Cyclic Nucleotide Phosphodiesterase 1 Regulates Lysosome-Dependent Type I Collagen Protein Degradation in Vascular Smooth Muscle Cells

Yujun Cai, Clint L. Miller, David J. Nagel, Kye-Im Jeon, Soyeon Lim, Pingjin Gao, Peter A. Knight, Chen Yan

Objective—The phenotypic modulation of vascular smooth muscle cells (VSMCs) to a synthetic phenotype is vital during pathological vascular remodeling and the development of various vascular diseases. An increase in type I collagen (collagen I) has been implicated in synthetic VSMCs, and cyclic nucleotide signaling is critical in collagen I regulation. Herein, we investigate the role and underlying mechanism of cyclic nucleotide phosphodiesterase 1 (PDE1) in regulating collagen I in synthetic VSMCs.

Methods and Results—The PDE1 inhibitor IC86340 significantly reduced collagen I in human saphenous vein explants undergoing spontaneous remodeling via ex vivo culture. In synthetic VSMCs, high basal levels of intracellular and extracellular collagen I protein were markedly decreased by IC86340. This attenuation was due to diminished protein but not mRNA. Inhibition of lysosome function abolished the effect of IC86340 effect on collagen I protein expression. PDE1C but not PDE1A is the major isoform responsible for mediating the effects of IC86340. Bicarbonate-sensitive soluble adenylyl cyclase/cAMP signaling was modulated by PDE1C, which is critical in collagen I degradation in VSMCs.

Conclusion—These data demonstrate that PDE1C regulates soluble adenylyl cyclase/cAMP signaling and lysosome-mediated collagen I protein degradation, and they suggest that PDE1C plays a critical role in regulating collagen homeostasis during pathological vascular remodeling.

Key Words: vascular smooth muscle cell ■ phosphodiesterase ■ collagen ■ lysosome ■ soluble adenylyl cyclase

During physiological states, vascular smooth muscle cells (VSMCs) residing in the media layer are quiescent and contractile. Their principal function is to maintain vascular tone. In response to biological and mechanical injury, VSMCs exhibit phenotypic plasticity and undergo modulation from a quiescent/contractile phenotype to an active synthetic one.1 Synthetic VSMCs contribute to vascular remodeling and dysfunction by downregulating contractile proteins and acquiring the capacity to proliferate, migrate, and produce extracellular matrix (ECM) proteins. Therefore, synthetic VSMCs play a key role in the pathogenesis of cardiovascular disorders such as atherosclerosis, postangioplasty restenosis, bypass vein graft failure, and cardiac allograft vasculopathy. Elucidating molecules that control the phenotypic changes may be critical to circumvent pathological vascular remodeling.

The major components of the ECM of the vessel wall are collagens.2 Several genetically distinct collagens are present in the vessel wall, including collagen types I, III, IV, V, and VIII.2 In normal vascular tissue, collagens play important roles in maintaining vascular structural integrity, regulating vascular mechanical properties (such as extensibility and stiffness), and regulating cellular function through receptor-mediated cell-collagen interaction.2 Synthetic VSMCs, in the atherosclerotic and neointimal lesions, produce abundant ECM, particularly type I collagen (collagen I). The ECM, together with cellular components in the lesions, is responsible for vessel wall thickening and eventual occlusion of the vessel lumen. In addition, collagen I in vascular lesions may also regulate VSMC proliferation/migration, monocyte activation, platelet circulation, lipid accumulation, calcification, and plaque stability.2

Cyclic nucleotide phosphodiesterases (PDEs), by catalyzing the hydrolysis of cAMP and cGMP, regulate the amplitude, duration, and compartmentalization of intracellular cyclic
nucleotide signaling. To date, more than 50 different isoforms have been identified and grouped into 11 broad families by differences in structure, kinetic and regulatory properties, and sensitivity to chemical inhibitors. The Ca\(^{2+}\)/calmodulin-stimulated PDE1 family enzymes are encoded by 3 distinct genes, PDE1A, PDE1B, and PDE1C. Both PDE1A and PDE1C have been previously shown to regulate synthetic VSMC growth.\(^5\)\(^6\) In the present study, we interrogated the role and underlying mechanism of PDE1 isozymes in regulating collagen I in synthetic VSMCs and defined a novel mechanism by which PDE1C/cAMP signaling regulates collagen I protein degradation through a lysosome-dependent mechanism.

**Materials and Methods**

IC86340 was provided by ICOS Corp, and the primary antibody against collagen I (LF-67) was kindly provided by Dr Fisher (National Institutes of Health, Bethesda, MD). Rat aortic VSMCs were prepared using enzymatic digestion of aortas as previously described.\(^6\) VSMCs (passages 7 to 12) were used for the experiments. Human saphenous veins (SVs), not required for surgery, were collected from patients after coronary artery bypass surgery. SVs were cultured for 7 days as previously described.\(^8\) The medium and drug were changed every other day.

An expanded Methods section is available in the online supplement (http://atvb.ahajournals.org).

**Results**

**Effects of PDE1 Inhibition on Collagen I Protein Levels in Human SV Explants**

Human SV is the most commonly used vessel to bypass blocked coronary arteries; however, late vein graft failure occurs because of the development of stenosis or occlusion.\(^9\) When human SVs are cultured in vitro, they spontaneously undergo remodeling, which predominantly involves smooth muscle cell growth and ECM deposition.\(^10\) As shown in Figure 1A (left panel), the SV wall can be divided into 3 zones, the internal zone (intima), the medial zone (inner and outer media), and the external zone (inner and outer adventitia).\(^11\)\(^12\) With Verhoeff Masson Trichrome combination staining, smooth muscles are stained with red, collagen with light blue or blue-green, and elastin with dark blue. When SVs were cultured in vitro for 1 week, SV remodeling occurred, revealing a larger amount of collagen and elastic fiber deposition and a less organized smooth muscle (Figure 1A, middle panel). This led to an increased thickness of the SV vessel wall. We found that collagen deposition was significantly reduced in human SV by the PDE1 inhibitor IC86340, accompanied by a significant reduction of remodeling (Figure 1A, right panel). Consistently, collagen I protein levels measured by Western blotting were increased in cultured SV compared with the same SV before culture. PDE1 inhibitor IC86340 significantly attenuated the increased collagen I protein levels (Figure 1B). According to the IC\(_{50}\) values of IC86340 on inhibiting different PDE family members (Supplemental Table), the doses of IC86340 used in this study should preferentially inhibit PDE1 isozymes in vascular cells. These results suggest that PDE1 is involved in regulating collagen I during SV remodeling. To further demonstrate the effects of PDE1 inhibition on collagen I accumulation in neointimal formation in vivo, we performed immunostaining of collagen I in a mouse arterial injury model induced by flow-cessation via carotid ligation.\(^4\) As shown in Supplemental Figure I, collagen I staining intensities are increased in the neointimal regions of the injured vessel, which is significantly reduced by PDE1 inhibitor IC86340.

