This Review is part of a thematic series on Cyclic GMP–Generating Enzymes and Cyclic GMP–Dependent Signaling, which includes the following articles:

- Regulation of Nitric Oxide–Sensitive Guanylyl Cyclase
- Cyclic GMP Phosphodiesterases and Regulation of Smooth Muscle Function
- Structure, Regulation, and Function of Membrane Guanylyl Cyclase Receptors, With a Focus on GC-A
- Cyclic GMP–Dependent Protein Kinases and the Cardiovascular System: Insights From Genetically Modified Mice
- Regulation of Gene Expression by Cyclic GMP
- Explaining the Phenomenon of Nitrate Tolerance

Rudi Busse, Editor

Cyclic GMP Phosphodiesterases and Regulation of Smooth Muscle Function

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Abstract—Cyclic GMP (cGMP) made in response to atrial natriuretic peptide (ANP) or nitric oxide (NO) is an important regulator of short-term changes in smooth muscle tone and longer-term responses to chronic drug treatment or proliferative signals. The ability of smooth muscle cells (SMCs) to utilize different combinations of phosphodiesterase (PDE) isozymes allows cGMP to mediate these multiple processes. For example, PDE5 as a major cGMP-hydrolyzing PDE effectively controls the development of smooth muscle relaxation. In order for contraction to occur, PDE5 is activated and cGMP falls. Conversely, blockade of PDE5 activity allows the relaxation cycle to be prolonged and enhanced. A recently shown direct activation of PDE5 by cGMP binding to the GAF A domain suggests that this regulatory site might be a target for new drug development. The calcium surge associated with vasoconstrictor initiated contraction also activates a calcium/calmodulin-dependent PDE (PDE1A). Together, PDE5 and PDE1A lower cGMP sufficiently to allow contraction. Longer term, both PDE5 and PDE1A mRNA are induced by chronic stimulation of guanylyl cyclase. This induction is a major cause of the tolerance that develops to NO-releasing drugs. Finally, high levels of cGMP or cAMP also act as a brake to attenuate the proliferative response of SMCs to many mitogens. After vessel damage, in order for SMC proliferation to occur, the levels of cGMP and cAMP must be decreased. In humans, this decrease is caused in large part by induction of another Ca⁺⁺/calmodulin-dependent PDE (PDE1C) that allows the brake to be released and proliferation to start. (Circ Res. 2003;93:280-291.)

Key Words: cyclic GMP • smooth muscle function

The cyclic nucleotide second messengers, cAMP and cGMP, have been shown to regulate a wide variety of processes in many different tissues of the body and have been suggested to regulate many more. In fact, they have been proposed to modulate so many different processes that, until recently, it has been difficult to understand how these simple, small, second messenger molecules could provide both the specificity of action and the diversity of function necessary for such regulation. Particularly problematic has been an understanding about how both very rapid and very slow processes can be modulated by the same mechanisms.

A major conceptual advance in our understanding of the mechanisms by which such temporally and spatially disparate processes can be controlled was the realization that many different isozymes for synthesis (cyclases) and degradation (phosphodiesterases, PDEs) of cAMP and cGMP are present...
in the organism. For example, at least 10 different adenylyl cyclase genes and nearly 20 different PDE genes have been identified in mammalian species. Any particular cell type might express two or three different cyclases and three or four different PDEs. More importantly, the number of possible combinations that can be expressed in any one compartment of the cell is very large. Therefore the differential expression and localization of unique combinations of synthetic and degradative enzymes provide each cell with molecular solutions for these problems.

In arterial smooth muscle, a number of processes are controlled by cAMP and cGMP. They include metabolic and mechanical events that are regulated on a relatively rapid time scale. The contractile tone of the muscle is perhaps the best example of this. Both cAMP and cGMP cause smooth muscle relaxation in large part through their effects to lower intracellular calcium or activate myosin phosphatase. Slower changes that are regulated by cyclic nucleotides include altered proliferation in response to injury or even longer-term desensitization to chronic stimulation by drugs or hormones.

As with all regulatory messengers, the amplitude and duration of the cyclic nucleotide signals are governed by their rates of synthesis and rates of degradation. In this article, we review some of the current thoughts about the regulation of smooth muscle function by cyclic nucleotides and especially the roles played by the phosphodiesterases that either hydrolyze or respond to cGMP.

Regulation of Smooth Muscle Function by Cyclic Nucleotide Phosphodiesterases

There are now known to be 11 different PDE gene families expressed in mammalian tissues (Figure 1). Most families contain more than one gene and most genes code for more than one mRNA (by alternative splicing or alternative transcriptional start sites). Depending on the species, the major phosphodiesterases present in arterial smooth muscle are PDEs1A, 1B, and 1C, PDEs3A and 3B, and PDE5. In a few species a substantial amount of PDE4 activity is also expressed in smooth muscle. As PDE4 is specific for cAMP and not regulated by cGMP, we will not discuss its role further in this review. Under basal conditions (ie, low calcium levels), it is thought that the most active cGMP-hydrolyzing PDE in smooth muscle is the cGMP-specific, cGMP-binding PDE, PDE5. Under higher calcium conditions (eg, during muscle contraction and possibly in cells being stimulated to divide), one or more of the PDE1 variants can become the predominant PDE. Although PDE3 does not have as great a total catalytic capacity as the other two, it may still play a role in controlling cAMP and perhaps cGMP in specific compartments of the cell. This enzyme is strongly inhibited by cGMP and has been termed the cGMP-inhibited PDE in many previous studies. In all cases, it must be remembered that these various PDEs do not necessarily share the same subcellular localization and therefore often subserve, at least in part, different functional compartments in the cell.

