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Phosphodiesterases and Cardiac cGMP: Evolving Roles and Controversies

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Abstract

Cyclic guanosine monophosphate (cGMP) and its primary target kinase –protein kinase G (PKG) are well-recognized modulators of cardiac function and chronic stress response. Their enhancement appears to serve as a myocardial brake, reducing maladaptive hypertrophy, enhancing cell survival signaling and mitochondrial function, protecting against ischemia/reperfusion injury, and blunting the stimulatory effects from catecholamines. Translation of these effects into a chronic treatment in patients with heart failure based on enhancing cGMP generation has be difficult however, with tolerance and hypotension effects from nitrates, and neutral responses to natriuretic peptides (at least B-type). Inhibition of cGMP-targeted phosphodiesterases such as PDE5A is an alternative approach, and one that appears to have more potent effects. Recent studies in experimental models and patients are revealing benefits in heart failure syndromes, and ongoing multicenter trials are specifically testing efficacy of PDE5A inhibition. Here we discuss recent research findings and controversies regarding the PDE/cGMP/PKG signaling pathway, and suggest directions for further research.

Introduction

By the end of each day, the average human heart beats >100, 000 times to fulfill its job of delivering nutrients and oxygen to the peripheral tissues. This requires the ability to both rapidly respond to changes in demand and adapt to chronic stresses that ensue from physiologic or pathological stimuli. Central to this regulation are the cyclic nucleotide 3', 5'-monophosphates cAMP and cGMP, and their respective effecter enzymes, protein kinase A and G (PKA and PKG). cAMP-PKA is a primary regulator of excitation-contraction associated with PKA-phosphorylation of the voltage-gated calcium channel, ryanodine receptor, phospholamban, and sarcomeric proteins to enhance and accelerate Ca^{2+} cycling through the sarcoplasmic reticulum and stimulate contraction and relaxation. cGMP/PKG by contrast is considered a sort of myocardial brake, countering cAMP stimulation as well as independently signaling alternative pathways to blunt contraction and growth, while still enhancing relaxation[1].

The centrality of cAMP and cGMP regulation has made them attractive targets for therapeutic intervention in heart failure. To date, activating cAMP has proven a difficult

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tactic. Although it acutely enhances cardiac function, chronic stimulation of cAMP can worsen outcome and increase mortality. New approaches based on targeting distal G-coupled protein receptors such as receptor kinase[2] or sarcoplasmic reticulum calcium ATPase(SERCA)[3, 4] could provide alternatives that are beneficial, though clinical testing is at very early stages or not yet started.

Cyclic GMP has also been targeted, principally by enhancing synthesis by nitric oxide (NO) or natriuretic peptide stimulation. NO-activates soluble guanylate cyclase, thereby inducing vasodilation, with cardiac effects primarily being modestly enhanced diastolic function; however, tachyphylaxis has limited its sustained use. B-type natriuretic peptide was introduced into clinical practice in 2001 for the treatment of acute decompensated heart failure based on its capacity to lower central vascular pressures rather than its renal or cardiac modifications[5, 6]. Subsequent studies raised nephrotoxicity concerns[7], though a recent trial found neutral results; no toxicity but also no benefits[8]. An alternative approach is to block specific phosphodiesterases (PDEs) that control cGMP hydrolysis. Yet as of early 2000, remarkably little was known about what enzymes accomplished this in the cardiac myocyte. This changed with evidence that PDE5A, the first discovered and still best understood cGMP-selective PDE, might be involved. Over the past decade, experimental and clinical studies have reported on the efficacy of PDE5A inhibition to treat various forms of heart disease. The work helped spawn the ongoing NIH-multicenter trial (RELAX: Evaluating the Effectiveness of Sildenafil at Improving Health Outcomes and Exercise Ability in People With Diastolic Heart Failure; NCT00763867) in patients with heart failure and a preserved ejection fraction (i.e. EF>50%).

Despite the recent work, plenty of questions and controversies persist. Are the drugs really targeting PDE5A, or are they inhibiting alternative PDEs, such as PDE1, which is more highly expressed in heart and also hydrolyzes cGMP? Is myocyte PDE5A important, or are cardiac effects seen from PDE5 inhibitors origniated from other cell types? Do PDEs selectively partner with specific intracellular cGMP pools, and if so, is this compartment altered by heart disease? How important is myocyte PKG activation to this process? And lastly, are effects of PDE5A inhibition similar in left ventricle (LV) and right ventricle (RV)? We discuss these issues and highlight areas for further research.

