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Ca^{2+}/calmodulin-stimulated PDE1 regulates the beta-catenin/TCF signaling through PP2A B56 gamma subunit in proliferating vascular smooth muscle cells

Kye-Im Jeon[1], **Hirofumi Jono**[2], **Clint L. Miller**[1], **Yujun Cai**[1], **Soyeon Lim**[1], **Xuan Liu**[3], **Pingjin Gao**[4], **Jun-Ichi Abe**[1], **Jian-Dong Li**[2], and **Chen Yan**[1]

[1] Aab Cardiovascular Research Institute, University of Rochester Medical Center, Rochester, NY 14642

[2] Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY14642

[3] Department of Biochemistry, University of California, Riverside, CA 92521

[4] Ruijin Hospital, Shanghai Institute of Hypertension, Shanghai, China

Abstract

The phenotypic change of vascular smooth muscle cells (VSMCs), from a “contractile” phenotype to “synthetic” phenotype, is crucial for pathogenic vascular remodeling in vascular diseases such as atherosclerosis and restenosis. Ca^{2+}-calmodulin stimulated phosphodiesterase 1 (PDE1) isozymes, including PDE1A and PDE1C, play integral roles in regulating the proliferation of synthetic VSMCs. However, the underlying molecular mechanism(s) remain unknown. In this study, we explore the role and mechanism of PDE1 isoforms in regulating β-catenin/TCF signaling in VSMCs, a pathway important for vascular remodeling through promoting VSMC growth and survival. We found that inhibition of PDE1 activity markedly attenuated β-catenin/TCF signaling by down-regulating β-catenin protein. The effect of PDE1 inhibition on β-catenin protein reduction is exerted via promoting GSK3β activation, β-catenin phosphorylation, and subsequent β-catenin protein degradation. Moreover, PDE1 inhibition specifically upregulated phosphatase PP2A B56γ subunit gene expression, which is responsible for the effects of PDE1 inhibition on GSK3β and β-catenin/TCF signaling. Further more, the effect of PDE1 inhibition on β-catenin was specifically mediated by PDE1A but not PDE1C isozyme. Interestingly, in synthetic VSMCs PP2A B56γ, phospho-GSK3β, and phospho-β-catenin were all found in the nucleus, suggesting that PDE1A regulates nuclear β-catenin protein stability through the nuclear PP2A-GSK3β-β-catenin signaling axis. Taken together these findings provide direct evidence for the first time that PP2A B56γ is a critical mediator for PDE1A in the regulation of β-catenin signaling in proliferating VSMCs.

Keywords

Phosphodiesterase; cyclic nucleotide; β-catenin; PP2A; VSMC

Address correspondence: Chen Yan, PhD, Department of Medicine, Aab Cardiovascular Research Institute, University of Rochester School of Medicine & Dentistry, 601 Elmwood Avenue, Box CVRI, Rochester, NY 14642, Phone: 585-276-9792, Fax: 585-276-9830, chen_yan@urmc.rochester.edu.

INTRODUCTION

Abnormal proliferation of VSMCs contributes to various cardiovascular proliferating disorders such as atherosclerosis, postangioplasty restenosis, bypass vein-graft failure, and cardiac allograft vasculopathy. The identification and characterization of molecules that control the phenotypic changes is critical for preventing VSMC proliferating diseases. Recent studies have shown that β-catenin plays a critical role in primary VSMC proliferation and vascular remodeling [1–4]. Wnt/β-catenin signaling pathway is present in adult vascular cells and found to be activated in the vascular lesions, which is likely involved in VSMC proliferation and apoptosis [5]. In addition to Wnt, growth factors such as PDGF and bFGF have also been shown to stimulate β-catenin nuclear translocation and activate β-catenin/TCF, which promotes VSMC proliferation [3,6].

β-catenin is a multifunctional protein involved in cell adhesion via membrane β-catenin and involved in transcriptional activation via nuclear β-catenin. It is well known that in non-vascular cells, stimulation of Wnt signaling pathway inhibits GSK3β-mediated phosphorylation of β-catenin and prevents β-catenin degradation [7]. β-catenin binds to DNA binding factor TCF/LEF (lymphoid enhancer factor), and induces the transcriptional activation of TCF-dependent gene transcription [7]. Thus accumulation of β-catenin in the nucleus and transactivation of TCF-target genes is crucial for promoting cell growth in carcinogenesis [8]. Increased evidence also shows that β-catenin stability can be regulated by GSK3β-independent mechanisms. For example, β-catenin degradation can be mediated by direct interaction with an ubiquitin ligase complex (SIP, Skip, and Ebi) via Siah [9]. Chibby (Cby) cooperates with 14-3-3 to facilitate nuclear export of β-catenin [10]. In addition to regulating β-catenin stability and nuclear accumulation, nuclear β-catenin/TCF activity is also positively or negatively modulated by various other nuclear proteins that interact with the β-catenin/TCF transcriptional complex [11]. However, the signaling mechanisms underlying β-catenin regulation in VSMC are still undefined.

Alteration of protein phosphorylation appears crucial for β-catenin regulation because most of the components in β-catenin degradation complex are regulated by phosphorylation [12]. The protein phosphatases involved in regulating β-catenin are less understood. Protein phosphatase 2A (PP2A) is one of four families of serine/threonine protein phosphatases (PP1, PP2A, PP2B and PP2C). PP2A is a complex molecule, composed of three different subunits, a conserved catalytic subunit ($PP2A_C$), an associated/scaffolding A subunit (PP2A/A), and a variable regulatory B subunit (PP2A/B) [13,14]. The C and A subunits form the core structure to which a B subunit binds. There are a variety of different PP2A/B subunits that are grouped into four families, termed B (or PR55), B′ (or B56), and B″ (or PR72), and B‴ (PR93/PR110) [13,14]. The B subunit composition determines the substrate specificity, catalytic activity, and subcellular localization of PP2A [14]. The regulation of expression and function of specific PP2A regulatory subunits in VSMCs remain largely unknown.

In VSMCs, cAMP and cGMP signaling regulates VSMC functions, including relaxation, proliferation, migration, extracellular matrix synthesis, and inflammatory response. Intracellular cAMP and cGMP is controlled by cyclases that mediate the synthesis, and phosphodiesterases (PDEs) that catalyze their degradation. PDEs comprise a large superfamily of enzymes grouped into 11 families distinguished by differences in structure, kinetic and regulatory properties, as well as sensitivity to chemical inhibitors [15]. Ca^{2+}/calmodulin-stimulated PDEs constitute a large family of enzymes (PDE1 family), encoded by three distinct genes, PDE1A, PDE1B and PDE1C. *In vitro*, the activity of all PDE1 family members can be stimulated 10-fold or more by Ca^{2+} in the presence of calmodulin [15]. However, they differ in their regulatory properties, substrate affinities, specific activities, Ca^{2+} sensitivities, and tissue/cell distribution [15]. *In vitro*, PDE1A and PDE1B

isozymes hydrolyze cGMP with much higher affinities than cAMP; however, PDE1C isozymes hydrolyze both cAMP and cGMP with equally high affinity [15]. *In vivo*, PDE1A and PDE1B appear to primarily regulate cGMP [16–18]. PDE1C, however, is able to regulate intracellular cAMP levels in a variety of cell types [19–21]. The role of PDE1C in regulating intracellular cGMP is not well characterized. Both PDE1A and PDE1C have been shown to regulate VSMC growth [16,19]. However, the underlying molecular mechanisms remain unknown. In this study, we provide direct evidence that PDE1A but not PDE1C regulates β-catenin/TCF signaling. More importantly we also demonstrate that inhibition of PDE1A specifically upregulated phosphatase PP2A B56γ subunit gene expression, which attenuates β-catenin signaling through regulation of β-catenin protein stability.