PDE5: cGMP-Specific, cGMP-Binding Phosphodiesterase

**PDE5 and Smooth Muscle Relaxation**

It is well established that nitric oxide (NO), atrial natriuretic peptide (ANP), and several other endogenous vasodilators regulate smooth muscle tone through activation of guanylyl cyclase, elevation of cGMP, and activation of cGMP-dependent protein kinase (PKG) (Figure 2). NO/cGMP effects on contraction in smooth muscle appear to be mediated specifically by PKG but not cAMP-dependent protein kinase (PKA), because in PKG-1-deficient mice, cGMP-induced relaxation of aortic smooth muscle is completely abolished, whereas cAMP-dependent relaxation is not affected. There are several specific physiological substrates for PKG in smooth muscle including the regulatory myosin-binding subunit of myosin phosphatase, calcium-activated maxi K (BKCa) channels, and IRAG (IP3 receptor associated cGMP kinase substrate). Phosphorylation of all of these targets contributes to a reduction of intracellular Ca2+ concentration or reduction in sensitivity to Ca2+ and thereby decreased smooth muscle tone.

PDE5, as a major cGMP-hydrolyzing PDE expressed in smooth muscle cells, is in a position to effectively control this cGMP/PKG signaling pathway, especially under conditions of low calcium. As discussed later, under conditions of higher calcium, PDE1s likely play an increasingly important role. PDE5 has been found in all types of vascular and visceral (uterus, small intestine) SMCs. The physiological importance
PDE5 and Pulmonary Vasculature

Recently, the treatment of pulmonary hypertension has emerged as a new potential area for clinical application of PDE5 inhibitors. Pulmonary hypertension is a life-threatening disease characterized by high pulmonary arterial pressure and vascular resistance. In the pulmonary vasculature, NO plays an important role as a vasorelaxant. Currently, inhaled nitric oxide is one of the more effective therapies for treatment of pulmonary hypertension. One of the advantages of exogenously administered NO is that it has little effect on systemic blood pressure. However, the half-life of NO is relatively short, and therefore, effective NO treatment requires its multiple administration. Unfortunately, tachyphylaxis commonly is seen within a few days.

The use of sildenafil for treatment of pulmonary hypertension has shown positive results in humans. A clinical study of patients with severe primary pulmonary hypertension showed a dramatic improvement of pulmonary systolic pressure after treatment with oral sildenafil. In another report sildenafil was found to be a potent pulmonary vasodilator and superior to inhaled NO in decreasing pulmonary artery pressure and reducing pulmonary vascular resistance. The combination of sildenafil and NO treatment produced an even larger, synergistic effect.

PDE5 and the Systemic Vasculature

Originally, sildenafil was tested as an antianginal drug, targeting PDE5 in the systemic vasculature. However, in early clinical trials it soon became evident that sildenafil had only a modest effect on reduction of systemic blood pressure. Nevertheless, this small effect may turn into severe hypotension for patients taking a combination of sildenafil and nitroglycerin or other organic nitrates. This is consistent with the widespread occurrence of PDE5 in all smooth muscle beds. Because sildenafil is able to greatly potentiate the effects of NO generating compounds, use of sildenafil is contraindicated for most patients also using any organic nitrate.

Thus, as with all PDE5 inhibitors, sildenafil is most effective when the NO/cGMP signaling pathway is activated, suggesting that the clinical use of these inhibitors might be potentially expanded to other diseases associated with changes in cGMP signaling.

Recently, the development of two other PDE5 specific inhibitors, tadalafil (Cialis, Lilly ICOS LLC) and vardenafil (Levitra, Bayer and GlaxoSmithKline), has been reported. Each is able to inhibit PDE5 activity with IC50s in the nmol/L concentration range, and currently, both are being used for treatment of erectile dysfunction. They each have very similar affinities for PDE5 but vary somewhat in their pharmacokinetics and selectivity toward other PDEs. It is not yet clear if these differences will provide any advantages in clinical efficacy or appearance of side effects.

PDE5 Splice Variants and Domain Organization

Three different isoforms of PDE5A have been reported, PDE5A1, PDE5A2, and PDE5A3 (Table). All PDE5 variants differ only at the N-terminal end. The first PDE5 to be purified to homogeneity was the soluble enzyme from bovine lung. Originally called cGMP-binding, cGMP-specific PDE, this isoform is now known as PDE5A1. It appears to be the predominant form expressed in most PDE5 containing tissues. Human PDE5A1 is very similar to bovine PDE5A1, except that it contains an additional 10 amino acid insert at the N-terminal end. Another variant, PDE5A2, contains a significantly shorter amino acid N-terminal fragment and also has been shown in several species. PDE5A3 has been reported only in human tissues based on RT-PCR data.

PDE5 is highly specific for cGMP hydrolysis and contains two homologous N-terminal regulatory domains, recently defined as GAF A and GAF B based on their sequence homology with similar regulatory motifs now known to be present in a large group of proteins. The initial members of this group included the cGMP-regulated phosphodiesterases (PDE2, PDE5 and PDE6), several adenylyl cyclases, and a bacterial transcription factor called FhA. The acronym, GAF, is derived from the first letters of these groups. A recent search of the database shows that nearly a thousand other isoforms of PDE5A have been reported.
proteins contain GAF domains. Many, however, are expressed only in prokaryotes.