Identifying the PDEs involved in cardiac funtion

Five PDEs are capable of hydrolyzing cGMP (PDE1, PDE2, PDE3, PDE5 and PDE9), and genes for each are expressed in heart[9]. However, debate exists as to which are most important. Most analysis of PDE activity is based on the use of pharmacologic inhibitors *in vitro*, but this might not duplicate the complex regulation of the PDEs *in vivo*, particularly for dual-esterases and/or those are positively or negatively regulated by the cyclic nucleotides themselves. Recent evidence suggests more than one is involved, each likely targeting a selective pool of cGMP to regulate signal transduction. Furthermore, the precise role played by each PDE may vary among mamallian species. The various cGMP-regulating PDEs and their proposed interactions with cGMP, cAMP, and PKG myocyte targets are summarized in Figure 1.

PDE1 is a Ca^{2+}/calmodulin stimulated dual-substrate esterase encoded by 3 genes with multiple splice variants. Humans express PDE1A and -1C, with PDE1C appearing to be the major isotype[10, 11], whereas in rodents, PDE1A seems the major isoform[12]. Levels can rise with chronic heart disease, as shown in mice exposed to isoproterenol or pressure-overload[12]. Understanding of PDE1 in the intact heart remains very limited, with only one study testing a selective inhibitor IC86340 (no longer available) showing reduced ISO-induced hypertrophy in mice[12]. A cGMP/PKG dependent anti-hypertrophic effect was

observed in cell culture from RNA silencing of PDE1A, and inhibiting both PDE1A and PDE5A yielded additive effects in these studies, supporting their regulation of different cGMP pools[12].

PDE2 is also a dual-substrate esterase that can hydrolyze cGMP *in vitro*, yet it also is stimulated by cGMP to increase its hydrolyze of cAMP, behavior observed in the presence of adrenergic stimulation[13]. Which action occurs *in vivo* and under what conditions is unknown, because selective PDE2 inhibitors active *in vivo* have not been developed, and genetic deletion studies not yet reported. PDE3 principally targets cAMP, and its inhibition was widely tested as a potential positive inotropic therapy, (e.g. milrinone). Although it can hydroylze cGMP as well, this occurs at a much lower Vmax, so cAMP is favored.

PDE5A was the first cGMP-selective PDE discovered, and it is also activated by cGMP which binds to its GAF regulatory domain[14]. It is expressed in vascular smooth muscle, endothelium, and fibroblasts. Many laboratories have also reported expression in cardiac myocytes, albeit at much lower levels as compared to lung. Immunoblots and confocal imaging employing different commercial[15–17] and custom generated/purified antibodies[18] have generally found myocyte PDE5a expression and its localization to Z-discs[16, 18, 19]. However, myocyte expression had historically been considered negligible, and there remains some controversy as a recent study again reported no protein[20]. Differences in the antibody reagents may underlie the discrepancy. Importantly, the specificity of immune detection has been tested and confirmed in rat neonatal and adult mouse cardiomyocytes in which PDE5a was also genetically silenced[21]. The expression and activity of PDE5 rises in human and mouse cardiac hypertrophy and heart failure[18, 22, 23], and could contribute to the pathophysiology by reducing cGMP and PKG activity. Such upregulation maybe coupled to myocardial oxidative stress[24].

PDE5a is regulated by a negative feedback loop where by increased levels of cGMP and PKG activity (via phosphorylation at S92) stimulate hydrolytic activity to reduce cGMP. Through this mechanism, PKG activation reduces cGMP generated by nitric oxide synthase-sGC pathways. However, a recent study found the opposite held for cGMP generated by natriuretic peptide coupled receptor GC, where PKG further stimulated cGMP generation[25]. The PKG target in the latter case remains unknown and the subject of ongoing research.

PDE9 (9A, 9B) is the newest and highest affinity cGMP-selective PDE discovered, and is expressed in testes, brain, skeletal muscle, and heart. Currently most studies are focused on the effect of PDE9 in neural syndromes such as affective disorders and memory loss[26–28]. Research into its cardiac role is at an early stage.