RESULTS

PDE1 inhibition decreased β-catenin/TCF-dependent transcriptional activity and β-catenin protein level in synthetic VSMCs

To understand the molecular mechanism by which PDE1 regulates VSMC growth, we studied the role of PDE1 in regulating β-catenin/TCF signaling. We first examined the effect of PDE1 inhibition on β-catenin/TCF signaling in rat aortic VSMCs by measuring the TCF responsive luciferase reporter gene using TOP-flash. TOP-flash contains three repeats of the TCF/LEF binding sites and has been widely used to detect β-catenin/TCF activation. Two different PDE1 inhibitors, IC86340 [16] and vinpocetine, were used. We found that PDE1 inhibitor IC86340 significantly decreased fetal bovine serum (FBS)-stimulated luciferase activity (Fig. 1A). Based on the IC_{50} values of IC86340 on various PDE family members (Supplemental Table 1) and the inhibitory curves of IC86340 for PDE1 recombinant protein versus VSMC lysates (Supplemental Fig. S1), the doses of IC86340 used should preferentially hydrolyze PDE1 family enzymes in VSMCs. Vinpocetine also inhibited TCF-luciferase activity similarly (Supplemental Fig. S2). Growth factor PDGF stimulated TCF-luciferace activity is similarly blocked by PDE1 inhibitor IC86340 (Fig. 1B). Overexpressing wild-type β-catenin further enhanced TCF-luciferase activity, which was also reduced upon IC86340 treatment (Fig. 1C). These results suggest that PDE1 is critical for the regulation of β-catenin/TCF signaling.

To elucidate the underlying mechanism by which PDE1 regulates β-catenin/TCF signaling, we first examined the effects of PDE1 inhibition on β-catenin protein expression. We found that FBS stimulation increased β-catenin protein levels in a time-dependent manner, which was abolished by PDE1 inhibitor IC86340 (Fig. 1D and E). We also found that decreased β-catenin protein levels were not due to decreased β-catenin mRNA levels (Supplemental Fig. S3A). Since β-catenin reduction by PDE1 inhibition was blocked by the proteasome inhibitor lactacystin or MG132 (Supplemental Fig. S3B and S3C), it suggests that the effect of PDE1 inhibition on β-catenin reduction involved ubiquitin-proteasomal degradation.

PDE1 inhibition stimulated GSK-3β activation and GSK-3β-mediated β-catenin phosphorylation

One mechanism underlying regulation of β-catenin stability is GSK3β-dependent phosphorylation of β-catenin. β-catenin is initially phosphorylated by priming kinase casein kinase I (CKI) at Ser45 and followed by GSK3β-mediated phosphorylation at Thr41, Ser37, and Ser33. The phosphorylated β-catenin is ultimately recognized by the E3 ligase complex, ubiquitinated, and degraded by the proteasome. Therefore, we determined if PDE1 inhibition regulates the multiple phosphorylation events using β-catenin phospho-antibodies that specifically recognize either β-catenin phosphorylated at S45 (p45) or at positions 33, 37, and 41 (p-33/37/41). As shown in Figure 2A, p-33/37/41 β-catenin was significantly increased by IC86340. In contrast, p45 β-catenin was not significantly altered. Moreover,

expression of β-catenin(WT) (wild-type) or β-catenin(SA) (a nondegradable mutant of β-catenin with Ser33 mutated to Ala) stimulated TCF-luciferase activity (Fig. 2B). IC86340 was only able to inhibit the TCF-luciferase activity induced by β-catenin(WT) but not β-catenin(SA) (Fig. 2B). These data suggest that the effect of PDE1 inhibition is likely mediated through GSK3β-mediated β-catenin phosphorylation and degradation.

Lithium chloride, a GSK inhibitor, blocked the inhibitory effect of IC86340 on TCF-luciferase activity, confirming the involvement of GSK3β (Fig. 2C). GSK3β activity is regulated by phosphorylation. GSK3β phosphorylation at Ser9 leads to GSK3β inactivation [22]. We found that serum significantly stimulated GSK3β phosphorylation and IC86340 significantly decreased GSK3β phosphorylation, thereby activating GSK3β (Fig. 2D). AKT is one of the kinases that can phosphorylate GSK3β. However, we found that the phosphorylation of AKT at Ser473, a major regulatory phosphorylation site in AKT activation, was not altered by IC86340 (data not shown). Collectively, these data suggest that the effect of IC86340 on β-catenin/TCF signaling is likely mediated through targeting GSK3β.

PP2A activity is critical for PDE1-mediated regulation of β-catenin/TCF signaling

To determine the molecular mechanism by which PDE1 inhibition decreases β-catenin phosphorylation, we focused on the phosphatase that dephosphorylates GSK3β. We found that okadaic acid (OA) dose-dependently blocked IC86340 effect on TCF-luciferase activity (Fig. 3A). OA also abrogated IC86340 effects on β-catenin protein reduction (Fig. 3B) and GSK3β phosphorylation (Fig. 3C). These data suggest that a phosphatase of the PP2A type is likely responsible for the effect of IC86340 on β-catenin/TCF signaling.

PDE1 inhibition specifically upregulated PP2A B56γ subunit expression, which is responsible for PDE1 inhibition-mediated attenuation of β-catenin/TCF signaling

PP2A is a heterotrimer with a catalytic (C) subunit, a scaffold (A, PR65) subunit, and a regulatory (B) subunit. More than a dozen regulatory B subunits have been identified and they confer the unique property to individual PP2A. Therefore, we examined the effect of IC86340 on the expression of various B56 subunits (alpha, beta, gamma, and epsilon based on the availability of rat sequences) by performing quantitative real-time RT-PCR analysis. We found that the major subunit of B56 expressed in rat aortic VSMCs is the B56γ subunit and IC86340 significantly induced the B56γ mRNA levels (Fig. 4A). The mRNA level of the PP2A catalytic subunit was not altered by PDE1 inhibitor IC86340 (data not shown). Knockdown of B56γ expression by B56γ siRNA significantly blocked the induction of B56γ mRNA and protein by IC86340, compared with control-scrambled siRNA (Fig. 4B and C).

In parallel, we also observed that knockdown of B56γ expression blocked the IC86340-mediated reduction of β-catenin protein levels (Fig. 5A) as well as GSK3β phosphorylation (Fig. 5B). In contrast, overexpression of B56γ considerably inhibited FBS-stimulated TCF-luciferase activity (Fig. 5C). Together these results suggest that induction of PP2A B56γ subunits is responsible for IC86340-mediated effects on regulating GSK3β and β-catenin.