The GAF A domain of PDE5 is most homologous to the GAF B domain of PDE2 that also binds cGMP with high specificity. Very recently the crystal structure of the GAF A/B domains of PDE2 with cGMP bound has been solved. 25 It is thought that the structure of the PDE5 GAF domains will be very similar.

**Mechanisms for Regulation of PDE5 Activity**

**PKG Induced Phosphorylation of PDE5**

Several years ago it was shown by in vitro studies that PDE5 is phosphorylated on its N-terminal part (serine 92) and that binding of cGMP to a noncatalytic GAF domain of PDE5 was necessary for maximal rates of phosphorylation by PKA or PKG.26,27 This phosphorylation site is conserved in all PDE5 isoforms including bovine, human, canine, mouse, and rat PDE5. Nevertheless, questions remained about whether phosphorylation had any physiological significance in vivo. It was soon shown that in cultured rat smooth muscle cells32 P could be incorporated into PDE5 after prelabeling with 32P-ATP and treatment with ANP.28

More recently, a more quantitative approach using antibodies specific for the phosphorylation site of PDE5 showed a good correlation of activity with increased phosphorylation by PKG but not PKA both in vitro and in intact cells.29 Addition of 8-Br-cGMP to cultured human smooth muscle cells led to a gradual accumulation of the phosphorylated form of PDE5 (Figure 3A). When phosphorylation of PDE5 was compared with phosphorylation of vasodilator-stimulated phosphoprotein (VASP), a well-characterized substrate for PKA and PKG, it was found that the time-course of PDE5 phosphorylation was similar to that of VASP. It was demonstrated that after PKG activation 25% to 30% of the total PDE5 was phosphorylated, and that immunoprecipitated PDE5 had 2 to 2.5 times higher activity over the nonphosphorylated basal form when assayed at a cGMP substrate concentration of 1 μmol/L.

To answer the question of protein kinase specificity for PDE5 in intact SMCs, aortic SMCs from mice having a disruption in the PKG I gene were used. Incubation of PKG I−/− cells with 8-Br-cGMP did not produce any phosphorylation of PDE5, whereas in PKG I+/− cells a significant phosphorylation of PDE5 was observed (Figure 3B). Addition of 8-Br-cAMP alone or a combination of both 8-Br-cAMP and 8-Br-cGMP did not stimulate PDE5 phosphorylation in PKG I−/− aortic SMCs. These experiments provide unambiguous evidence that PDE5 phosphorylation in vivo is predominately mediated through the cGMP/PKG I, and not through the cAMP/PKA pathway.29

In related studies, PDE5 has been shown to be an important regulator of cGMP signaling in platelets. In these cells NO donors like GSNO (S-nitrosoglutathione) produce an extremely rapid increase followed by a rapid decline of intracellular cGMP. A time-dependent change of PDE5 activity

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Figure 3. In intact cells, activation of PKG, but not PKA, leads to PDE5 phosphorylation. A, 1 mmol/L 8-Br-cGMP was added to the cultured human uterine smooth muscle cells, and cells were harvested at different times after addition of 8-Br-cGMP. Phospho-PDE5 accumulation was detected by Western blot analysis using a phospho-specific PDE5 antibody. Phosphorylation of PKG-preferable site on VASP, serine 239, was detected by using monoclonal phospho-serine 239-specific VASP antibody. B, Mouse aortic smooth muscle cells from control mice (+/−) and PKG I−/− deficient mice (−/−) were incubated with 1 mmol/L 8-Br-cGMP (1), 1 mmol/L 8-Br-cAMP (2), or both (3) for 30 and 60 minutes. Phospho-PDE5 accumulation was detected by Western blot analysis using a phospho-specific PDE5 antibody. Adapted from Rybalkin SD, Rybalkina IG, Feil R, Hofmann F, Beavo JA. Regulation of cGMP-specific phosphodiesterase (PDE5) phosphorylation in smooth muscle cells. J Biol Chem. 2002;277:3310–3317.
substrate for the catalytic domain. However, activation of which bound poorly to the GAF domain but was a reasonable mAb/P3B2), or with mAb/P3B2 for 30 minutes on ice (Figure 4A). PDE5 activity was assayed with 0.1 μmol/L cGMP for 5 minutes at 30°C. An additional portion of 50 μmol/L cGMP was added to the preincubation mixture at 60 minutes after the start of preincubation as indicated by an arrow (open triangles). PDE5 activity was expressed as pmol min⁻¹ μg⁻¹ of protein. B, Pretreatment of PDE5 with mAb/P3B2 blocks cGMP-induced PDE5 activation and lowers basal PDE5 activity. PDE5 activity was measured at 0.1 μmol/L, 1.0 μmol/L, or 10 μmol/L cGMP. Samples were analyzed without any treatments (control) or after preincubation with 50 μmol/L cGMP on ice (+cGMP), or after preincubation with mAb/P3B2 for 30 minutes on ice (+mAb/P3B2), or with mAb/P3B2 for 30 minutes and then with 50 μmol/L cGMP (+mAb/P3B2 – cGMP). PDE5 activity was expressed as percent of control, and control (nonstimulated) PDE5 activity was defined as 100%. Adapted from Rybaklin SD, Rybakina IG, Shimizu-Albergine M, Tang X-B, Beavo JA. PDE5 is converted to an activated state upon cGMP binding to the GAF A domain. EMBO J. 2003;22:469–478.

and phosphorylation correlated with this rapid decline although no desensitization of guanylyl cyclase was observed.

cGMP Binding to the GAF Domain Directly Activates PDE5 Catalytic Activity

Because cGMP binding to PDE2 causes activation of its catalytic activity, the question arose whether or not cGMP binding to the GAF domain of PDE5 causes a similar activation. Although suspected, for many years no evidence had been found to support this idea.