**Upregulation of Collagen I Protein in Synthetic VSMCs**

VSMCs in vitro show distinct phenotypes in response to different types of extracellular matrices. When cultured on dishes coated with polymerized collagen, VSMCs elicit a “contractile”-like phenotype and mimic many characteristics of VSMCs in the normal medial layer in vivo.\(^13\) In contrast, VSMCs cultured on monomer collagen or noncoated plastic dishes have synthetic phenotype and retain many properties of VSMCs in developing vascular lesions.\(^13\) We thus compared collagen I protein levels in contractile-like and synthetic VSMCs (Figure 1C). As expected, we observed that contractile-like VSMCs (on polymerized collagen) expressed much higher levels of contractile marker proteins, such as smooth muscle calponin, compared with synthetic VSMCs.
(on monomeric collagen). In contrast, the collagen I protein levels were much higher in the synthetic VSMCs, consistent with the previous finding that synthetic rabbit VSMCs have significantly higher collagen I than contractile VSMCs. Interestingly, PDE1 inhibition blocked collagen I upregulation in synthetic VSMCs (Figure 1D), further supporting the role of PDE1 in the regulation of collagen I in synthetic VSMCs.

**Mechanism of Collagen I Regulation by PDE1 in Synthetic VSMCs**

As shown in Supplemental Figure II, synthetic VSMCs had a high basal level of collagen I. Stimulation with serum or transforming growth factor-β further increased collagen I protein (Supplemental Figure IIA) and mRNA (Supplemental Figure IIB). Interestingly, PDE1 inhibition IC86340 inhibited not only agonist-stimulated collagen I protein but also the basal collagen I protein to a great extent (Supplemental Figure IIIA). In contrast, inhibiting PDE1 reduced only the amount of agonist-stimulated collagen I mRNA, not basal mRNA levels (Supplemental Figure IIB). These observations suggest that PDE1 regulates both basal and agonist-stimulated collagen I in synthetic VSMCs, very likely via distinct mechanisms. For instance, PDE1 regulates the basal collagen I at the protein level and the agonist-stimulated collagen I at the mRNA level. Because the role of cyclic nucleotide-mediated signaling in agonist-stimulated collagen I expression is well described, in this study we specifically focused on the function and underlying mechanism of PDE1 in regulating phenotype-associated basal collagen I in synthetic VSMCs.

Using synthetic VSMCs in the absence of stimulation, we found that PDE1 inhibitor IC86340 caused a significant decrease in intracellular procollagen I protein levels in a dose- and time-dependent manner (Figure 2A and 2B). Because collagen I is a secretory protein and the major component of interstitial connective tissue, we also analyzed extracellular collagen I. As shown in Figure 2C, the extracellular secreted collagen I protein in the culture medium was much higher than intracellular collagen I. There were also large amounts of cleaved collagen I fragments in the extracellular fraction, consistent with reports stating that extracellular collagen I undergoes fragmentation via free radicals and proteinases. We consistently observed concomitantly reduced intracellular and extracellular collagen I (Figure 2C). Immunofluorescent staining also revealed that the collagen I staining intensities are decreased by IC86340 in both intracellular (Figure 2D) and extracellular space (Figure 2E). Taken together, these results provide support that PDE1 plays a critical role in regulating collagen I protein levels in synthetic VSMCs.

**Role of Lysosome in PDE1-Mediated Regulation of Collagen I Protein Levels**

To further confirm that the basal collagen I protein reduction by PDE1 inhibition is not due to decreased collagen I gene expression, we measured mRNA levels by reverse transcription-polymerase chain reaction. As expected, basal collagen I mRNA levels were not significantly altered by IC86340 (Supplemental Figure IIIA). We next determined whether IC86340 treatment causes proteasome-mediated collagen degradation and found that the proteasome inhibitor MG132 did not significantly influence IC86340-mediated collagen I protein reduction (Supplemental Figure IIIB). This suggests that a proteasome-dependent mechanism is likely not involved.

Because lysosome-dependent degradation of collagen I is an important mechanism in fibroblast-like cells, we determined the role of lysosomes in IC86340-mediated regulation of collagen I in VSMCs. Within lysosomes, digestive enzymes function in an acidic condition (around pH 5.0), which is maintained by vacuolar-type H(+) ATPase (V-ATPase). Therefore, the V-ATPase inhibitor or lysosomal pH neutralizer is commonly used to inhibit lysosome function. As shown in Figure 3A, bafilomycin A1 (a specific inhibitor of the vacuolar type H(+)-ATPase) significantly blocked intracellular collagen I reduction by IC86340. Similarly, chloroquine and NH4Cl (neutralizing lysosomal pH and thus decreasing the lysosomal function) also prevented intracellular collagen I reduction (Figure 3B and 3C). The reduction of extracellular collagen I levels was also blocked by lysosome inhibitor bafilomycin A1 (Figure 3D) and NH4Cl (Figure 3E). A similar observation was also made in human VSMCs (Supplemental Figure IIIC). These results suggest that PDE1 inhibition stimulates lysosome-mediated degradation of col-
lagen I, which leads to a decrease of intracellular and extracellular collagen I protein levels.

To determine the role of PDE1 on lysosome regulation, we first performed cytochemical analysis of lysosomes with LysoTracker Red DND-99, a fluorescent acidotropic probe for labeling and tracking acidic organelles in live cells. We found that the number and fluorescent intensity of lysosome-like organelles in the perinuclear regions of VSMCs are significantly increased on PDE1 inhibition (Figure 3F and 3G). In addition, we also analyzed a LysoSensor Yellow/Blue DND-160, a fluorescent pH indicator used to detect active lysosomes. We observed a similar increase of fluorescence intensity in the presence of IC86340 (Figure 3F and 3H). These observations suggest that PDE1 may regulate lysosome function in an as-yet-unknown manner.