Evidence and Controversy regarding myocardial PDE5A regulation

The introduction of sildenafil' in 1998 for treating erectile dysfunction represented a major fork in the road for a drug being tested against hypertension and angina. A decade later, it was approved for pulmonary hypertension, but cardiac indications were still considered unlikely given its modest impact on arterial tone and low expression and activity in resting myocytes. However, in hearts under various forms of stress, the situation appeared quite different. Initial studies showed in intact dog, mouse, and human, that PDE5 inhibition blunted acute beta-adrenergic stimulated contractility coupled to protein kinase G activation. Kukreja and colleagues reported sildenafil acts in hearts and myocytes to suppress reperfusion injury and apoptosis[15, 29, 30], and in 2005, Takimoto et al[16] reported sildenafil had anti-hypertrophic effects in mice with pressure-overload in the absence of vascular unloading. The latter could be achieved well after pre-existing pathology was established[31]. These effects coexist with PKG activation, and targets such as RGS2[32], calcineurin-NFAT-TRPC6[23, 33, 34], ERK and GSK3β[35] and mitochondrial K_{ATP} channels[36–38] have been

identified to be contributors. New work in the *mdx* mouse model of Duchenne muscular dystrophy found sildenafil improves cardiac function, sarcolemmal integrity, and suppresses abnormal fetal gene expression[35, 39], effects coupled to reduced Ca^{2+} loading and mitochondrial Ca^{2+} uptake, preventing excessive mitochondrial permeability transition pore opening[40]. This work gave rise to a recently initiated clinical trial of sildenafil (REVERSE-DMD, NCT01168908) to treat Duchenne's patients with cardiac disease, which is currently recruiting patients at the Johns Hopkins Medical Institutions.

These studies have mostly employed sildenafil, which is relatively but not fully selective for PDE5A, and some have argued the doses used might also target PDE1[41]. Moreover, as the drug affects all cell types, myocytes might not be the primary target. However, PDE5 gene silencing blocks hypertrophy in neonatal myocytes[21], and myocyte PDE5 overexpression worsens LV remodeling after infarction[18]. We recently developed a bi-directional PDE5 overexpression model, showing that myocyte PDE5 gene up- or down-regulation potently modulates cardiac responses to pressure-overload (worsens or ameliorates, respectively) while orchestrating extracellular matrix remodeling as well[42]. Reducing myocyte PDE5 gene expression/activity after establishing cardiac disease improved heart function and fibrosis, a response similar to that from sildenafil therapy. Other evidence for selectivity comes from studies with another PDE5 inhibitor-tadalafil, which is much specific for PDE5A over PDE1, yet also protects against myocardial ischemia/reperfusion injury[43] and improves LV function and prevents apoptosis in doxorubicin-induced acute cardiomyopathy[44]. Admittedly, the role of PDE5 versus PDE1 could differ in humans. However, recent clinical studies in heart failure patients using sildenafil at doses considered fairly specific to PDE5A support benefits not only on exercise performance and symptoms[45–47], but recently on both cardiac systolic and diastolic function and remodeling[47].

Microdomains couple PDEs to specific guanylate cyclases—Compartmentation of cAMP is well established and thought regulated by anchoring proteins (AKAPs) that coordinate the cyclase with relevant PDE and PKA within a given microdonain. Growing evidence supports the existence of cGMP compartmentation as well, and although GKAP equivalents have not been found, localized PDE regulation appears important. In cardiac myocytes, PDE1C1 localizes to Z-discs and M-lines in a striated pattern[10], and has been suggested to provide the majority of cAMP and cGMP hydrolytic activity in soluble fractions from human myocardium, and majority of cGMP hydrolytic activity in microsomal fractions[10]. This is less the case in rodent hearts[12]; but again these conclusions are based on *in vitro* assays. PDE2 localizes to the plasma membrane (particularly at cell-cell junctions) and Z-disc[13]. At the membrane, it resides along with beta-adrenergic receptors (β-AR), adenylate cyclase (AC), and nitric oxide synthase (NOS), all located in caveolin-rich lipid rafts. This localization could underlie its reported coupling to β3AR-dependent NOS/cGMP-activation of the PDE to hydrolyze cAMP and blunt adrenergic tone[13]. In isolated myocytes, PDE2 was found to selectively target the NP-receptor-coupled cGMP pool[48]. Yet even if PDE2 is inhibited, the cGMP generated from NP stimulation does not appear to replicate myocyte contractile regulation effects (e.g. blunt β-AR stimulation) as compared to that observed with NO-stimulation or PDE5 inhibition[49]. Something yet unknown appears to be constraining the NP signal.