One approach to this problem made use of a fluorescent analog of cGMP (Ant-cGMP-2′-O-anthraniloyl cGMP), which bound poorly to the GAF domain but was a reasonable substrate for the catalytic domain. However, activation of catalytic activity by cGMP binding was not directly demonstrated.

More recently, direct PDE5 activation upon cGMP binding to the regulatory GAF A domain was reported in a study using recombinant PDE5. When PDE5 was preincubated with cGMP, a large (up to 10-fold), time-dependent activation of PDE5 was observed (Figure 4A). PDE5 could also be reactivated after addition of another portion of cGMP to the preincubation mixture. cGMP-induced PDE5 activation was the highest when PDE5 activity was assayed at 0.1 μmol/L cGMP (9 to 11 times). At 1.0 μmol/L cGMP, PDE5 activation was only 2- to 3-fold, and at 10 μmol/L cGMP, no activation was detected (Figure 4B). The observed activation of PDE5 was not a result of PDE5 phosphorylation because mutation of the phosphorylation site (serine 92) to alanine did not change the pattern of PDE5 activation by cGMP.

To verify that the effect of PDE5 activation was due to a direct effect of cGMP occupancy of the cGMP-binding sites, a mouse monoclonal antibody (mAb/P3B2), generated against the GAF domain of PDE5, was found to be able to substantially block cGMP binding to the GAF A domain of PDE5. When this cGMP-blocking monoclonal antibody was applied before the cGMP preincubation, it completely prevented PDE5 activation and greatly reduced the hydrolytic activity of PDE5 (Figure 4B). These data strongly indicate that cGMP bound to its GAF domain exhibits a large stimulatory effect on the PDE5 catalytic domain, and without such an effect PDE5 has only a very low intrinsic hydrolytic activity.

Binding of cGMP appears to be necessary and sufficient to achieve full activation of PDE5, because in vitro phosphorylation of the activated PDE5 did not show any additional activation. It is known that cGMP binding to the GAF domain is necessary for phosphorylation by PKG and that phosphorylation increases the apparent affinity for cGMP binding. Therefore, it appears that what phosphorylation of PDE5 really does in vivo is to stabilize the cGMP-bound, activated state of the enzyme. This probably explains why most studies show 2-fold or less activation when assayed at 1 μmol/L substrate. Moreover, because PDE5 is a selective substrate for PKG, the phosphorylation status of PDE5, which is positively correlated with changes in PKG activity, can be used as an in vivo specific marker for PKG activation in tissues such as smooth muscle, platelets, and cerebellum that contain both PKG and PDE5.

Together, these data suggest that PDE5 can exist in at least two different conformational states in vivo: nonactivated and activated upon cGMP-binding (Figure 5). The nonactivated PDE5 is in a state with low intrinsic catalytic activity that can be converted reversibly to an activated state upon cGMP binding. These reversible conformational states of PDE5 possess different kinetic and inhibitory properties. For example, cGMP-activated PDE5 demonstrated a higher sensitivity toward the PDE5 specific inhibitor sildenafil, compared with nonactivated PDE5. The IC₅₀ for sildenafil inhibition declined from 2.1 to 0.63 nmol/L when PDE5 activity was assayed at a substrate concentration of 0.1 μmol/L cGMP.

Very recently, it has been shown that cGMP binding to the cGMP-binding sites could increase ³H-sildenafil binding to the catalytic site. Using a ³H-sildenafil exchange dissociation method two KD values (12 and 0.83 nmol/L) were found, implying the existence of two “conformers” of the PDE5 catalytic site.

Interestingly, it also has been found that the ability of PDE5 to be directly activated by cGMP was limited to relatively fresh preparations. PDE5 gradually lost its re-
PDE5, can still inhibit the photoreceptor PDE6 in the nmol/L range of concentration. It is likely that this inhibition may explain the visual side effects some patients report after clinical use of sildenafil.18 Tadalafil, a newer PDE5 inhibitor, has been reported as much more specific for PDE5 than for PDE6, but it is a relatively good inhibitor of PDE11, with an IC50 of 37 nmol/L. One of the newest PDE5 selective inhibitors, vardenafil, has an IC50 for PDE11 inhibition of 162 nmol/L. Sildenafil inhibited PDE11 with an IC50 of 2730 nmol/L.17

Therefore, one possible advantage of targeting cGMP binding to the GAF A domain of PDE5 (cGMP antagonists) might be a different profile of selectivity than achieved with the catalytic site antagonists. Conversely, a cGMP binding site agonist might also be possible. Such an agent would be expected to keep intracellular cGMP levels very low. This might be advantageous, for example, in treatment of neuronal excitotoxicity or toxicity associated with ischemia and reperfusion.