Role of cGMP-Dependent Protein Kinase, cAMP-Dependent Protein Kinase, and Exchange Protein Activated by cAMP in PDE1-Mediated Regulation of Collagen I Protein

PDE1 isozymes are dual specificity enzymes, which hydrolyze both cAMP and cGMP in vitro. We then examined the effects of several common cyclic nucleotide effector molecules on PDE1 inhibition-mediated collagen I reduction, including cGMP-dependent protein kinase (PKG), cAMP-dependent protein kinase (PKA), and exchange protein activated by cAMP (Epac). As shown in Supplemental Figure IVA, when PKG protein levels were significantly downregulated with 2 different PKG-I short interfering RNA (siRNA) duplexes, the effects of IC86340 on collagen I protein were not altered. The fact that downregulation of PKG by siRNA blocked PKG-mediated VASP Ser239 phosphorylation (Supplemental Figure IVB) indicates that the VSMCs have functional PKG. We obtained very similar results when using the PKG inhibitor DT-3 or Rp-8-Br-PET-cGMPs to inhibit PKG activity (Supplemental Figure IVC). These results suggest that activation of PKG is not involved in PDE1-mediated regulation of collagen I protein degradation.

We next examined the role of PKA by expressing the PKA inhibitor PKI. As shown in Supplemental Figure VA, adeno-viral mediated PKI expression did not significantly alter IC86340-mediated reduction of collagen I compared with control or LacZ expression. However, expression of PKI inhibited PKA-dependent phosphorylation of Ser157 of VASP induced by cAMP analog Sp-8-CPT-cAMPs (Supplemental Figure VIB), suggesting that PKI is functional in blocking PKA activity.

Because cAMP can also activate Epac, we examined the effects of Epac stimulation and inhibition on the ability of IC86340 to reduce collagen I protein. We found 2 Epac-specific activators, 8-CPT-2′-O-Me-cAMP and 8-pCPT-2′-O-Me-cAMP-AM, neither altered collagen I reduction in response to IC86340 (Supplemental Figure VIA). In addition, knocking down Epac1 using siRNA had no effect on IC86340-mediated collagen I reduction (Supplemental Figure VIB and VIC). These data suggest that Epac1 is also not likely involved.
Role of Cyclic Nucleotide Gated Channel in PDE1-Mediated Regulation of Collagen I Protein

CNG channels inhibitors have been reported in vascular cells; however, their specific function in the regulation of collagen I degradation is unclear. Therefore, we investigated the potential involvement of CNG using pharmacological CNG inhibitors. As shown in Figure 4A, 2',4'-dichlorobenzamil, a nonspecific CNG channel blocker, prevented the effect of IC86340 on collagen I reduction. In addition, another, more specific CNG channel blocker, L-cis-diltiazem, almost completely reversed the effect of IC86340 on collagen I (Figure 4B). The fact that 2 very different chemical inhibitors had similar results in IC86340-mediated collagen I reduction strongly supports the role of CNG channels in the regulation of collagen I degradation. Consistently, CNG channel blockers also abolished the effects of IC86340 on extracellular collagen I (Figure 4C and 4D).

Role of Bicarbonate-Sensitive Soluble Adenylyl Cyclase in PDE1-Mediated Regulation of Collagen I Protein

Bicarbonate-sensitive soluble adenylyl cyclase (sAC) has been shown to regulate V-ATPase in germs cells and renal epithelial cells. Given that V-ATPase is critical for lysosome function, we determined the role of PDE1 in the regulation of sAC signaling and collagen I reduction. We found that sAC stimulus with bicarbonate at low doses (5 and 10 \(\mu\)mol/L) did not affect collagen I protein, whereas high doses of bicarbonate (25 and 50 mmol/L) gradually reduced collagen I protein (Figure 5A). In the absence of bicarbonate, 5 \(\mu\)mol/L of IC86340 itself had no effect on collagen I protein. Interestingly, bicarbonate and IC86340 together synergistically reduced collagen I protein. The synergistic effect of bicarbonate and IC86340 was blocked by the sAC inhibitor KH7 (Figure 5B). In addition, in the normal bicarbonate-containing medium, the effect of IC86340 on collagen reduction was diminished by KH7 (Figure 5C). Similar effects of KH7 on IC86340-mediated regulation of collagen I protein were also observed in cultured human VSMCs (Supplemental Figure VIIA), as well as in human SV explants cultured ex vivo (Supplemental Figure VIIIB). These results suggest that the effect of PDE1 inhibitor IC86340 on collagen I reduction likely require the activation of sAC. We detected sAC expression in VSMCs via reverse transcription–polymerase chain reaction. As shown in Supplemental Figure VIIIA, rat aortic VSMCs express sAC at a level that is comparable to PC12 cells, in which sAC has been shown to be functionally important. To specifically target sAC, we used sAC siRNA. We found that downregulation of sAC with sAC siRNA (Figure 5D, right) significantly attenuated the effects of IC86340 on collagen I protein, similar to KH7 (Figure 5D, left).

To further confirm the involvement of CAMP, we used nonhydrolyzable cAMP analogs. We found that 2 different cAMP analogs, Db-cAMP and Sp-8-CPT-cAMPS, significantly reduced collagen I protein levels, similar to the PDE1 inhibitor IC86340 (Figure 5E). In contrast, a cGMP analog (8-pCPT-cGMP) and a nitric oxide donor (SNAP) had no effect on collagen I protein (Supplemental Figure VIIIB). These data together suggest that cAMP but not cGMP regulates collagen I protein in VSMCs, consistent with the role of sAC.

To examine the effect of PDE1 inhibition on intracellular cAMP, we analyzed cellular cAMP via radioimmunoassay. We found that without bicarbonate stimulation, IC86340 did not significantly change intracellular cAMP levels (Figure 5F), which is consistent with our previous observation that IC86340 does not significant alter basal cAMP in VSMCs. However, with bicarbonate stimulation, IC86340 significantly elevated cAMP levels (Figure 5F).