PDE5 normally localizes to z-disc[16, 19, 50, 51] where it modulates soluble but not particulate cGMP[48], and this localization is also important for its contractile modulation. Cyclic GMP enhanced by PDE5 inhibition (but not NP-cGMP) blocks β-AR stimulation, an effect requiring β3-AR stimulated NOS3-sGC activation[16, 49, 50, 52, 53]. Intriguingly, PDE5 can move away from z-discs in myocytes from the failing or eNOS deficient heart[16, 50, 51]. The chaperone(s) involved and pathophysiological significance of this translocation, for example

whether it alters that targeting to another cGMP pool, is under active investigation. Whether this applies to other cGMP-PDEs has not yet been reported.

Localized regulation could also be a function of post-translational modifications of a given cyclase or PDE by PKG. For example, Castro *et al*.[54] recently showed that PKG activation in adult cardiomyocytes limits cGMP accumulation from NO stimuli by phosphorylating and activating PDE5, but increases cGMP generation via pGC-NP in a positive feed back loop. The protein and residue target for the latter modulation remains unknown. PDE localization and regulation within microdomains means that analyzing their activity from homogenized cell/tissues using labeled cyclic nucleotides may not provide accurate insight as to what their behavior would be *in vivo*. Fluorescent-based sensors and mutant membrane bound cGMP-stimulated channels (CNG) have been used[48, 54, 55], though these remain challenging particularly for identifying non-membrane pools.

Is myocyte PKG important?

PKG is thought to be the primary downstream effecter of cGMP signaling pathway, and has long been thought to play a role in myocardial remodeling. The strongest evidence for this has been in cell culture studies in which selective PKG inhibitors, expression of dominant negative kinase (or gene silencing), or overexpression is used[56, 57]. *In vivo* evidence has been more “guilt by association”, that is a given maneuver coupled to cGMP generation improves heart disease and PKG activation also rises[58–60]. Global PKG gene silencing induces early lethality (largely from gut immotility), and conditional cardiac targeted knock-down models have yet to be successfully generated. The relevance of PKG activation as an anti-hypertrophic modulator also appears to depend upon the activation of particular target enzymes induced by the stress that can be in turn modulated by PKG. If these are not activated, PKG might have only minimal influence[61].

A recent study has challenged the role of myocyte PKG by subjecting mice with global PKG-1 knockout and concomitant smooth muscle-targeted PKGIβ overexpression to both hormonal and mechanical stress[20]. Mice lacking myocyte PKG exposed to 7-days of intravenous isoproterenol infusion or transaortic constriction developed similar levels of ventricular hypertrophy as did controls. Fetal gene recapitulation including a fall in myosin heavy chain α/β ratio and rise in natriuretic peptide were also similar in both groups. However, the level of stress induced in both models was modest and the existence of maladaptive signaling known to be targeted by PKG was not shown. This could be important, as a prior study had shown that enhancing PKG activity during moderate pressure-overload stress had little impact, whereas similar activation with more severe stress was effective[61]. This correlated with the presence of PKG-targeted cascades with the latter but not former condition.

An alternative approach to selectively manipulating PKG activity was recently reported using a doxycycline-controllable (Tet-off) myocyte-targeted PDE5 overexpressor mouse[42]. When myocyte PDE5 was upregulated, PKG activity became depressed, and when combined with pressure-overload, resulted in marked worsening of cardiac hypertrophy, dysfunction, and fibrosis. Importantly, after inducing this pathophysiology, targeted suppression purely of myocyte PDE5 led to a rise in PKG activity, and subsequent improvement all aspects of the pathophysiology. Neither approach attacks the problem directly, and a further resolution awaits the development of selective and inducible PKG-knock down models, which are hopefully underway.

What about the right ventricle?

One might have presumed that, given the initial focus on PDE5 inhibition for pulmonary disease, studies regarding its impact on the RV would have come early. However, the concomitant decline in vascular afterload made this difficult to assess. Several experimental studies have since appeared, and the results seem quite different to those for the LV. In two models in which pulmonary arterial banding was used, and contrasted to a model of pulmonary vascular hypertension, sildenafil benefited the RV only in the latter setting[62, 63]. In a third study, investigators found that PDE5 inhibitors augmented contractility in RV hypertrophied rat hearts (unlike controls), accompanied by a rise in cAMP level similar to that from a direct β-agonist[22]. This was ascribed to cGMP-inhibition of PDE3 to elevated cAMP. This has not been reported for the LV, nor to our knowledge, in other studies on the RV. Still, more and more data supports fundamental differences in developmental biology, molecular and cellular physiology of RV versus LV, and their response to stress, so differences in cGMP/PDE/PKG signaling may well exist. This area is ripe for more detailed mechanistic analysis, and is important given the role cGMP-PDE inhibitors play in clinical pulmonary vascular disease.

Concluding remarks

Nearly two decades after the first clinical trials of a novel PDE5 inhibitor, and 12 years after it was turned into an drug for erectile dysfunction, therapeutic efforts to treat heart disease by this approach have been reignited. By the end of 2011, we should have important data on its efficacy in HFpEF patients, there is movement to develop a large scale trial of sildenafil in dilated cardiomyopathy, and other indications such as for muscular dystrophy syndromes may be forthcoming. In the meantime, much remains to be learned about the molecular targets, role of PKG, and importance of other cGMP PDEs and capacity of their selective inhibitors to ameliorate cardiac disease. New conditional and targeted mouse models are needed to selectively modify PDEs and confirm the specificity for observed changes with small molecule inhibitors. Limitations due to the inability to often target specific splice variants and thus compartmentalized PDE regulation need to be better understood or surmounted. One key lesson we have learned, and one that applies broadly to the field, is that expression levels of a given PDE do not directly translated to physiologic importance. Some are expressed at very low levels yet can exert a great effect by regulating a defined microdomain. In vitro activity removed from all of the localized signaling and regulation may not identify what is happening in tissues. The past decade has seen explosive interest in how PDE modulation can be used to impact cGMP/PKG signaling in the heart. The next one will undoubtedly reveal new actors and story lines that will hopefully further point to useful therapies for heart disease patients.

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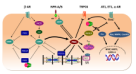

Figure 1. Schematic diagram showing PDE/PKG regulation of cardiac myocytes

Generation of cGMP is shown from both a nitric oxide synthase (NOS) –soluble guanylate cyclase (sGC) pathway, and natriuretic peptide receptor-receptor GC pathway. These are two very different pools, and appear to regulate different aspects of cardiac myocyte function and stress adapatation. Their stimulated PKG is also likely compartmentalized though this remains to be clarified and is depicted here for simplicity as a common pool. Once generated, cGMP can stimulate its effecter kinase PKG, and also regulates and is regulated by phosphodiesterases. Four PDEs (PDE1a, c; PDE2, PDE3a, and PDE5a) have been identified as contributing to hydrolytic regulation and/or signaling in myocytes. PDE1 is calcium-calmodulin (CaM) dependent and is a dual esterase, PDE2 is a cGMP-stimulated cAMP esterase, while PDE3A is principally a cAMP esterase that can be competitively inhibited by cGMP. PDE5a is the only selective cGMP esterase of this group. It has several post-translational activation mechanisms; cGMP binding to N-terminus GAF domains, and PKG phosphorylation at serine 92.

On the right of the figure are the targets of PKG. PKG can activate PDE5a – to lower cGMP, but may also target the rGC/NPR complex to stimulate cGMP generation, PKG also phosphorylates phospholamban (PLB) to enhance calcium uptake by the sarcoplasmic reticulum (SR), and mitochondrial proteins to regulate K_{ATP} channel opening and provide cardioprotection. Recent studies have also shown revealed it phosphoryates and binds to regulator of G-coupled protein signaling RGS2 and RGS4, both acting to suppress Gαq-coupled signaling cascades (e.g. Ras, MAP kinase, CamKII, and associated transcription factors, NFAT, MEF2 and GATA-4) Studies have also shown PKG directly phosphorylates and suppresses transient receptor potential channel 6, reducing the trigger calcium that has been linked to calcineurin (Cn) activation and subsequent dephosphorylation of NFAT, leading to the latter's nuclear translocation.