PDE1: Calmodulin-Stimulated Cyclic Nucleotide Phosphodiesterases

PDE1 Splice Variants and Domain Organization
Ca2+/calmodulin-stimulated PDEs (CaM-PDEs) constitute a large family of enzymes, encoded by three genes, PDE1A, PDE1B, and PDE1C.36 Multiple amino-terminal or carboxy-terminal splice variants have been identified within each gene. CaM-PDEs contain two Ca2+/calmodulin binding domains and binding of both Ca2+ and calmodulin is required for full activation of these PDEs. The degree of activation by Ca2+/CaM varies from 3- to 10-fold or even higher depending on the source, tissue, and purity of the enzyme preparation. In vitro, a basal nonstimulated state of PDE1s is obtained by removing Ca2+ with a Ca2+ chelator (eg, EGTA).

CaM-PDEs are able to hydrolyze both cGMP and cAMP, but substrate specificity differs among the different genes. PDE1A and PDE1B share the same high affinity for cGMP, but have different and lower affinities for cAMP. The affinity of PDE1B for cAMP is higher than that of PDE1A. PDE1C differs from both PDE1A and PDE1B by its ability to hydrolyze cGMP and cAMP equally well. Splice variants from the same gene family retain similar substrate characteristics. However, variations at the amino-terminal end have been found to have profound effects on the ability of calmodulin to induce PDE1 activation. For example, bovine PDE1A1 and PDE1A2 share identical protein sequence except for the very N-terminal 18 amino acids, but half maximal activation of PDE1A1 by calmodulin is 0.1 nmol/L, whereas for PDE1A2 it is 10 times higher.37

PDE1A and Nitrate Tolerance
Ca2+/CaM-stimulated PDE enzyme activity has been shown previously to be important for the regulation of vascular cGMP levels and reactivity (Figure 2).38 Most vasconstrictors, such as norepinephrine (NE), angiotensin II (Ang II), and endothelin-1 (ET-1) increase intracellular Ca2+, which is thought to be the major mechanism of vasconstrictor-mediated smooth muscle contraction. Accordingly, a Ca2+/CaM-stimulated PDE was found to be the major enzyme responsible for hydrolysis of cGMP in rabbit aorta stimulated...
with vasoconstrictors such as NE.\textsuperscript{39} Activation of PDE1A1 by increases in Ca\textsuperscript{2+} concentration has been demonstrated in cultured rat aortic SMCs. For example, it has been found that Ang II stimulates PDE1A1 activity in rat aortic SMCs, probably via an Ang II-mediated increase in Ca\textsuperscript{2+} concentration.\textsuperscript{40} Inhibition of PDE1A1 blocked the Ang II-mediated attenuation of ANP-evoked cGMP accumulation, suggesting that PDE1A1 mediates the inhibitory effect of Ang II on cGMP accumulation.

In addition to the rapid allosteric regulation of PDE1A1 and PDE5A1 by Ca\textsuperscript{2+} and cGMP, respectively, the longer-term expression levels of PDE1A1 and 5A1 are regulated by various pharmacological reagents or pathophysiological settings. For example, PDE1A1 enzyme activity, protein level, and mRNA expression are selectively upregulated in the nitrate tolerant rat model induced by chronic nitroglycerin (NTG) treatment.\textsuperscript{40} NTG remains one of the foremost drugs in the treatment of stable and unstable angina pectoris.\textsuperscript{41} When given acutely, NTG has potent vasodilator capacities on arteries, veins, and coronary collateral vessels. Chronic administration of NTG, however, is limited due to the rapid development of nitrate tolerance.\textsuperscript{42,43} Several mechanisms have been proposed to account for this phenomenon such as neurohormonal counter regulation (so-called pseudotolerance),\textsuperscript{44} or mechanisms intrinsic to the vascular tissue itself such as intracellular SH-group depletion, desensitization of the soluble guanylyl cyclase (sGC),\textsuperscript{45} increases in vascular production of reactive oxygen species,\textsuperscript{46} or increases in PDE activity (so-called true vascular tolerance).\textsuperscript{37} Chronic NTG treatment also has been shown to be associated with an increase in sensitivity to vasoconstrictors such as catecholamines, Ang II, KCl, and serotonin\textsuperscript{48} all of which may compromise the vasodilator capacity of NTG, thereby contributing to tolerance.

NTG induces vasorelaxation by releasing NO. NO can activate sGC and increase tissue levels of cGMP.\textsuperscript{49} As described earlier, cGMP in turn activates PKG, which has been shown to mediate vasorelaxation via phosphorylation of proteins that regulate contractility. Regardless of the mechanisms of tolerance, it appears to be associated not only with diminished cGMP elevation in response to subsequent nitrate exposure but also in response to vasoconstrictors such as NE.\textsuperscript{45} Interestingly, the functional consequences of decreased intracellular cGMP levels would nicely explain both phenomena observed in the setting of tolerance, ie, decreased sensitivity to NTG as well as increased sensitivity to vasoconstrictors. Thus, as mentioned earlier, the activity and expression of Ca\textsuperscript{2+}/CaM-stimulated PDE1A1 but not PDE5A1 was selectively induced in rat aortas treated for 3 days with a clinically relevant dose (10 \( \mu \text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \)) of NTG.\textsuperscript{40} The PDE inhibitor vinpocetine partially restored the sensitivity of the tolerant vasculature to subsequent NTG exposure. In the vasculature, PDE1A1 is primarily present in SMCs.\textsuperscript{50} Therefore, changes in PDE1A1 expression in intact aortas after NTG treatment most likely occurs in the SMCs. In nitrate tolerant vessels, an increase in sensitivity to NE has been found to be due to a greater cGMP lowering effect of NE in nitrate tolerant vessels.\textsuperscript{45} These observations together strongly support the idea that induction of PDE1A1 in nitrate tolerant vessels may be one of the mechanisms by which NO/cGMP-mediated vasodilation is desensitized and Ca\textsuperscript{2+}-mediated vasoconstriction is sensitized.\textsuperscript{40} Selective inhibition of PDE1A1 expression and/or activity therefore could be a novel therapeutic approach to limit nitrate tolerance.