In synthetic VSMCs, PP2A B56γ is primarily expressed in nucleus, which is consistent with the previous findings [23–25]. Interesting, phospho-GSK3β, and phospho-β-catenin were all found in the nucleus (Fig. 5D), suggesting that PDE1A regulates nuclear β-catenin protein stability through the nuclear PP2A-GSK3β-β-catenin signaling axis.

PDE1A but not PDE1C regulates β-catenin/TCF signaling in VSMCs

There are three PDE1 family members (1A, 1B, and 1C), all of which can be inhibited by IC86340. Thus, we examined the expression of PDE1 isoforms in the rat aortic VSMCs

relative to known PDE1-expressing tissue such as brain using RT-PCR. We found that in low-passage rat aortic VSMCs, both PDE1A and PDE1C expression were detected although PDE1C mRNA level appears to be lower than PDE1A (Supplemental Fig. S4A). PDE1B was almost not detectable. To determine the role of individual PDE1 isoforms in regulating β-catenin/TCF activity, we used isozymes- specific RNA interference. PDE1A and PDE1C shRNA selectively downregulated PDE1A and PDE1C mRNA, protein, and PDE 1 activity, respectively (Supplemental Fig. S4B, S4C, and S4D). Interestingly, we found that downregulation of PDE1A expression by PDE1A shRNA significantly reduced serum-stimulated TCF-luciferase activity, similar to PDE1 inhibitor IC86340 (Fig. 6A). However, downregulating PDE1C expression by PDE1C shRNA did not have any significant effect (Fig. 6B). As expected, PDE1B siRNA had no effect (Supplemental Fig. S4E). When PDE1A was knocked down by shRNA, IC86340 no longer further inhibited the TCF-luciferase activity (Fig. 6C), suggesting that IC86340 effect is mainly mediated by PDE1A inhibition. In addition, overexpressing wild-type PDE1A1 significantly enhanced TCF-luciferase activity compared with expressing LacZ (Fig. 6D). Similar to IC86340, PDE1A shRNA-mediated decrease of TCF-luciferase activity was also reversed in the presence of PP2A inhibitor OA (Fig. 6E). Furthermore, we showed that knockdown of PDE1A expression by adenovirus mediated expression of PDE1A shRNA reduced FBS-induced β-catenin protein (Fig. 6F) as well as B56γ protein expression (Fig. 6G) as compared with expression of control lacZ shRNA. Together these data provide strong evidence for the role of PDE1A in regulating PP2A B56γ and β-catenin/TCF signaling, thereby supporting that the inhibitory effect of PDE1 inhibitor IC86340 on β-catenin/TCF signaling is mainly attributed to PDE1A inhibition.

PDE1A is a Ca^{2+}/calmodulin-stimulated enzyme and has been shown to primarily hydrolyze cGMP in VSMCs [16] and cardiomyocytes [17]. Therefore, we hypothesize that serum via elevation of Ca^{2+} activates PDE1A, decreases cGMP, and thus promotes β-catenin/TCF signaling. To test the hypothesis, we first examined the role of Ca^{2+} in β-catenin/TCF regulation. As shown in Fig. 7A, preventing intracellular Ca^{2+} increase by intracellular Ca^{2+} chelator BAPTA-AM significantly reduced the serum effect on TCF-luciferase activity, indicating that the serum-stimulated β-catenin/TCF signaling is at least in part dependent on intracellular Ca^{2+}. We next examined the effects of serum and PDE1A in the regulation of total intracellular cGMP using RIA (radioimmunoassay). As shown in Fig. 7B and C, FBS stimulation significantly decreased intracellular cGMP levels. Inhibition of PDE1 activity with inhibitor IC86340 (Fig. 7B) or downregulation of PDE1A via shRNA (Fig. 7C) prevented the cGMP reduction by FBS. To determine the effect of cGMP on β-catenin/TCF-luciferase, we used cGMP analog and nitric oxide donor (SNAP). We found that similar to IC86340 cGMP analog 8-CPT-cGMP and SNAP significantly reduced TCF-luciferase activity (Fig 7D), as well as increased B56γ mRNA (Fig. 7E). Although the cAMP analog and cAMP elevator forskolin also inhibited FBS-stimulated TCF-luciferase activity (Supplemental Fig. S5A), the cAMP analog did not alter PP2A B56γ mRNA expression (Supplemental Fig. S5B), suggesting that cAMP inhibits β-catenin/TCF signaling via a distinct mechanism that is independent of B56γ induction. We observed a greater effect of SNAP on TCF-luc activity than B56γ mRNA (Fig. 7D versus 7E), which might be explained by the fact that SNAP is also able to stimulate cAMP signaling in addition to cGMP [26]. Taken together, these results imply that PDE1A plays a critical role in regulating cGMP and β-catenin/TCF signaling in VSMCs.

DISCUSSION

Previous studies have shown that PDE1 family members, PDE1A and PDE1C, play critical roles in VSMC growth [16,19]. However, the underlying molecular mechanisms are not known. In this study, we demonstrated that PDE1, particularly PDE1A, regulates β-catenin/

TCF signaling through PP2A B56γ-dependent regulation of GSK-3β phosphorylation, β-catenin phosphorylation, and β-catenin protein degradation. As shown by the model in Fig. 8, growth factors promotes TCF-dependent gene expression via increasing GSK3β phosphorylation (GSK3β inactivation), decreasing β-catenin phosphorylation, increasing β-catenin stability and protein level. A specific cGMP signaling (controlled by PDE1A) negatively regulates β-catenin/TCF signaling through upregulating PP2A B56γ and decreasing GSK3β phosphorylation (GSK3β activation), which leads to β-catenin degradation. Growth factors, probably via increasing intracellular Ca^{2+} and activating PDE1A, attenuates cGMP accumulation and thus promotes β-catenin/TCF signaling.

There are two major novel findings of this study. First, we demonstrated that PP2A B56γ is the major PP2A B subunit expressed in VSMCs, which is regulated by PDE1. PDE1 inhibition upregulates PP2A B56γ expression, which is responsible for the effect of PDE1 inhibition on attenuation of β-catenin/TCF signaling. PP2A is highly regulated [14,27]. The regulatory B subunit determines the substrate specificity, catalytic activity, and subcellular localization of PP2A [14,27]. Several previous studies using chemical inhibitors of PP2A support the important role of PP2A activity in a variety of cellular processes, such as regulation of ion channel properties [28], cell growth [29], migration [30], and survival [31]. However, the regulation and function of specific PP2A regulatory subunits in VSMCs have not yet been well documented. In the present study, we examined the expression of various PP2A B56 subunits and demonstrated for the first time that rat aortic VSMCs express relatively high levels of B56γ subunit. More importantly, we showed that PDE1 inhibition specifically upregulates PP2A B56γ expression, which leads to β-catenin degradation. Thus upregulation of PP2A and downregulation of β-catenin/TCF signaling may represent one mechanism by which PDE1A inhibition reduces VSMC proliferation. Our finding is in line with a previous report showing that PP2A activity was significantly reduced in the animal vascular injury model, and inhibition of vascular wall remodeling restored PP2A activity [32]. This suggests that modification of PP2A activity is a potential regulatory mechanism underlying arterial wall response to injury.

