-type VASP to the 50 kDa form, indicative of phosphorylation of Ser157 on VASP (Fig. 4B).

4. Discussion

A role for VASP in the regulation and modulation of focal adhesions and the actin cytoskeleton has been suggested. In this study, we show that serum treatment induces phosphorylation of VASP on Ser157 in rat vascular SMCs, and demonstrate this phosphorylation event to be mediated by PKC. We further show that PKCα directly phosphorylates VASP, introducing potential novel kinase involved in the regulation of VASP.

In rat vascular SMCs, serum-induced VASP phosphorylation is not inhibited by blockade of PKA or PKG, the known VASP kinases to date, but is prevented by inhibition of PKC (Figs. 1–3). Additionally, activation of PKC with the phorbol ester, PMA, induces phosphorylation of Ser157 on VASP, in a GF109203X-sensitive manner (Fig. 2B). In HEK 293 cells, PMA-induced phosphorylation of VASP is inhibited by blockade of PKG [33]. However, in our study, inhibition of PKG does not prevent PMA-induced VASP phosphorylation of rat vascular SMCs, which may indicate cell-type specific regulation of VASP (data not shown).

PKC mediates the formation and regulation of focal adhesions in response to cell adhesion and growth factor stimulation [22,24,25]. Our data in rat vascular SMCs suggest that a classical, diacylglycerol-dependent isoform of PKC mediates serum-induced VASP phosphorylation. The α isoform of PKC has been shown to account for the majority of PKC activity in rat vascular SMCs [26]. Thus, we examined PKCα-induced phosphorylation of VASP, but do not exclude the possibility that the β isoform(s) of PKC plays a role in VASP regulation (PKCγ is not found in detectable levels in rat vascular SMCs [26]). The in vitro phosphorylation of recombinant human VASP by PKCα results in the incorporation of radiolabeled phosphate at the apparent molecular weights of 46 and 50 kDa during SDS–PAGE (Fig. 4A). The upper 50 kDa band corresponds to that of VASP phosphorylated on Ser157. However, the significance of the radiolabeled phosphate incorporation at the position of the lower 46 kDa band is unclear, as it may indicate phosphorylation of additional sites on VASP, such as Thr278 or a yet unidentified site.

PKC is established to modulate focal adhesions and stress fibers through numerous mechanisms. The α isoform of PKC is translocated to focal adhesions upon integrin $\alpha_{IIb}\beta_3$ activation of the fibronectin receptor, and is reported to phosphorylate focal adhesion proteins such as vinculin and talin [23–25,34]. Additionally, PKC modulates the cellular cytoskeleton through the regulation of intermediate filament and stress fiber-related proteins, including vimentin, CPI-17, myosin light chain kinase, tau and numerous others [24,35]. The results of this study suggest that PKCα phosphorylates VASP, demonstrating a novel cytoskeleton-associated substrate for PKC and a potential additional mechanism of PKC-mediated focal adhesion/stress fiber regulation.

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