Finally, in cultured rat SMCs, Ang II treatment rapidly and transiently upregulates both PDE1A1 and PDE5A1 mRNA expression, which is followed by increased protein levels and enzyme activities.\textsuperscript{51} The molecular mechanism for Ang II regulation of PDE1A1 and PDE5A1 expression appears very similar to that of the immediate early gene, c-fos. These observations suggest that alteration of PDE1A1 and PDE5A1 mRNA expression is an important mechanism for regulation of the cGMP-hydrolyzing activity in vasculature SMCs under physiological and pathological conditions. However, as discussed in the next section, human and nonhuman (eg, rat or monkey) SMCs have different PDE expression profiles. Thus, because nitrate tolerant models are mostly based on experiments with nonhuman tissues, studies in human vessels need to be conducted.

**PDE1C and Human Arterial Smooth Muscle Cell Proliferation**

**PDE1C Is Induced in Proliferating Human SMCs**

As mentioned earlier, all of the PDE1 isoforms, PDE1A, PDE1B, and PDE1C, can be expressed in arterial SMCs under different conditions and in different species. Expression of PDE1A and PDE1B, however, does not appear to be regulated by the proliferative state of the SMCs.\textsuperscript{52} This section will focus on PDE1C, an isoform that is activated by Ca\textsuperscript{2+}/calmodulin like all other PDE1s but, unlike PDE1A and PDE1B, has the ability to hydrolyze both cGMP and cAMP with equal efficiency.\textsuperscript{53}

Smooth muscle of intact human thoracic aorta from adult or newborn donors does not express PDE1C.\textsuperscript{52} In contrast, PDE1C is highly expressed in smooth muscle of intact human fetal aorta, which contains abundant proliferating cells as measured by expression of proliferating cell nuclear antigen (Figure 2).\textsuperscript{54} Furthermore, there is a marked induction of PDE1C expression and activity in human adult and newborn aortic SMCs that have been stimulated to proliferate in culture.\textsuperscript{52} Thus, in striking contrast to the intact adult or newborn human aorta, when SMCs were cultured and analyzed by HPLC, high activity of PDE1C was observed (Figure 6A). As expected, PDE1C hydrolyzes both cGMP and cAMP in the presence of Ca\textsuperscript{2+}/calmodulin (denoted by solid symbols in Figure 6A), and the PDE1C protein is recognized by antibodies generated against the C-terminus of recombinant PDE1C (Figure 6A), but not by antibodies against PDE1A or PDE1B. The PDE1C induced in proliferative human SMCs in culture constitutes as much as 85% of the total cGMP-hydrolyzing activity and 80% of the total cAMP-hydrolyzing activity (at low substrate concentrations) in the presence of Ca\textsuperscript{2+}/calmodulin. The presence of high PDE1C activity in cultured human aortic SMCs has recently been confirmed by Palmer and Maurice.\textsuperscript{55} Abundant PDE1C activity also has been found in cultured human SMCs isolated from the carotid artery, and a number of human proliferating SMCs from different smooth muscle containing organs,
showing that PDE1C induction is not restricted to cultured aortic SMCs.52

The above studies showed a clear correlation between induction of PDE1C expression and activity, on the one hand, and the proliferative capacity of human SMCs in fetal aorta and cultured human SMCs. To further verify that PDE1C expression is regulated by the proliferative state of the cell, human SMCs were plated on fibrillar collagen type I. This type of collagen results in complete growth arrest of SMCs56 and a phenotype similar to SMCs found in the intact aorta.57 Consistent with the hypothesis that PDE1C expression is regulated by the proliferative state, cultured human SMCs plated onto fibrillar collagen type I have a markedly reduced PDE1C expression.54 On the other hand, cultured human SMCs exposed to low serum conditions do not downregulate PDE1C expression to a significant extent. Interestingly, the quiescence induced by fibrillar collagen is more pronounced than that induced by serum withdrawal. Thus, cells that have been plated onto fibrillar collagen require more time to enter S phase after release from the fibrillar collagen compared with cells that have not been previously exposed to fibrillar collagen. It is likely that the fibrillar structure of the collagen results in exit of the cells from the cell cycle (most cells would be in G0), whereas serum withdrawal results in G1 arrest. Thus, PDE1C expression is downregulated first when the SMC exits the cell cycle to G0.

Finally, there is a clear correlation between time required for induction of DNA synthesis and induction of PDE1C expression in SMCs that have been released from fibrillar collagen type I and replated onto tissue culture plastic in the presence of serum (Figure 7A). Together, these experiments show that PDE1C expression not only is upregulated when SMCs enter the cell cycle, but also is downregulated when cellular quiescence is induced, and suggest that either complete quiescence or exit of SMCs from the cell cycle into G0 is required to reduce PDE1C expression.