These results together suggest that activation of sAC/cAMP signaling is essential for collagen I degradation. PDE1 may represent a major PDE activity that terminates sAC/cAMP signaling. Inhibition of PDE1 potentiates sAC/cAMP signaling and synergistically promotes collagen I degradation.

**PDE1C but Not PDE1A Regulates Collagen I Protein in VSMCs**

Both PDE1A and PDE1C were detected in growing rat aortic and human SV VSMCs, whereas PDE1B was minimally detected (Supplemental Figure IXA and IXB). To determine the specific function of PDE1 isoform in the regulation of collagen I protein degradation, we used isoform-specific short hairpin RNA (shRNA) via the adenooviral expression system. As expected, PDE1A and PDE1C shRNA selectively knocked down PDE1A and PDE1C gene expression, respectively (Supplemental Figure IXC). However, PDE1C shRNA but not PDE1A shRNA significantly reduced the collagen I protein level (Figure 6A). Consistent with IC89340, basal collagen I mRNA was not affected by PDE1A and PDE1C shRNA (Supplemental Figure IXD). In addition, similar to...
IC86340, the effect of PDE1C shRNA on collagen I protein was blocked by the lysosome inhibitor bafilomycin A1 or NH₄Cl (Figure 6B), in the absence of sAC activator bicarbonate (Figure 6C), or in the presence of sAC inhibitor KH7 (Figure 6D). Consistent with the role of PDE1C in synthetic VSMCs, we observed an upregulation of PDE1C expression in VSMCs cultured on monomeric collagen relative to polymeric collagen (Supplemental Figure IXE), and in cultured human SV explants as compared with noncultured explants (Supplemental Figure IXF). These results demonstrate that PDE1C but not PDE1A inhibition contributed to the effect of IC86340 on collagen I reduction in synthetic VSMCs.

Discussion

In this study, we demonstrated that synthetic VSMCs associate with a high basal level of collagen I. PDE1 plays a critical role in regulating the phenotype-associated basal collagen I protein level via modulating lysosome-mediated collagen I protein degradation. Besides agonist-stimulated collagen I gene expression, our finding suggests that regulation of lysosome-mediated collagen I protein degradation represents one of the important mechanisms for controlling collagen I homeostasis in synthetic VSMC. Both PDE1A and PDE1C are expressed in synthetic VSMCs and have been shown to regulate VSMC growth. However, here we demonstrate that PDE1C but not PDE1A is specifically involved in lysosome-mediated collagen I degradation. Together with our unpublished observation that PDE1A but not PDE1C specifically regulates agonist-stimulated collagen I mRNA expression in cardiac myofibroblasts (Miller et al, unpublished data), these data suggest that PDE1A and PDE1C differentially regulate collagen I, probably through modulating distinctly compartmentalized cyclic nucleotide pools.

In addition, we also demonstrate that the effect of PDE1 inhibition on collagen I degradation appears to involve CNG channel activation. The expression of CNGs has been reported in endothelial cells and VSMCs in vitro and in vivo; however the function of these channels in the vasculature is unknown. Our finding of CNG in collagen I degradation indicates for the first time that the CNG channel(s) may play important roles in regulating the nonsensory function. Although the mechanism by which CNG channel(s)
involves lysosome-mediated collagen I degradation remains to be investigated, it is known that CNG channel activation leads to intracellular Ca\(^{2+}\) elevation, and Ca\(^{2+}\) might be critical in phagosome-lysosome fusion and lysosome exocytosis. Finally, we demonstrated that the activation of sAC/cAMP signaling is essential for collagen I degradation. PDE1C specifically regulates sAC/cAMP signaling, which identifies PDE1C as the first PDE isoform shown to modulate sAC/cAMP signaling. Taken together, our findings reveal a novel mechanism for the regulation of collagen I in synthetic VSMCs by PDE1C/cAMP through lysosome-dependent degradation of collagen I protein (Supplemental Figure XI) and imply that PDE1C is an important regulator of collagen I metabolism and a potential therapeutic target for vascular remodeling. It should be noted that despite these novel findings, how sAC-PDE1C-cAMP signaling regulates lysosome function through CNG channels remains to be investigated in the future.

Collagen I levels are controlled by both its biosynthesis and degradation. Biosynthesis of collagen I is regulated mainly at the transcription level by growth factors and cytokines. Accumulating evidence demonstrates that degradation of collagen I is an important process in both physiological (during developmental growth and wound healing) and pathological circumstances. For example, excessive collagen I degradation causes tissue destruction, whereas insufficient degradation leads to scar formation and fibrosis. Collagen I degradation can occur either before or after secretion from cells. The pathways for degradation of intracellular procollagen I molecules differ from those for extracellular fibrillar collagen I. The intracellular degradation before secretion can occur in 2 different sites: the Golgi apparatus and the lysosome. Also, secreted collagen I is degraded mainly by 2 consecutive routes: proteolytic and phagocytotic. Proteolytic degradation occurs mainly through matrix metalloproteinase–mediated cleavage. The resultant collagen I fragments are phagocytosed by cells and degraded in lysosomes within the cell. It is believed that during ECM remodeling, phagocytosis of collagen I fibrils by fibroblasts appears to be a continuous process. In addition to the intracellular lysosome, evidence has also revealed the existence of Ca\(^{2+}\)-dependent exocytosis of lysosomes in many different tissues and cell types and that the extracellular lysosome enzymes contribute to degradation of connective tissues. Although matrix metalloproteinase–mediated cleavage appears to be important for collagen phagocytosis and degradation by lysosome, we found that the effect of PDE1 inhibition on collagen I degradation is unlikely through regulating matrix metalloproteinase (Supplemental Figure X). Taken together, the lysosome appears to be a central player for collagen I degradation, regardless of intracellular or extracellular mechanisms. This may provide an explanation for our findings that intracellular and extracellular collagen I is concomitantly regulated by the inhibitors of PDE1, CNG channel, and lysosome.

**Sources of Funding**

This research was supported by grants from the National Institutes of Health (HL077789 and HL088400) and the American Heart Association (0740021N).

**Disclosures**

None.