Another novel finding is that PDE1A and PDE1C play distinct roles in β-catenin/TCF signaling. Specifically, we showed that PDE1A but not PDE1C regulates β-catenin/TCF signaling. Although both PDE1A and PDE1C have been shown to regulate VSMC growth [16,19], our findings suggest that their mechanisms of action on VSMC growth are different. PDE1A is likely through a cGMP-mediated and PP2A-depedent regulation of β-catenin/TCF signaling. *In vitro*, PDE1C hydrolyzes both cAMP and cGMP with equally high affinity. *In vivo*, PDE1C is able to regulate intracellular cAMP levels in a variety of cell types including VSMC [19], pancreatic β-cells [20], and glioblastoma cell [21]. PDE1A is expressed in both contractile and proliferating [16]. PDE1A is predominantly cytoplasmic in “contractile” VSMCs but is nuclear in “synthetic” VSMCs both *in vitro* and *in vivo* [16]. Nuclear PDE1A controls the growth and survival of synthetic VSMCs, whereas cytosolic PDE1A may regulate the contractility of VSMCs [16]. PDE1C was reported to be not expressed in contractile VSMCs but induced in growing VSMCs [33]. These observations again suggest different PDE1 isozymes have unique functions in VSMCs, probably through regulating distinct pools of cyclic nucleotides. Similar to PDE1A, PDE1B has a much higher affinity for cGMP than for cAMP *in vitro* and preferentially hydrolyzes cGMP *in vivo*. We found that PDE1B is almost undetectable in rat aortic VSMCs (Supplemental Fig. S4A), and PDE1B siRNA does not affect β-catenin/TCF-dependent gene expression (Supplemental Fig. S4E). However, PDE1B has been shown to regulate cell survival in other cell types. For example, PDE1B expression is induced in a human lymphoblastoid B-cell line (RPMI-8392) and PDE1B inhibition induces apoptosis [34]. The fact that PDE1B does not regulate β-catenin-TCF signaling might be due to the lack of PDE1B expression in VSMCs.

The role of other cGMP hydrolyzing PDEs in regulating β-catenin signaling has been reported previously in studies of anti-colon cancer drugs. However, the molecular mechanism is quite different from PDE1. It has been shown that anti-cancer drugs such as exisulind and analogs stimulate cGMP elevation and PKGIβ activation/induction in the cancer cells probably via inhibition of PDE5 and PDE2 [35,36]. It was hypothesized that these drugs stimulate β-catenin degradation through a mechanism that does not involve GSK3β-mediated phosphorylation [37]. PKG I was shown to directly phosphorylate β-catenin at the C-terminus (different from GSK3β phosphorylation sites), which may lead to β-catenin degradation by exisulind and analogs [36,37]. Differently, in the current study, we found that PDE1 inhibition reduced GSK3β phosphorylation (activated GSK3β) and promoted β-catenin degradation via GSK3β-dependent phosphorylation of β-catenin. These indicate that the molecular mechanism by which PDE1 inhibition induces β-catenin degradation in VSMC is clearly different from the PDE2/PDE5 inhibition in cancer cells. PDE5A and PDE1A enzymes in VSMCs are likely coupled to distinct intracellular cGMP signaling pathways and have different regulatory mechanisms of activation. For example, PDE5A activity is primarily stimulated upon NO induced cGMP/PKG activation, which leads to a rapid decline in intracellular cGMP. Thus PDE5 plays an important role in the negative feedback regulation of NO/cGMP signaling [38]. However, PDE1A activity is stimulated by Ca^{2+}-elevating stimuli (such as Ang II, ET-1, and α-AR agonists), and PDE1A-dependent cGMP hydrolyzing activity is predominant during elevations in intracellular [Ca^{2+}] [39]. Thus PDE1A plays a critical role in the Ca^{2+}-mediated negative regulation of cGMP signaling [38]. Indeed in this study, we found that serum stimulation attenuates cGMP accumulation, which is mediated by PDE1A (Fig. 7). In addition, PDE5A and PDE1A are most likely localized to distinct subcellular compartments. For example, in proliferating VSMCs, we found that PDE5A was primarily distributed in the cytosolic compartment while PDE1A was localized in the nucleus [16]. Thus, PDE5A and PDE1A regulate distinct pools of cGMP, which differentially modulate β-catenin signaling.

In addition, previous studies have also shown a non-canonical Wnt-Ca^{2+}-cGMP pathway mediated by Frizzled-2 [40–42]. Activation of this pathway causes Ca^{2+} increase and cGMP decrease via the activation of phospholipase C (PLC) and PDE6, respectively, which leads to the activation of several down-stream Ca^{2+}-sensitive targets including protein kinase C (PKC), Ca^{2+}-calmodulin-dependent protein kinase II (CamKII), and Ca^{2+}-sensitive phosphatase calcineurin [43]. Activation of PDE and suppression of cGMP is essential for the stimulation of Ca^{2+} signaling pathway [42]. However, the role of this pathway in the regulation of β-catenin signaling is not clear. The effects of cAMP on β-catenin/TCF signaling appear to be more complicated and cell type-specific. For example, it has been shown that cAMP/PKA is able to mediate β-catenin priming phosphorylation at Ser45, which promotes the subsequent β-catenin phosphorylation at 33/37/41 and β-catenin degradation in embryonic stem cells [44]. We also found that the cAMP analog inhibits TCF-luc activity (Fig. S5). However, it has been also shown that elevation of cAMP increases β-catenin, which is mediated by PKA-dependent β-catenin phosphorylation at Ser675 [45]. This phosphorylation stabilizes β-catenin through inhibition of its ubiquitination. In contrast, cAMP elevation via PDE7 silencing has been shown to modestly increase β-catenin mRNA level in human mesenchymal stem cell-derived osteoblasts [46]. Nonetheless, our findings suggest that PDE1A modulates β-catenin/TCF signaling in synthetic VSMCs through cGMP mediated regulation of PP2A B56γ expression.

MATERIAL AND METHODS

Reagents

IC86340 was kindly provided by Vincent Florio (ICOS, Bothell, WA). Okadaic acid and Lactacystin were purchased from Calbiochem. Epac activator 8-pCPT-2′-O-Me-cAMP was

purchased from Alexis Biochemicals (AXXORA). The PDE1A-specific polyclonal antibody was produced by immunizing rabbits with mouse PDE1A1 polypeptide (aa471 - aa565) fused to glutathione S-transferase (GST) as described previously [16]. The antibodies recognizing p-GSK3 β (p-ser9), total-GSK3β, p33/37/41-β-catenin, and p45- β-catenin were purchased from Cell Signaling. β-catenin monoclonal antibody and polyclonal were purchased from BD Transduction (Pharmingen) and Santa Cruz, respectively. Tubulin antibody (sigma) and PKG I antibody (Stressgen) were also used. PP2A B56 isoform antibody was purchased from Upstate (Millipore). PP2A B56γ antibody and PP2A B56 gamma1 and gamma3 plasmids were described previously [47]. pTOP-FLASH were kind gifts from Dr. Kikuchi (Hiroshima University, Hiroshima, Japan).