**PDE1C Regulates Proliferation of Human SMCs**

The studies described above show that PDE1C expression is markedly regulated by the cell cycle in human SMCs. Does this mean that induction of PDE1C is required for cell cycle progression and cell proliferation? Currently, there are two means available to address this question: inhibition of PDE1C activity using pharmacological inhibitors and reduction of PDE1C expression by PDE1C antisense or RNA interference.
techniques. Specific PDE isoforms show different responsiveness to pharmacological inhibitors.53 However, because specific PDE1C inhibitors were not available, 8-methoxymethyl 3-isobutyl-1-methylxanthine (8MM-IBMX) at concentrations of 10 to 30 μmol/L, was used to inhibit PDE1C activity. Treatment of cultured human SMCs with 8MM-IBMX resulted in a significant reduction of DNA synthesis.54 These effects of 8MM-IBMX are likely to be due to PDE1C inhibition, because the same concentrations do not significantly inhibit the other cAMP PDEs (PDE3 and PDE4). Furthermore, the effects of 8MM-IBMX were not mimicked by PDE5 inhibitors. To further verify a regulatory role for PDE1C in human SMC proliferation, PDE1C antisense studies were performed. Treatment with PDE1C antisense oligonucleotides resulted in decreased expression of PDE1C without affecting expression of PDE5, and also significantly inhibited SMC proliferation (Figure 7B). Together, these results strongly suggest that induction of PDE1C promotes proliferation of human arterial SMCs and may be required for it to occur.

Is the effect of PDE1C on SMC proliferation mediated by hydrolysis of cAMP or cGMP, or both? In vivo, SMCs are believed to be exposed to both cAMP- and cGMP-elevating agents released by the endothelium. Prostacyclin (PGI2) is an example of a cAMP-inducing agent released from the endothelium, whereas NO acts, at least in part, by elevating cGMP levels in target cells (Figure 2). Both cGMP and cAMP are known to inhibit SMC proliferation in vitro and in vivo.58 Accordingly, human SMC proliferation can be inhibited by pharmacological inhibitors selective for cAMP-hydrolyzing PDEs or for cGMP-hydrolyzing PDEs.54 The effect of cGMP on SMC proliferation appears to be more complex than that of cAMP.59,60 In contrast to the complete suppression of cell cycle traverse induced by 8-Br-cAMP (a cAMP analog partially resistant to PDE-mediated hydrolysis), 8-Br-cGMP appears to delay, but not block, the G1-to-S transition in human aortic smooth muscle SMCs.60 These cells express both PKA and PKG, effectors of cAMP and cGMP signaling. Thus, the molecular pathways regulated by cAMP and cGMP may be distinct. This concept was further supported by the findings that both 8-Br-cAMP and 8-Br-cGMP completely suppressed platelet-derived growth factor (PDGF)-stimulated cdk4 activity, but that only 8-Br-cAMP resulted in induction of the cdk inhibitor p27kip1 and a sustained suppression of cdk2 activation.60 Alternatively, 8-Br-cAMP and 8-Br-cGMP may be hydrolyzed at different rates by PDEs expressed in the human umbilical artery SMCs, resulting in a more transient effect of 8-Br-cGMP as compared with 8-Br-cAMP. In this context, it may also be relevant that cAMP analogs and subsequent activation of PKA can exert a negative feedback on PDE1C activity.61

In human SMCs, PDE1C inhibition results in increased levels of both cAMP and cGMP,54 as would be expected based on the enzymatic characteristics of PDE1C. In fact, the actions of cAMP and cGMP are not always unrelated. For example, a recent study shows that PDE5 inhibitors reduce proliferation of bovine coronary artery SMCs via cGMP elevation and subsequent inhibition of PDE3, a cGMP-inhibitable cAMP-hydrolyzing PDE.62 Through this mechanism, increased intracellular levels of cGMP can result in elevation of cAMP levels (see section on PDE3). The effects of PDE1C on SMC proliferation may therefore be due to hydrolysis of both cGMP and cAMP.

Why Is PDE1C Induced in Proliferating Human SMCs but not in SMCs From Other Species?
To date, detectable PDE1C activity has only been observed in proliferating human SMCs and, curiously, not in proliferating aortic SMCs from nonhuman primates (pigtail monkey, Macaca nemestrina, and baboon), bovine SMCs, porcine SMCs, rat SMCs, or ovine SMCs.52,63 On the other hand, all of these species express PDE1A and/or PDE1B. As shown by Figure 6B, the PDE activity profile in proliferating aortic SMCs isolated from pigtail monkey is strikingly different from that of SMCs isolated from human aorta (Figure 6A). Both human and monkey SMCs express PDE5 and PDE3/4, but whereas human SMCs express PDE1C, monkey SMCs express PDE1B (Figure 6). These findings show that there are marked differences in expression and activities of PDE1C in SMCs from different species. We can only speculate on the reason for induction of PDE1C in proliferating human SMC, as opposed to SMCs derived from other species. It is possible that human SMCs have the need for a more extensive and controlled cAMP and cGMP hydrolysis during cell cycle progression than SMC from many other species. Intracellular calcium levels are tightly regulated during cell cycle progression,64 and induction of PDE1C may serve as a means to coordinate mitogenic calcium signaling with a concomitant decrease in levels of the growth-inhibiting actions of both cGMP and cAMP. Regardless of the reason for the induction of PDE1C in proliferating human SMCs, special care must be taken when extrapolating results obtained with selective PDE inhibitors in animal studies to clinical trials in humans.