**References**


Cyclic Nucleotide Phosphodiesterase 1 Regulates Lysosome-Dependent Type I Collagen Protein Degradation in Vascular Smooth Muscle Cells
Yujun Cai, Clint L. Miller, David J. Nagel, Kye-Im Jeon, Soyeon Lim, Pingjin Gao, Peter A. Knight and Chen Yan

Arterioscler Thromb Vasc Biol. published online December 9, 2010;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2010/12/09/ATVBAHA.110.212621.citation

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2010/12/08/ATVBAHA.110.212621.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Materials and Methods

Reagents
IC86340 was provided by ICOS Corporation. 8-pCPT-2'-O-Me-cAMP-AM, L-cis Diltiazem HCl, 2’,4’-Dichlorobenzamil, Rp-8-Br-PET-cGMPS, 8-pCPT-cGMP, Sp-8-CPT-cAMPs, Dibutyryl-cAMP (Db-cAMP), were purchased from Biomol (Plymouth Meeting, PA), while the PKG inhibitors DT-3 was purchased from Biolog. 8-CPT-2'-O-Me-cAMP and 8-CPT-2'-O-Me-cAMP-AM were purchased from ALEXIS. Bafilomycine A1, Chloroquine diphosphate salt, NH4Cl, and other reagents were purchased from Sigma. Adenovirus expressing the highly selective endogenous PKA inhibitor (PKI) was described previously. Adenovirus expressing the LacZ gene was used as a negative control.

Ex vivo culture of human saphenous veins
Human saphenous veins, not required for surgery, were collected from patients after coronary artery by-pass surgery. Saphenous veins were cultured as previously described. Briefly, the vein segments were opened longitudinally and cut transversely into 0.5 cm lengths. The segments were pinned onto a Mersilene mesh (Ethicon) with luminal surface facing up. The vein segments were cultured individually with luminal surface facing up in 12-well plates in RPMI 1640 medium supplemented with 30% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 100ug/ml streptomycin for 7 days at 37ºC with 5% CO2. The culture media and drug were changed every other day. At the end of the culture, the vein segments were washed in PBS and then used for western blot or histological immunostaining. For histological analysis, human saphenous vein segments were washed with PBS, and fixed with 10% phosphate-buffered formalin for 24 h, embedded in paraffin, and cross-sections (6 µm) were prepared. The paraffin sections were stained with Verhoeff Masson Trichrome combination method.

Isolation and culture of human VSMCs from saphenous veins
VSMCs from human saphenous veins were prepared with an explant method as described previously. Briefly, human saphenous veins segments were opened longitudinally and cut into small fragments, and then scraped the intima and removed adventitia. The fragments were chopped into small pieces and maintained in RPMI 1640 medium supplemented with 10% FBS at 37ºC in a humidified incubator in 5% CO2. The medium was changed every 2 days. After two weeks, cells were passaged and used for experiments under 3 passages. Human VSMCs from saphenous vein were identified by typical “hill and valley” growing pattern and smooth muscle α-actin staining.

Rat aortic VSMC preparation and culture
Rat aortic VSMCs were prepared using enzymatic digestion of aortas from 10-week-old Sprague-Dawley rats as previously described. VSMCs were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) in a humidified incubator (37º, 5% CO2). VSMCs (passages 7 to 12) were used for the experiments.

Collagen Matrices
To prepare two-dimensional monomeric collagen matrices, tissue culture dishes were coated with 0.1 mg/ml type I collagen solution (PureCol, INAMED, CA) in 0.1 M acetic acid for at least 3 hr at room temperature and then washed and stored in DMEM. Three-dimensional
polymerized collagen matrices (final concentration, 2 mg/ml) were prepared by neutralizing the collagen-I solution with 1/6 volume of 6×DMEM and diluting to a final volume with 1×DMEM to which human plasma-derived serum (PDS) was added at a final concentration of 1% PDS. Gels formed following incubation of this solution at 37 °C for overnight as described previously. VSMCs, cultured in 1% PDS/DMEM for 2 days, were plated in 1% PDS/DMEM onto 60mm dishes coated with monomer or polymerized collagen, and continually cultured for 48 h. To harvest cultures on polymerized collagen, cells were digested with 2.5mg/ml type I collagenase in DMEM for 30 min until the cells dispersed completely; for cells on monomer collagen, cells were washed twice with phosphate-buffered saline (PBS: 145 mM NaCl, 5 mM KCl, 10 mM sodium phosphate, pH 7.4) and digested with 0.01% trypsin, 0.1 mM EDTA. Dispersed cells were washed twice with PBS, and then used for Western blot.

**Carotid artery ligation injury model**

All animal studies were approved by University of Rochester Animal Care and Use Committee. FVB/NJ mice were purchased from Jackson Laboratories. 8-week-old male mice were used all animal experiments. Complete common carotid artery ligation was performed as previously described. Mice were anesthetized with isoflurane. The left common carotid artery was dissected through a small midline incision in the neck and ligated with 6-0 silk sutures. In the treatment group, 50 µl 20 % F-127 pluronic gel (BASF) containing 30 µM PDE1 inhibitor IC86340 was applied around the carotid artery. In the right common carotid artery (sham), the suture was passed under the exposed carotid artery but not ligated. The remodeling was carried out at 14 day after operation. The arteries were embedded in paraffin, and cross sections were cut at 200 μm intervals. For collagen I immunohistochemistry, the sections were treated with 3 % H2O2, followed by blocking with Dako serum-free blocking solution (Dako). The primary antibody polyclonal anti-collagen-I type I (LF-67) was incubated at 4°C overnight, followed by incubation with biotinylated secondary antibody for 30 minutes. ABC complex and DAB Substrate Chromogen system (Dako) were used for detection. The sections were counterstained with hematoxylin.