Cell Culture

Primary rat aortic VSMCs were prepared as previously described using enzymatic digestion of aortas from Sprague-Dawley rats [16]. VSMCs were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). VSMCs (passages 7 to 15) were used for the experiments.

Western blot analysis

VSMCs were seeded at 5×10^5 cells/per 60-mm dish. After culturing overnight, the cells were serum-starved with serum-free DMEM for 48hours. The cells were pretreated with IC86340 at indicated does for 1hour, followed by stimulation with 10% FBS for 24 hours. Cells were harvested and cell lysates prepared in RIPA buffer and subjected for Western blotting analysis as previously described [16].

Small Interfering RNA Transfection

siRNA were designed through siDESIGN Center with Dharmacon RNAi Technologies or purchased from Ambion. The siRNA was transfected into VSMCs by electroporation using Bio-Rad Genepulser Xcell. After attachment, cells were serum-starved for 48h, followed by treatment and 10% FBS stimulation for 24 hours. The targeting sequences are as follows:

PP2A B56 gamma subunit: ACACACAACCGGAACGUAAUU (Dharmacon)

PDE1B: duplex GGAUGUUCCGGAGAACGUA and UACGUUCUCCGGAACAUCC (Sigma)

PDE1A shRNA: ATCACATGGTTGGTTTGACAT

PDE1C shRNA: GCTGGAGATCTTTGCAATA

Immunostaining

Immunostaining was performed as described previously [16]. Briefly, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% PBS-Triton, and stained with indicated primary antibodies, followed by the secondary antibodies (goat anti-rabbit Alexa Fluor 488 or anti-mouse 546 (Molecular Probes)) as indicated. Nuclei were stained with DAPI (Sigma). Cells were visualized with an Olympus (BX-51) fluorescent microscope.

RNA isolation, Semi-quantitative RT-PCR, Quantitative Real-time RT-PCR

Total cellular RNA was isolated from VSMCs using the RNeasy Mini Kit (Qiagen). β-catenin (and PDE1, PKG, etc) mRNA level was measured by semi-quantitative RT-PCR using RT-PCR kit (Qiagen) according to the manufacturer's instruction. The mRNA levels of PP2A isoforms were measured by real-time RT-PCR. The primer sequences were as following:

β-catenin: Forward primer 5′-TGCCATCTGTGCTCTTCGTCATCT-3′

Reverse primer 5′-GCTCAACCGAAAGCCGTTTCTTGT-3′

PDE1A: Forward primer 5′-CTAAAGATGACTGGAGGGATCTTCGGAAC-3′

Reverse primer 5′-TGGAGAAAATGGAAGCCCTAATTCAGC-3′

PDE1B: Forward primer 5′-CCTCCACCTTCACCCAGCAG-3′

Reverse primer 5′-CACTGTGGGAATCTTGAAGCGGCTGATG-3′

PDE1C: Forward primer 5′-ATGGTTGGGCTGAGCTATCCACC-3′

Reverse primer 5′-CCAGTTTGCCACTCCTGTCTTATAAAGGAGG-3′

PP2A B56α subunit: Forward primer 5′-TTCCTCCAAGTGATAACCCAGACT-3′

Reverse primer 5′-CTGTATGTGAGGCCAAGAGGC-3′

PP2A B56β subunit: Forward primer 5′-TGATGCTCCTGGAGCTATTTGA-3′

Reverse primer 5′-CGATGCAAGATGGTCTTGAGG-3′

PP2A B56ε subunit: Forward primer 5′-GCGCAGCCACAGATCATCT -3′

Reverse primer 5′-TTGGTGCTGAGGACATATCCC -3′

PP2A B56γ subunit: Forward primer 5′-CGTAATCACGGAGCCCATTT-3′

Reverse primer 5′-TGGCAAGGTTCGAAACATGTT-3′

GAPDH: used as an endogenous control.

Luciferase assay

TCF-luciferase reporter constructs were transfected via electroporation. The day before electroporation, cells were seeded and grown in T-75 flasks. On the day of transfection, cells at about 70% confluence were harvested by trypsinization, centrifugation and washed twice with PBS. Cells were then transfected via electroporation with the indicated siRNA or plasmid DNA in optimum media containing 1.25% DMSO [48]. After the electroporation, cells were reseeded in 12-well plates. Upon attachment cells were serum starved for 48h. Cells were then pretreated with indicated drugs for 1h and stimulated with 10% FBS in triplicate. After 24h cells were lysed with passive lysis buffer (Promega) and luciferase activity was measured using a luciferase assay system (Promega). β-galactosidase was used as the internal control.

Statistical analysis

Statistical tests were performed using either two-way analysis of variance (ANOVA), followed by Fisher's LSD post hoc test, or Student's t-test where appropriate. All experimental data are presented as mean±SD. Statistically significant data points had a p value less than 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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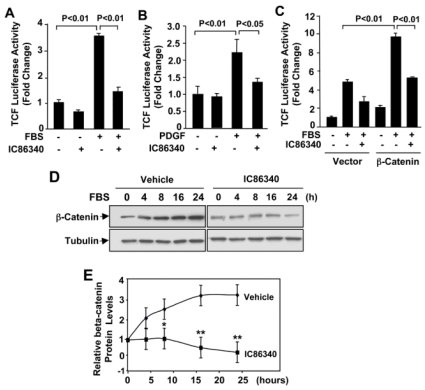

Figure 1. PDE1 inhibition decreased β-catenin/TCF-dependent transcriptional activity and β-catenin protein levels in synthetic VSMCs

(**A and B**) PDE1 inhibitor IC86340 decreases serum-stimulated (A) or PDGF-stimulated (B) TCF-luc activity. Rat aortic VSMCs were transfected with TCF-luc reporter plasmid, serum starved for two days. Cells were pretreated with IC86340 (30 μM) or vehicle, and followed by 0%, 10% FBS, or 50ng/ml PDGF stimulation for 24 hours. (**C**) PDE1 inhibitor IC86340 decreases β-catenin induced TCF-luc activity. Rat aortic VSMCs were transfected with TCF-luc reporter plasmid together with control vector DNA or wild-type β-catenin plasmid DNA. After serum starvation for two days, cells were pretreated with vehicle or IC86340 (30 μM) and followed with 0% or 10% FBS stimulation for 24 hours. Data were normalized to the sample (vehicle+0%FBS) that was arbitrarily set to 1.0. Values are mean±SD of triplicates from the same experiment. Similar results were obtained from at least three independent experiments. (**D**) Role of PDE1 inβ-catenin protein expression. Western blots showing that PDE1 inhibitor IC86340 blocked FBS stimulated upregulation of β-catenin protein expression. VSMCs were serum starved for two days, pretreated with IC86340 (30 μM) or vehicle, and followed by 10% FBS stimulation for indicated time. Western blotting analyses were performed with anti-β-catenin antibody. Tubulin was used to normalize for protein loading. (**E**) The quantitative results of blots were analyzed by densitometry, showing β-catenin levels relative to the loading control Tubulin. Data were normalized to time at zero that was arbitrarily set to 1.0. Values are mean±SD of at least three independent experiments performed in triplicates. *p<0.05,**p<0.01 vs. vehicle.

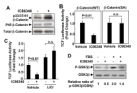

Figure 2. PDE1 inhibition stimulated β-catenin phosphorylation and GSK3β activation

(**A**) Representative Western blot showing that PDE1 inhibition by IC86340 enhanced GSK3β-meidated β-catenin phosphorylation. Serum starved VSMCs were pretreated with IC86340 or vehicle, and followed by 10% FBS stimulation for 24 hours in the presence of 5 μM of MG132 (proteasome inhibitor) to block β-catenin degradation. p-33/37/41, p45, and total β-catenin levels were measured by Western blotting analysis. (**B**) Effects of IC86340 on the TCF-luc activity induced by wild-type (WT) β-catenin or non-degradable β-catenin mutant (β-catenin(SA)). VSMCs were co-transfected with TCF-luc reporter plasmid together with β-catenin(WT) or β-catenin(SA) DNA. The cells were then pretreated with 30μM IC86340 or vehicle for 24 hours. (**C**) Lithium chloride (LiCl) abolished the inhibitory effects of IC86340 on TCF-luc activity. VSMCs were transfected with TCF-luc reporter plasmid and serum-starved for two days. The cells were then pretreated with 20 mM LiCl or vehicle and 30μM IC86340 or vehicle, and followed by 10% FBS stimulation of 24 hours. Data were normalized to the sample (vehicle alone) that was arbitrarily set to 1.0. Values are mean±SD of triplicate from one experiment. (**D**) Representative Western blots showing PDE1 inhibition by IC86340 reduced FBS-stimulated GSK3β phosphorylation. Cells were serum starved for two days, pretreated with 30 μM IC86340 or vehicle, and followed by stimulation with 0% or 10% FBS for 24 hours. Phosphorylated GSK3β was measured by Western blotting analysis using anti-Ser9 GSK3β or total GSK3β antibody. The blots were analyzed by densitometry and the relative fold changes were shown below the blots. Similar results were obtained from two to three independent experiments. n.s: no significant difference.

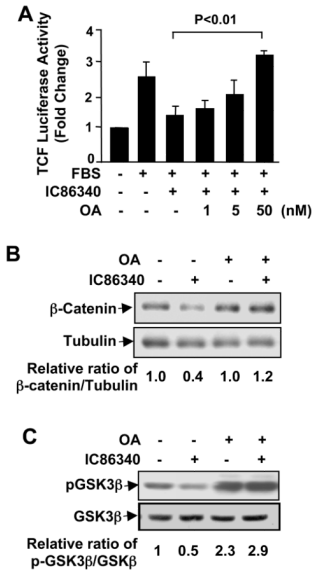

Figure 3. Effects of phosphatase inhibitor okadaic acid on PDE1-mediated regulation β-catenin/TCF signaling

(**A**) Okadaic acid (OA) dose-dependently blocked the inhibitory effect of IC86340 on TCF-luc activity. VSMCs were transfected with TCF-luc reporter plasmid and serum-starved for 2 days. The cells were then pretreated with various does of OA as indicated, 30μM IC86340 or vehicle, and followed with or without 10% FBS stimulation of 24 hours. Data were normalized to the sample (vehicle alone) that was arbitrarily set to 1.0. Values are mean±SD of at least three independent experiments performed in triplicates. (**B–C**) Representative Western blots showing that OA blocked IC86340-mediated reduction of β-catenin or p-GSK3β. Cells were serum starved for two days and pretreated with or without 50 nM OA, 30 μM IC86340 or vehicle, and followed with 10% FBS stimulation for 24 hours. β-catenin, Ser9 phospho-GSK3β, total GSK3β, and loading control tubulin were measured by Western blotting analyses. The blots were analyzed by densitometry and the relative fold changes were shown below the blots. Similar observations were obtained from at two to three independent experiments.

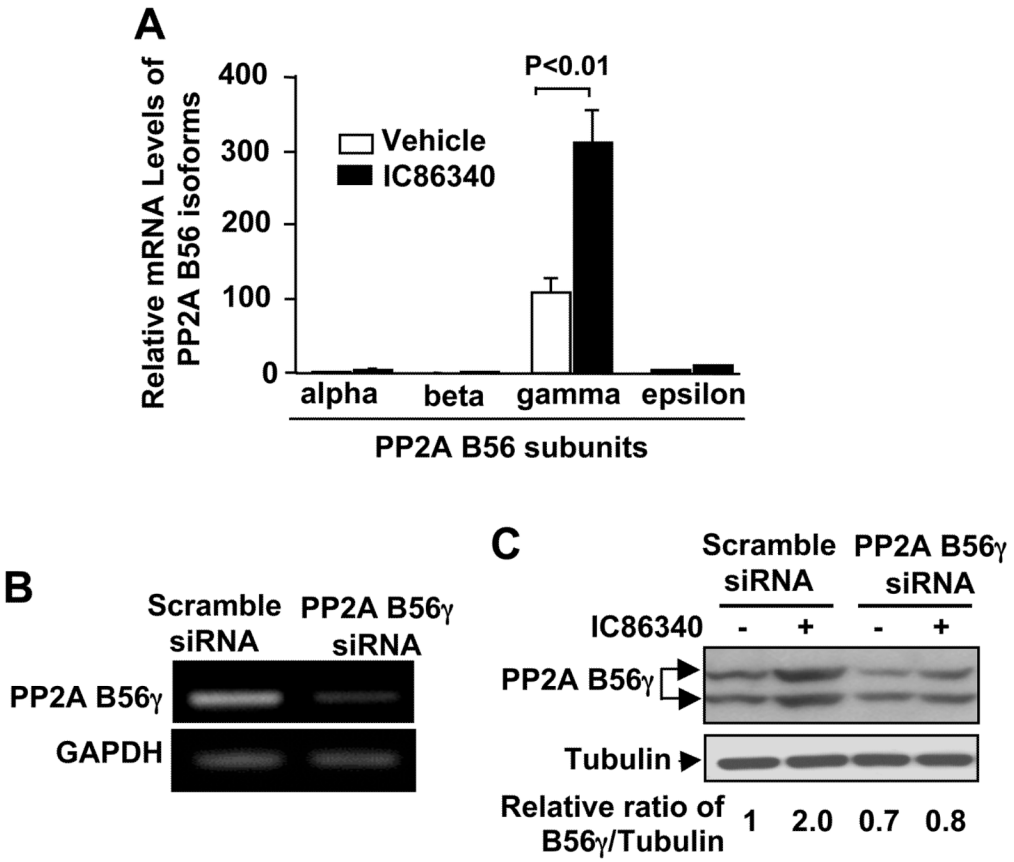

Figure 4. PDE1 inhibition specifically upregulated PP2A B56γ subunit expression

(**A**) Real-time RT-PCR data showing IC86340 induced PP2A B56γ subunit mRNA expression. Serum-starved VSMC were pretreated with 30μM IC86340 or vehicle, and followed by 10% FBS stimulation of 24 hours. mRNA levels of various PP2A B56 subunits were measured by real-time RT-PCR using subunit specific primers. Data were normalized to the sample (α subunit with vehicle) that was arbitrarily set to 1.0. (**B**) Effects of PP2A B56γ siRNA on B56γ mRNA expression. VSMCs were transfected with PP2A B56γ siRNA or control-scramble siRNA and kept at serum free condition for three days. PP2A B56γ and control GAPDH mRNA levels were analyzed by RT-PCR. (**C**) Representative Western blots showing that PP2A B56γ siRNA blocked the upregulation of PP2A B56γ subunit proteins induced by IC86340 (30 μM). VSMCs were transfected with the control or B56γ siRNA for three days, pretreated with 30 μM IC86340 or vehicle, and followed by serum stimulation for 24 hours. Cell lysates were subjected to Western blotting using antibodies recognizing PP2A B56γ or Tubulin. The blots were analyzed by densitometry and the relative fold changes were shown below the blots. Similar observations were obtained from at least three independent experiments.

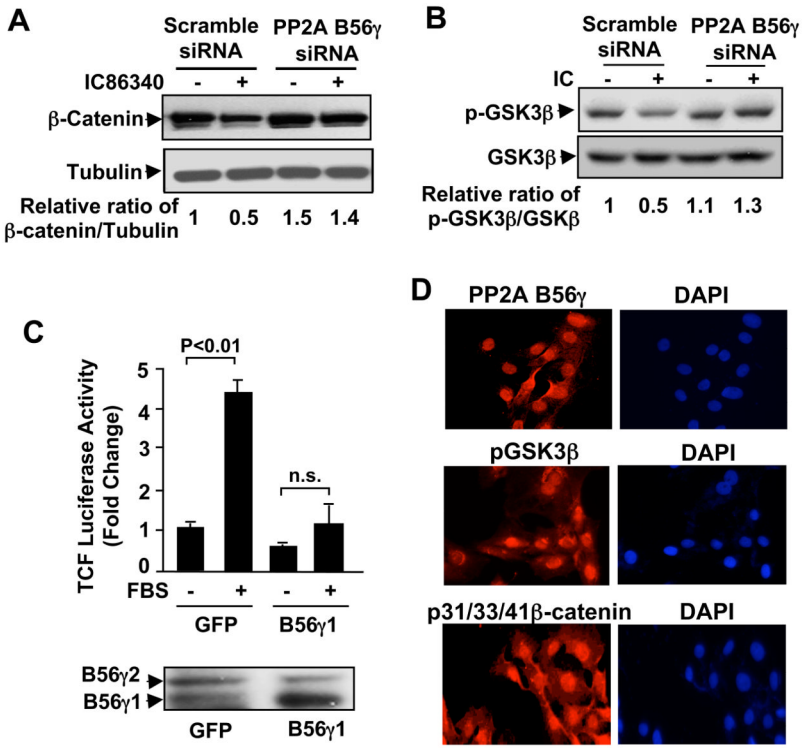

Figure 5. Role of PP2A B56γ subunit in regulating β-catenin/TCF signaling

(**A**–**B**) Representative Western blots showing that preventing PP3A B56γ subunit upregulation blocked the effects of IC86340 on β-catenin reduction (A) and p-GSK3β (B). VSMCs were transfected with PP2A B56γ siRNA or control-scramble siRNA and kept at serum free condition for three days. Cells were then pretreated with 30 μM IC86340 or vehicle, and followed by serum stimulation for 24 hours. β-catenin, Ser9 phospho-GSK3β, total GSK3β, and loading control tubulin were measured by Western blotting analyses. The blots were analyzed by densitometry and the relative fold changes were shown below the blots. (**C**) Ectopically expressing PP2A B56γ1 reduced TCF-luc activity. VSMCs were transfected with TCF-luc reporter plasmid together with PP2A B56γ1 or control GFP plasmid and kept in serum free condition for two days, followed with or without 10% FBS for 24 hours. Values are mean±SD of at least three independent experiments performed in triplicates. Lower panel: Western blot showing B56γ1 overexpression. (**D**) Representative immunostaining images showing the subcellular localization of PP2A B56, or phosphorylated GSK3β or p31/37/41 β-catenin. Similar results were obtained from at least three independent experiments.

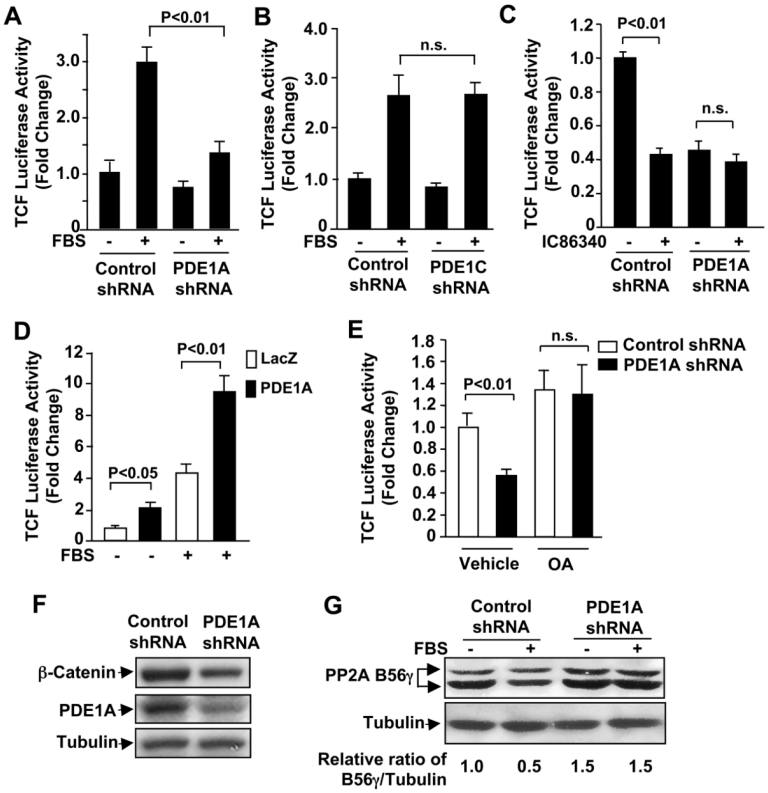

Figure 6. Role of PDE1A in regulating β-catenin/TCF-dependent transcriptional activity

(**A–B**) Effects of PDE1A shRNA (A) and PDE1C shRNA (B) on TCF-luc activity. Rat aortic VSMCs were transduced with adenovirus encoding PDE1A shRNA, PDE1C shRNA, or control lacZ shRNA and transfected with the TCF-luc reporter plasmid for two days under the serum-free condition for three days, and followed with 0% or 10% FBS stimulation for 24 hours. Data were normalized to the sample (control shRNA + 0%FBS) that was arbitrarily set to 1.0. (**C**) Effects of PDE1A knockdown and PDE1 inhibitor IC86340 on TCF-luc activity. Transfected VSMCs were stimulated with 10% FBS in the presence or absence of IC86340. Data were normalized to the sample (control shRNA, no IC86340) that was arbitrarily set to 1.0. (**D**) Ectopic expression of PDE1A enhanced TCF-luc activity. VSMCs were transfected with TCF-luc reporter plasmid together with LacZ or wild type PDE1A plasmid under the serum-free condition for two days followed with 0% or 10% FBS stimulation for 24 hours. Data were normalized to the sample (LacZ+0%FBS) that was arbitrarily set to 1.0. Values are mean±SD of triplicates from the same experiment. The similar results were obtained from at least three independent experiments. (**E**) Okadaic acid (OA) blocked the inhibitory effect of PDE1A knockdown on TCF-luc activity. VSMCs were treated similarly as described in Fig. 6A and B. The cells were then pretreated with 50 nM OA and followed with 10% FBS stimulation of 24 hours. Data were normalized to the sample (control shRNA + vehicle) that was arbitrarily set to 1.0. Values are mean±SD of three samples from the same experiment. The similar results were obtained from at least two independent experiments. (**F–G**) Representative Western blots showing knockdown of PDE1A with PDE1A shRNA decreased the protein levels of β-catenin (F) and P2A B56γ (G). Cells were treated as described above. β-catenin, PP2A B56γ, PDE1A, and loading control tubulin were measured by Western blotting analyses. The blots were analyzed by densitometry and the average of relative fold changes were shown below the blots. Similar results were obtained from at least three independent experiments.

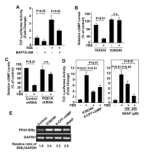

Figure 7. Role of PDE1A in the regulation of intracellular cGMP

(**A**) The effect of intracellular Ca^{2+} on serum stimulates TCF-luc activity. Transfected and serum-starved cells were pretreated with BAPTA-AM (30 μM) and EGTA (2 mM) or vehicle, and followed by 0% or 10% FBS stimulation for 24 hours. (**B and C**) The effect of PDE1 inhibitor IC86340 or PDE1A shRNA on intracellular cGMP. Total cGMP levels measured by radioimmunoassay (RIA) in VSMCs pretreated with IC86340 (15 μM) for 30 minutes (B) or separately transfected with PDE1A shRNA or control LacZ shRNA for three days (C), followed by 0% or 10% FBS stimulation for 1 minutes. Data were normalized to the sample (with vehicle or control shRNA plus 0% FBS) that was arbitrarily set to 1.0. Values are means SD of at least 3 repeats performed in the same experiment. Similar observations were obtained from at least three independent experiments. (**D**) Effects of the cGMP analog and NO donor SNAP on TCF-luc activity. Transfected and serum-starved cells were treated with 30 μM IC86340, 200 μM 8-CPT-cGMP, 100 or 200 μM SNAP, and followed with 0% or 10% FBS stimulation for 24 hours. Data were normalized to the sample (0% + vehicle) that was arbitrarily set to 1.0. Values are mean±SD of three samples from the same experiment. The similar results were obtained from at least two independent experiments. (**E**) Representative RT-PCR gel image showing the effects of the cGMP analog and NO donor SNAP on PP2A B56γ expression. Transfected and serum-starved cells were treated with 30 μM IC86340, 200 μM 8-CPT-cGMP, or 200 μM SNAP, and followed with 10% FBS stimulation for 24 hours. The images were analyzed by densitometry and the relative fold changes were shown below. Similar results were obtained from at least three independent experiments.

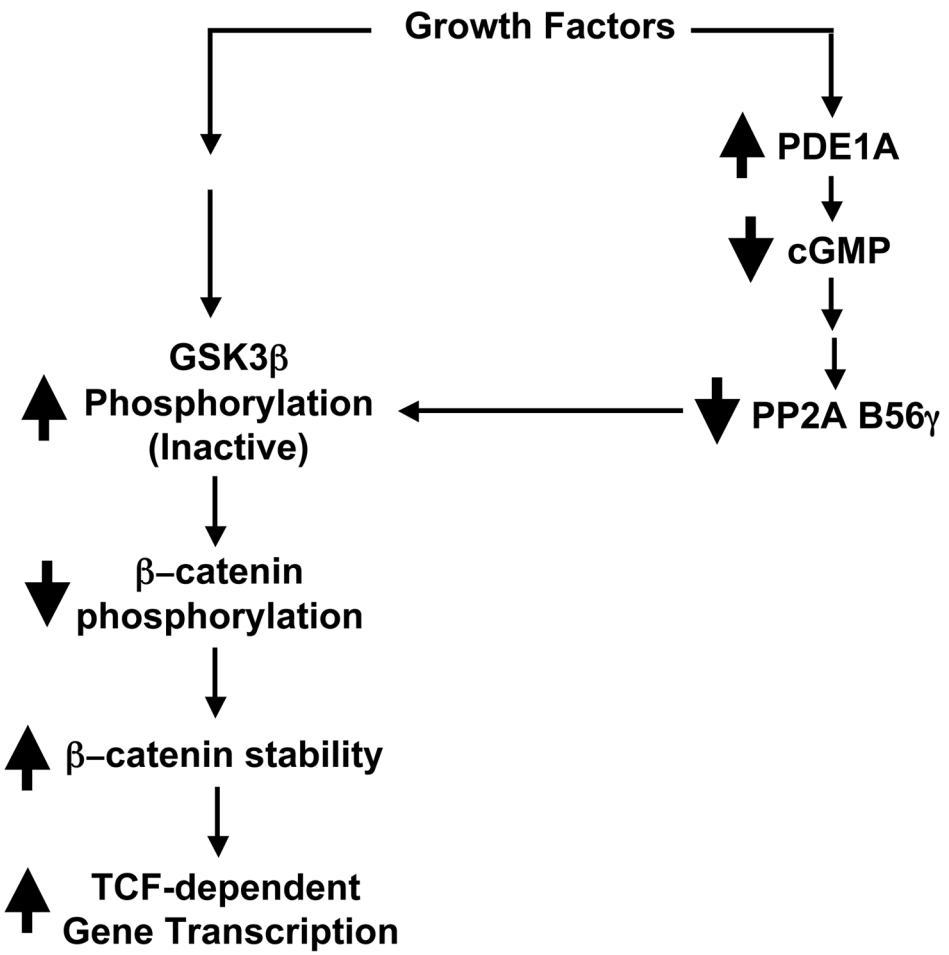

Figure 8. Schematic diagram showing the role of PDE1A in the regulation of β-catenin/TCF signaling in proliferating VSMCs

As indicated, FBS inactivates GSK3β (by increasing its phosphorylation), leading to decreasing β-catenin phosphorylation and increasing β-catenin stability. Accumulation of β-catenin promotes TCF-dependent gene expression. FBS stimulates PDE1A activity by increasing intracellular Ca^{2+} and in turn reduces the cGMP level, which leads to the reduced level of PP2A B56γ expression. Low level of PP2A activity maintains GSK3β at the phosphorylated (inactive) state, which is critical for FBS-stimulated activation of β-catenin/TCF signaling and gene expression. PDE1A inhibition restored intracellular cGMP and PP2A B56γ expression, leading to inhibition of β-catenin/TCF signaling.