Given the key role apparently played by PDE1C in the regulation of human smooth muscle proliferation, it would not be surprising if therapeutic agents can be developed that target PDE1C activity. Such agents would be expected to minimize the excess smooth muscle proliferative response that occurs in response to injury and inflammation that is caused by balloon angioplasty or stenting and perhaps even hypertension. They likely also would be less toxic than many currently used agents.

PDE3: cGMP-Inhibited PDE
Both PDE3A and 3B are expressed in most vascular smooth muscle beds. Although they may not be the predominant contributors to hydrolysis of cGMP in these vascular beds, or for that matter in the cardiocyte, the PDE3s probably are regulated by cGMP in vivo. PDE3s do not have separate allosteric cGMP-binding domains, and are not regulated by cGMP in the same way as the GAF domain containing PDEs (PDE2 and PDE5). However, the PDE3 catalytic sites have similar high affinity for cAMP and cGMP, but the Vmax for cAMP is much higher (4 to 10 times) than for cGMP. Therefore, the mechanism by which cGMP inhibits PDE3 catalytic activity is through competition with cAMP at the catalytic site. Thus, cGMP acts as a transient switch causing
inhibition of cAMP hydrolytic activity by PDE3 until it is itself hydrolyzed.

**PDE3 Domain Organization and Function**

The PDE3 family contains two genes, PDE3A and PDE3B. The domain organization of both is quite similar and includes a conserved catalytic domain, a divergent N-terminal region with its membrane association domain and a C-terminal hydrophilic end (Figure 1). PDE3A and PDE3B share most homology in the catalytic domain, including a unique 44 amino acid insertion, not found in the catalytic domains of PDEs from any other family. However, these isoforms differ greatly at both their N-terminal and C-terminal ends. Studies of different truncated PDE3 forms revealed that the N-terminal end was not required for maintaining full catalytic activity and sensitivity to PDE3 specific inhibitors, although it might be important for localization.

The two PDE3 isoforms are differentially expressed. PDE3A is expressed in vascular smooth muscle, platelets, cardiocytes, and oocytes. In rat and human vascular smooth muscle, both PDE3A and PDE3B are expressed, but they have distinct subcellular localizations. PDE3A was found mostly in the soluble fraction, whereas PDE3B was associated with the particulate fractions. PDE3B also is highly expressed in adipose cells, hepatocytes, and spermatocytes.

PDE3 is usually thought to mediate mostly cAMP regulated processes such as cardiac contractility, platelet aggregation, smooth muscle relaxation, and hormonal regulation. Much less is known about PDE3 involvement in regulation of cGMP signaling. Some studies have shown that cGMP elevating agents can produce an increase in cAMP levels by inhibiting PDE3 activity. For example, NO-induced inhibition of rabbit platelet aggregation was caused in part by cAMP accumulation as a result of PDE3 inhibition. It also has been suggested that in human atrial myocytes, the stimulatory effects of NO-onors on cardiac calcium current were due to cGMP inhibition of PDE3 activity. PDE3 has also been suggested as an important determinate of NO effects on renal vasculature.

However, recent studies ofPKG I–deficient mice showed that high concentrations of NO donors were able to produce enough cGMP to get direct PKA activation, whereas low concentrations of cGMP induced smooth muscle relaxation exclusively through the cGMP signaling pathway. Therefore, further studies are needed to determine under what conditions cGMP enhances PKA activity by direct activation as opposed to indirectly through PDE3 inhibition.

**PDE3 and Cardiovascular Drug Development**

The first generation of PDE3 inhibitors (milrinone, vasodilator, exenatide) were found to have significant vasodilatory and inotropic effects in vitro and in animal studies. In initial clinical trials these inhibitors were believed to have a positive effect in the treatment of chronic congestive heart failure. However, long-term effects of oral administration of milrinone revealed an increase the morbidity and mortality of patients with severe chronic heart failure. Although it is not known if the correct doses were used or even if the cardiotoxic effect was due only to PDE3 inhibition, this unsuccessful clinical trial presented an additional challenge for the development of PDE3 inhibitors. Despite these results, short-term clinical use of milrinone has been approved for the treatment of patients with acute decompensated heart failure. Intravenous injection of milrinone lactate (Primacor, Sanofi-Synthelabo Inc) can provide a significant, but strongly time-limited clinical effect (no longer than 48 hours) and requires close observation of the electrocardiographic parameters of these patients.

Another PDE3 inhibitor, cilostazol (Pletal, Otsuka America Pharmaceutical, Inc/Pharmacia Corporation), has been approved for the treatment of intermittent claudication, a vascular disease characterized by pain in the legs. Again, this drug is contraindicated for patients with congestive heart failure. Newer generations of PDE3 specific inhibitors probably will have to demonstrate tissue or isoform specificity that should minimize effects on cardiac tissue in order to meet approval.

**Conclusions and Perspective**

It is expected that in the next few years we will learn much more about what specific roles individual PDE isozymes play in smooth muscle function. We also should be able to identify how the transcription of these PDEs is regulated, what role(s) may be played by some of the newly discovered PDEs (eg, PDE9), and particularly how different species utilize different combinations of PDEs to regulate their cyclic nucleotide dependent processes. It seems quite possible that new uses for the “traditional” smooth muscle PDE inhibitors like Viagra will be identified and perhaps new drugs will be developed that act on other sites of PDE5 or on the active sites of other PDEs expressed in vascular smooth muscle.

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**References**


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