**Recombinant adenoviruses**

Adenovirus vectors encoding PDE1A, PDE1C, or LacZ shRNA were constructed using BLOCK-iT Adenoviral shRNA Expression System (Invitrogen) according to the manufacturer's instructions. The expression of the shRNA is controlled by the human U6 promoter. The PDE1AshRNA and PDE1C shRNA were designed using web-based BLOCK-iT RNAi designer. One ssDNA oligonucleotide with an additional sequence CACC (PDE1A: 5’-CACCATCACATGGTTGTTTGCATCGAAATGTCAAACCATGTGATA-3’; PDE1C: 5’-CACCGCTATCCACCGGCTGTAATCCGAAATTACAGCCCGGTGATAGC-3’), the other encoding complementary sequence with an additional sequence AAAA (PDE1A: 5’-AAAATCACATGGTTGTTTGCATCGAAATGTCAAACCATGTGATA-3’; PDE1C: 5’-AAAAGCTATCCACCGGCTGTAATCCGAAATTACAGCCCGGTGATAGC-3’), were annealed and then the generated dsDNA oligonucleotide was cloned into pENTR/U6 vector by using BLOCK-iT U6 RNAi Entry Vector kit (Invitrogen). The U6 RNAi cassette in the pENTR/U6 PDE1AshRNA or pENTR/U6 PDE1CshRNA plasmid was performed an LR recombination reaction with pAd/PL-DEST Gateway vector by using Gateway LR Clonase II Enzyme Mix to generate pAd/PL-PDE1AshRNA and pAd/PL-PDE1CshRNA. The adenoviral expression plasmids were digested with Pacl to expose the inverted terminal repeats and
transfected into HEK 293A cells using Lipofectamine 2000 (Invitrogen) to produce crude adenoviral stocks (Ad-PDE1AshRNA and Ad-PDE1CshRNA). Large-scale implication of adenoviral vectors was conducted in HEK 293A cell as described previously. The titer of the purified virus was determined and expressed as plaque-forming units (pfu/ml). The adenoviral shRNA against β-galactosidase was used as control shRNA (Ad-lacZ shRNA).

Small Interfering RNA Transfection
Small Interfering RNA (siRNA) were designed through siDESIGN Center with Dharmacon RNAi Technologies or purchased from Ambion. The targeting sequences are as follows: PKGI: GCGUUCGGAAAGUUCACUA and CCAUACAAGUCUACUUAAG (Ambion). Epac1: CCACAGAGCAUGUGCACAAG (Dharmacon). sAC: CCAAGUGUAUGGCCUCAUTT (Sigma). VSMCs were transfected with the siRNA using a Gene Pulser Xcell Electroporation system (Bio-Rad) according to the manufacturer’s instructions. Briefly, 1x10⁶ VSMCs were mixed with 400 ng of siRNA in 250 µl of siRNA electroporation buffer in cuvettes. The electroporation was performed at 300 V, 500 µf. After an additional incubation for 15 min at room temperature, cells were plated on six-well plates. Electroporated cells were allowed to recover for 24 hours in complete medium before doing further treatment.

RNA isolation, Semi-quantitative RT-PCR
Total cellular RNA was isolated from VSMCs using the RNeasy Mini Kit (Qiagen). mRNA levels of various gene products were measured by semi-quantitative RT-PCR using Reverse Transcription System (Promega, A3500) and GoTaq Green Master Mix (Promega, M7123), according to the manufacturer’s instruction. The primer sequences were as following:

PDE1A:
- Forward primer 5’-CTAAAGATGACTGGAGGGATCTTCGGGAAAG-3’
- Reverse primer 5’-TGGAGAAAAATGGAAGGCCCTAATTCCAGC-3’

PDE1B:
- Forward primer 5’-CCTCCACCTTCACCCAGCAG-3’
- Reverse primer 5’- CACCTGTGGGAATCTTGAAGCGTGATG-3’

PDE1C:
- Forward primer 5’-ATGGCTTGGCTGAGCTATCCACC-3’
- Reverse primer 5’-CCAGTTGCGCCACTCTGTCTTATAAGGAG-3’

Collagen-I:
- Forward primer 5’- AACGATGGTGCCAAGGGTGAT-3’
- Reverse primer 5’-ATTCTTGCCAGCAGCAAC-3’

Epac1:
- Forward primer 5’-GATTCCAGTGCTCATGAACACCACCAG-3’
- Reverse primer 5’-CACCCAGTCTGAGGTTCTGGAGG-3’

sAC:
- Forward primer 5’-GATGATGTTTCATCCTAGAGAAGGCTG-3’
- Reverse primer 5’-GGAAACATTCTGGAGAAGCTTCACTTC-3’

GAPDH:
- Forward primer 5’-TCAAGAAGGATGTCAGGAG-3’
- Reverse primer 5’-TTGGAATGCTGAGGACTC-3’

Western blot analysis
Western blot analysis was performed as previously described. Briefly, lysates were prepared in RIPA buffer with protease inhibitor cocktail (Sigma). The total lysates were loaded on SDS-PAGE, electrotransferred into PVDF membrane. The primary antibodies against collagen-I (LF-67, gifted from Dr. Fisher, NIH, Bethesda, MD), PKG1 (Stressgen, KAP-PK005), VASP (ALEXIS, ALX-210-898), pSer239 VASP (ALEXIS, ALX-804-240-C200), pSer157 VASP (ALEXIS, ALX-804-403), and β-Actin (Santa Cruz Biotechnology, Inc, sc-1616) were used.
**Immunostaining**

Immunofluorescence was performed as previously described. Briefly, VSMCs were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-100, and stained with polyclonal anti-collagen-I (LF-67) antibody followed by secondary antibody (goat anti-rabbit Alexa Fluor 488 (Molecular Probes) as indicated. In order to measure extracellular collagen-I, the cells were not permeabilized. Nuclei were stained with DAPI (Sigma). Cells were visualized with an Olympus (BX-51) fluorescent microscope.

**Microscopic analysis of lysosomes**

Lysotracker red DND-99 (Molecular Probes) and LysoSensor Yellow/blue DND-160 (Molecular Probes) were used to visualize lysosomes. Lysotracker red DND-99 is a fluorescent acidotropic probe for labeling and tracking acidic organelles in live cells. LysoSensor Yellow/Blue DND-160 is a fluorescent pH indicator used to detect active lysosomes in live cells. Active lysosomes with pH < 4.8 elicit yellow fluorescence labeled with the LysoSensor. Rat aortic VSMCs were treated with IC86340 for 24 h, and then washed in PBS, and 25 nM Lysotracker or 10 µM LysoSensor were added to the cells medium. Images were collected using FV1000 Olympus Laser Scanning Confocal Microscope within 10 min after placement of the dye. To quantify changes of lysosomes, the fluorescent intensity of lysosomes in each field was analyzed using Image J software. At least 5 fields per condition from three independent experiments were measured.

**Cyclic AMP Radioimmunoassay (RIA)**

Cyclic nucleotide levels were measured as previously described (Miller et al 2009). Briefly, VSMCs were lysed in 0.1 N HCl solution, scraped with a rubber policeman, and centrifuged. Acid precipitates were solubilized and protein levels were measured by the Bradford method. Supernatants were dried under a vacuum using a Savant Speed-Vac concentrator, resuspended in 0.05 N sodium acetate, centrifuged and supernatants were used directly in a non-acetylated [125I]-labeled cAMP radioimmunoassay (Biomedical Technologies; Stoughton, MA) following the manufacturer’s instructions. Cyclic nucleotide levels were calculated against a standard curve using logarithmic analysis and defined as pmol/mg protein. Due to interassay variation cyclic nucleotide levels were presented as the fold change of control samples.

**Statistical analysis.**

Quantitative results are expressed as mean ± SD. All of the experiments were from at least three independent experiments. Comparisons between two groups were performed using paired Student’s t-test. P-values <0.05 were considered statistically significant.

**References:**


Supplemental Figure I. Effect of PDE1 inhibition on collagen-I expression in mouse vascular injury in vivo. Immunostaining of collagen-I in a mouse vascular injury model induced by flow cessation via complete carotid ligation. Collagen-I staining was revealed by diaminobenzidine (brown) and nuclei were counterstained with hematoxylin (purple/blue). Panel a: mouse carotid artery with sham operation; panel b: mouse carotid artery with ligation treated with vehicle ; panel c: mouse carotid artery with ligation treated with 30 µM IC86340. Vehicle or IC86340 was applied perivascularly via pluronic gel. Collagen-I staining is predominantly found in the adventitial of normal and ligated arteries, as well as in the neointimal region of injured artery in the absence of IC86340. The neointimal collagen-I level was significantly reduced by IC86340 treatment. Similar results were observed in at least three different mouse vessel samples. Adv: adventitia; Med: media; Neo: neointima.
Supplemental Figure II

Supplemental Figure II. Effects of PDE1 inhibitor IC86340 on serum and TGFβ stimulated collagen-I protein (A) and mRNA (B) levels. Rat aortic VSMCs were growth arrested in serum free medium (SF) for 48 hours, and followed by stimulating with 10% FBS or 10ng/ml TGFβ for 24 hours in the presence of vehicle or 15 µM IC86340. Collagen-I protein levels were measured by immunoblotting and mRNA levels were measured by semi-quantitative RT-PCR. The intensities of bands were analyzed by densitometry. Fold changes normalized to the first lane are shown below the blots (n=2-3).
Supplemental Figure III. (A) inhibition of PDE1 by IC86340 did not significantly alter basal collagen-I mRNA levels in synthetic VSMCs. Rat aortic VSMCs were cultured for indicated time points in the presence of 15 µM IC86340, and mRNA levels were measured by semi-quantitative RT-PCR. The intensities of bands were analyzed by densitometry. Values are mean ± SD (n=3), (B) MG132 did not alter the effects of IC86340 on collagen-I protein reduction. VSMCs were pretreated with 5 µM MG132 for 30 min, followed by vehicle or 15 µM IC86340 for 24 h. Protein levels were detected by Western blot. (C) Effects of lysosome inhibitor on intracellular collagen-I protein in human VSMCs. Human VSMCs were isolated from the medial layer of human saphenous vein and cultured in vitro. Cells were pretreated with vehicle or lysosome inhibitor 50 nM bafilomycin A1 (Baf A1) for 30 min, followed by treatment with vehicle or 30 µM IC86340 for 24 hours. Cells were lysed and intracellular collagen-I expression was analyzed by Western blotting. Band intensities were quantified and values are mean ± SD of three independent experiments. ns: no significant difference.
Supplemental Figure VI. Role of PKG in PDE1 inhibition mediated collagen-I protein reduction.

(A) Knocking down PKG-I did not alter IC86340-mediated reduction of collagen-I protein. Rat aortic VSMCs were transfected with 100 nM control siRNA or PKG-I siRNA1 and siRNA2 via electroporation, and cultured for 48 h, followed by treatment with vehicle or 15 μM IC86340 for 24 hours. Collagen-I and PKG-I protein were analyzed by Western blotting.

(B) Knocking down PKG-I via siRNA significantly reduced PKG-mediated VASP phosphorylation. VSMCs were transfected with 100 nM control siRNA or PKG-I siRNA1 for 48 hours, followed by stimulation with or without 100 μM 8-pCPT-cGMP for 30 min. PKG-dependent phosphorylation of VASP was analyzed by the antibody specifically recognizing ser239 phosphorylated VASP.

(C) PKG inhibitor did not alter IC86340 effects on collagen-I protein reduction. VSMCs were treated with 100 μM Rp-8-Br-PET-cGMPs or 250 nM DT-3 for 30 min, followed by vehicle or 15 μM IC86340 for 24 h. These results suggest that activation of PKG is not involved in IC86340-mediated regulation of collagen-I protein degradation.
Supplemental Figure V. Role of PKA in PDE1 inhibition mediated collagen-I protein reduction.

(A) Inhibition of PKA by PKI did not block IC86340-mediated collagen-I reduction. VSMCs were transduced with 100 MOI adenovirus expressing PKI or LacZ for 48 h, followed by treatment with vehicle or 15 µM IC86340 for 24 h. Collagen-I protein levels were analyzed by Western blotting.

(B) Inhibition of PKA by PKI blocked PKA-mediated VASP phosphorylation. VSMCs were transduced with 100 MOI adenovirus expressing PKI or LacZ for 48 h, followed by treatment with 100 µM Sp-8-CPT-cAMPs for 30 min. PKA-dependent phosphorylation of VASP was analyzed by the antibody specifically recognizing ser157 phosphorylated VASP. These results suggest that activation of PKA is not involved in IC86340-mediated regulation of collagen-I protein degradation.
Supplemental Figure VI. Role of Epac in PDE1 inhibition mediated collagen-I protein reduction. (A) Activation of Epac did not influence IC86340-mediated collagen-I degradation. VSMCs were pretreated with Epac activators 100 µM 8-CPT-2′-O-Me-cAMP or 10 µM 8-pCPT-2′-O-Me-cAMP-AM for 30 min, followed by treatment with vehicle or 15 µM IC86340 for 24 h. The expression of collagen-I was evaluated by Western blotting. (B) Knocking down Epac1 by Epac1-specific siRNA. (C) Knocking down Epac1 did not affect IC86340-mediated regulation of collagen-I protein degradation. VSMCs were trasfected with Epac siRNA or control siRNA via electroporation. After 48 h, cells were treated with vehicle or 15 µM IC86340 for 24 h. These results suggest that activation of Epac is not involved in IC86340-mediated regulation of collagen-I protein degradation.
Supplemental Figure VII. Role of bicarbonate-sensitive soluble AC in PDE1-mediated regulation of collagen-I protein in human VSMCs and saphenous vein explants. (A) Effects of KH7 on IC86340 induced collagen-I reduction in human VSMCs. Human saphenous vein VSMCs cultured in normal bicarbonate-containing medium were treated with vehicle or 30 µM IC86340 in the presence of vehicle or 10 µM KH7 for 24 hours. (B) Effects of KH7 on IC86340 induced collagen-I reduction in human saphenous vein cultured ex vivo. Human saphenous vein segments from the same patients cultured in normal bicarbonate-containing medium were treated with vehicle or 30 µM IC86340 in the presence of vehicle or 10 µM KH7 for 7 days. The expression of collagen-I was evaluated by Western blotting. Equal loading was confirmed by immunoblotting for β-Actin. The blots were analyzed by densitometry. Band intensities were quantified and values are mean ± SD of three independent experiments.
Supplemental Figure VIII

(A) RT-PCR showing expression of sAC in VSMCs, PC12 cells, kidney, and testis. Although sAC is expressed much more abundantly in testis, sAC mRNA and/or proteins were also detected in many other somatic tissues and cell lines.\(^1\)\(^2\) sAC has been shown to regulate a variety of somatic cell functions, such as Na\(^+\) transport in kidney collecting duct cells,\(^3\) osteoclast formation,\(^4\) as well as PC12 cell migration.\(^5\) Therefore, we included PC12 cells, kidney and testis as positive controls.

(B) Role of cGMP in collagen-I protein reduction. VSMCs were treated with vehicle, 100 \(\mu\)M SNAP, or 100 \(\mu\)M 8-pCPT-cGMP for 24 hours. Levels of collagen-I were analyzed by Western blotting. Equal loading was confirmed by immunoblotting for \(\beta\)-actin.

References:
Supplemental Figure IX. (A-B) Expression of PDE1 isoforms in cultured rat aortic (A) and human saphenous vein (B) VSMCs. Brain, a known PDE1-expressing tissue, was used as a positive control. The mRNA levels of PDE1A, 1B, and 1C were measured by relative semi-quantitative RT-PCR with isoform-specific primers. Both PDE1A and PDE1C were detected in cultured rat aortic and human vein VSMCs, and PDE1B was hardly detected. (C) PDE1A and PDE1C shRNA specifically reduced PDE1A and PDE1C gene expression, respectively. Top panels: gel images; Bottom graph: quantitative data. (D) PDE1A and PDE1C shRNA does not alter collagen-I mRNA levels. Rat aortic VSMCs were transduced with or without 100 MOI adenovirus encoding shRNA for lacZ, PDE1A, or PDE1C for 72 hours. PDE1A, PDE1C, collagen-I, and GAPDH mRNA levels were analyzed by semi-quantitative RT-PCR. Band intensities were quantified and values are mean ± SD of three independent experiments. *p<0.01 vs. no virus. (E-F) PDE1C expression was upregulated in rat aortic VSMCs cultured on monomeric collagen compared with polymerized collagen (C), as well as human saphenous vein explants cultured ex vivo for 7 days compared with those without culture (D).
Supplemental Figure X. Effects of PDE1 inhibitor IC86340 on MMP expression. (A) RT-PCR results showing MMP2 and MMP9 mRNA levels in rat aortic VSMCs. The quantitative data are the average values from two independent experiments. (B) Gelatin Zymography showing pro-MMP2 and activated-MMP2 protein levels. Band intensities were quantified and values are mean ± SD of three experiments. VSMCs were growth arrested and stimulated with 100 nM Ang II for 24 hours. Cells were collected for isolation of RNA and the supernatants were collected for zymographic analysis of MMP proteins. We found that the basal level of MMP2 is much higher than MMP9 in VSMCs, which is consistent with the notion that MMP2 is constitutively expressed in VSMCs and MMP9 expression is dependent on stimulation. It appears that PDE1 inhibition did not affect basal MMP2 and MMP9 mRNA levels, but significantly decreased Ang II-stimulated MMP2 and MMP9 mRNA. In addition, PDE1 inhibition reduced the basal Pro-MMP2 but not the active-MMP2 level. The Ang II-stimulated increases of MMP2 proteins (both Pro and active forms) were completely abolished by PDE1 inhibition. The MMP-9 level was too low to be detected in VSMCs. Because PDE1 inhibition has no change or decreases MMP levels, this suggests that the effect of PDE1 inhibition on collagen-I degradation is unlikely through regulating MMPs.

References:
Supplemental Figure XI. Proposed model. Schematic diagram showing the potential mechanism by which PDE1C/cAMP regulates collagen-I protein degradation. Elevation of cAMP generated by sAC activates CNG, which in turn elevates Ca\(^{2+}\). Ca\(^{2+}\) increases lysosome function in an as-yet-unknown manner, which promotes lysosome-mediated collagen-I degradation and subsequently reduced the collagen-I protein level. In synthetic VSMCs, induction of PDE1C expression causes increased cAMP hydrolysis and reduced cAMP levels, and thus blocks the effect of cAMP signaling on collagen-I degradation. PDE1C inhibition leads to cAMP elevation and consequent collagen-I degradation.

In addition to our current findings, other previous findings supporting the proposed model include: (1) cAMP-stimulator PGE\(_2\) is involved in a lysosomal degradation of connective tissue\(^1\); (2) cAMP generated by sAC stimulates the assembly, accumulation, and recycling of V-ATPase that is critical for the lysosome function\(^2-4\). (3) cAMP regulates Ca\(^{2+}\)-dependent lysosomal functions\(^5\); (4) Ca\(^{2+}\) is critical for lysosome functions such as phagosome-lysosome fusion and lysosome exocytosis\(^6-7\).

References: