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Role of Phosphodiesterases in Cyclic Nucleotide Compartmentation in Cardiac Myocytes

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A current challenge in cellular signaling is to decipher the complex intracellular spatiotemporal organization that any given cell type has concocted to be able to discriminate among different external stimuli acting via a common signaling pathway. This obviously applies to cAMP and cGMP signaling in the heart where these cyclic nucleotides determine the regulation of cardiac function by many hormones and neuromediators.\textsuperscript{1-5} Recent studies have identified phosphodiesterases (PDEs) as key actors in limiting the spread of cAMP and cGMP, and in shaping and organizing intracellular signaling microdomains. With this new role, PDEs have been promoted from the rank of a housekeeping attendant to that of an executive officer.

\section*{1. CYCLIC NUCLEOTIDES REGULATE CARDIAC FUNCTION}

\subsection*{1.1. Cardiac excitation-contraction coupling (ECC)}

When a myocyte is depolarized by an action potential, Ca\textsuperscript{2+} ions enter the cell through L-type Ca\textsuperscript{2+} channels (LTCCs) located on the sarcolemma and generate an inward current, I_{Ca,L}. I_{Ca,L} triggers a subsequent release of Ca\textsuperscript{2+} from the sarcoplasmic reticulum (SR) through ryanodine receptors Ca\textsuperscript{2+}-release channels (RyR2), a mechanism known as “Ca\textsuperscript{2+}-induced-Ca\textsuperscript{2+}-release” (CICR).\textsuperscript{6} Free Ca\textsuperscript{2+} ions bind to contractile proteins, such as troponin I (TnI), and induce myofilament contraction.\textsuperscript{7} During relaxation, part of cytosolic Ca\textsuperscript{2+} is sequestered into the SR by an ATP-dependent Ca\textsuperscript{2+} pump (SERCA, sarco-endoplasmic reticulum Ca\textsuperscript{2+}-ATPase) which is controlled by phospholamban (PLB), thus lowering the cytosolic Ca\textsuperscript{2+} concentration and removing Ca\textsuperscript{2+} from contractile proteins (Fig. 1).\textsuperscript{7}

\subsection*{1.2. Regulation of cardiac function by cAMP pathways}

Force of contraction (inotropy) and beating frequency (chronotropy) are under the dual control of the sympathetic and parasympathetic systems. Both systems control in an opposite manner the synthesis of cAMP, and, hence, the activity of the cAMP effectors (Fig. 1). Catecholamines released into the synaptic cleft at sympathetic nerve terminals bind to \( \beta_1 \)-adrenergic receptors (\( \beta_1 \)-ARs) on the cardiac sarcolemma, activate stimulatory G proteins (G\(_S\)), which in turn activate two isoforms of adenylyl cyclase (AC), AC5 and AC6, that catalyze the conversion of ATP to cAMP. cAMP then activates the cAMP-dependent protein kinase (PKA) which initiates a series of phosphorylation processes activating ECC.
Phosphorylation of LTCC and RyR2 enhances their open probability resulting in higher cytosolic Ca\(^{2+}\) concentrations and increased contractility.\(^7,8\) Additionally, PKA phosphorylates PLB, hence stimulating SERCA activity and increasing Ca\(^{2+}\) reuptake into the SR.\(^9\) Phosphorylation of TnI proteins decreases their sensitivity to Ca\(^{2+}\).\(^10\) These two latter events contribute to accelerate relaxation (lusitropic effect) during β-adrenergic stimulation. PKA also controls metabolic (glycogen synthase, phosphorylase kinase) and transcriptional activities (via CREB) in cardiac myocytes.\(^11\) cAMP can also act in cardiomyocytes in a PKA-independent manner, through direct activation of Epac,\(^12\) a guanine nucleotide exchange factor for the small GTPase Rap1,\(^13\) and HCN cyclic nucleotide-gated ion channels.\(^14\) As a mirror image, acetylcholine released into the synaptic cleft at parasympathetic nerve terminals binds to the muscarinic M2 receptors and induces AC inhibition via inhibitory G protein (Gi), thus decreasing PKA activation (Fig. 1).\(^15\)

The level of intracellular cAMP is regulated by the balance between the activity of AC and the cyclic nucleotide PDEs that degrade cAMP to 5’AMP. PDE activity is found not only in cytosol, but also in a variety of membrane, nuclear and cytoskeletal locations.\(^16\) Cardiac PDEs fall into at least five families: PDE1, which is activated by Ca\(^{2+}\)-calmodulin; PDE2, which is stimulated by cGMP; PDE3, which is inhibited by cGMP; PDE4 and PDE5. Whereas PDE1 and PDE2 can hydrolyze both cAMP and cGMP, PDE3 preferentially hydrolyzes cAMP; PDE4 and PDE 5 are specific for cAMP and cGMP, respectively.

1.3. Regulation of cardiac function by cGMP pathways

It is generally accepted that cGMP opposes the effect of cAMP on cardiac function.\(^4,17\) Intracellular cGMP production is achieved by two different forms of guanylyl cyclases: a soluble form (sGC) which is activated by NO; a particulate form (pGC) which is activated by natriuretic peptides, such as ANP, BNP and CNP.

1.3.1. Cardiac NO signalling

In mammalian heart, NO can be produced by three different NO-synthase (NOS) isoenzymes which are expressed in various cell types, including cardiomyocytes: the neuronal (nNOS or NOS1), the endothelial (eNOS or NOS3) and the inducible (iNOS or NOS2) isoforms.\(^18,19\) Both eNOS and nNOS are constitutively expressed in cardiomyocytes. eNOS is located within membrane caveolae\(^20\) whereas nNOS is normally located in sarcoplasmic
reticulum. These isoforms undergo specific responses in the heart. For example, NO produced in SR by nNOS exerts a facilitatory effect on SERCA activity and stimulate Ca\(^{2+}\) influx trough LTCC, while eNOS attenuates the inotropic response to β-adrenergic stimulation. Most of the physiological effects of NO depend on cGMP synthesis that follows the binding of NO to the heme moiety of sGC. However, a direct reaction between NO and reactive thiol residues in several proteins, like RyR2, has also been shown to have significant functional consequences.

### 1.3.2. Cardiac Natriuretic Peptides Signaling

ANP and BNP are primarily synthesized in cardiac atria and ventricles, respectively, while CNP is predominantly located in central nervous system, pituitary, kidney and vascular endothelial cells. CNP is secreted by endothelial cells in the heart, but its role in myocardial function is less clear than that of ANP. Natriuretic peptides exert their effects by three single transmembrane natriuretic peptide receptors: NPR-A, NPR-B and NPR-C. Both NPR-A and NPR-B have intrinsic enzymatic activity in their cytosolic domain and catalyze the synthesis of cGMP from GTP. NPR-C lacks enzymatic activity, but controls local concentrations of natriuretic peptide through constitutive receptor-mediated internalization and degradation and acts via G\(_i\)-dependent mechanisms. All receptors can be activated by the three natriuretic peptides but NPR-A has a higher affinity for ANP and BNP while NPR-B is more specific for CNP.

### 1.3.3. cGMP Signaling

Intracardiac cGMP acts via three main enzymes: PDE2, PDE3 and PKG. Activation of PKG by cGMP reduces cardiac contractility by inhibition of LTCC activity and reduction of myofilament Ca\(^{2+}\) sensitivity. At submicromolar concentrations, cGMP increases heart rate and contractility by inhibition of PDE3 and increase in cAMP levels; at higher concentrations, cGMP exerts the opposite effect due to PDE2 activation and decrease in cAMP levels. Due to these multiple mechanisms, the overall effect of a rise in cGMP via NO or natriuretic peptides will depend on the level of expression, the location and the activity of PKG, PDE2 and PDE3, which may vary considerably depending on the animal species, the cardiac tissue, and the basal and stimulated levels of cAMP and cGMP.
2. COMPARTMENTATION OF CYCLIC NUCLEOTIDES SIGNALING

A prevailing molecular view of the receptor/enzyme/effecter interaction in cells is based upon the random collision theory, according to which proteins are floating randomly on the lipid membrane surface and contact with the target-protein interaction. A major limitation of this theory, however, is that a rapid signaling cascade involving multiple proteins may not be efficiently processed, particularly when these signaling molecules are scarce. Several observations have suggested that some components within the cyclic nucleotide-signaling pathway are colocalized to discrete regions of the plasma membrane such as caveolae41-44 and transverse tubules45,46, thereby allowing rapid and preferential modulation of cAMP and cGMP production within a defined microenvironment. With the discovery of AKAP (A Kinase Anchoring Protein)47,48, it has become apparent that intracellular targeting of PKA as well as the preassembly of components of signaling pathways in clusters or on scaffolds are important for the speed and organization of cAMP signal transduction events. However, one would wonder how specificity is maintained when small diffusible molecules such as cAMP and cGMP are generated during signaling cascade. Localized cyclic nucleotide signals may be generated by interplay between discrete production sites and restricted diffusion within the cytoplasm. In addition to specialized membrane structures that may circumvent cAMP and cGMP spreading,1,49 degradation of these cyclic nucleotides by PDEs appears critical for the formation of dynamic microdomains that confer specificity of the response.50-53

The first evidence for a compartmentation of cAMP signaling in heart comes from experiments made almost 30 years ago in isolated perfused hearts.54-56 Important differences were observed when comparing hearts perfused with different agonists activating the cAMP cascade, particularly via β1-AR and prostaglandin E1 receptor (PGE1-R): with isoproterenol (ISO), cAMP is elevated, the force of contraction is enhanced, soluble and particulate PKA are activated, and the activity of phosphorylase kinase and glycogen phosphorylase is increased; with PGE1, cAMP content and soluble PKA activity are also increased, but there is no change in contractile activity or in the activities of PKA substrates that regulate glycogen metabolism.56 Similar results were reproduced in isolated myocytes.57 The situation is even more complex if one considers that a given cardiac myocyte expresses many other Gs-coupled receptors, besides β1-ARs and PGE1-Rs, that increase cAMP but produce different effects. For instance, adult rat ventricular myocytes also express β2-ARs, glucagon receptors (Glu-Rs) and glucagon-like peptide-1 receptors (GLP1-Rs). β2-AR stimulation increases contractile force but does not activate glycogen phosphorylase58 and does not accelerate relaxation59,60 (but see...
Glu-R stimulation activates phosphorylase and exerts positive inotropic and lusitropic effects, but the contractile effects fade with time; GLP₁-R stimulation exerts a modest negative inotropic effect despite an increase in total cAMP comparable to that elicited by a β₁-adrenergic stimulation.

These results clearly show that the cell is able to distinguish between different stimuli acting on a common signaling cascade. One possible way to achieve that distinction is to confine the cAMP signalling cascade to distinct intracellular compartments which may differ depending on the stimulus used.

In the same context, several studies have shown that cGMP produced by either sGC or pGC produces different functional effects in various cell types. For instance, in human endothelial cells from umbilical vein, activation of sGC induces a more efficient relaxation than does pGC activation. In airway smooth muscle cells from pig, stimulation of pGC induces relaxation exclusively by decreasing intracellular Ca²⁺ concentration, whereas sGC stimulation decreases both Ca²⁺ concentration and sensitivity of the myofilaments. In human embryonic kidney, ANP, but not SNAP induces a recruitment of PKG to plasma membrane and amplifies GC-A activity. Differences between sGC and pGC activation have also been reported in cardiac preparations. For instance, in frog ventricular myocytes, sGC activation causes a pronounced inhibition of I_{Ca,L} upon cAMP stimulation, while pGC activation has little effect. In rabbit atria, pGC activation caused a larger cAMP accumulation (via PDE3 inhibition), cGMP efflux and ANP release than activation of sGC. In mouse ventricular myocytes, both pGC and sGC activation exerted similar negative inotropic effects. These effects on cell contraction were mediated by cGMP dependent pathway involving PKG and PDEs. However, pGC activation decreased Ca²⁺ transients, whereas sGC activation had marginal effects, similarly to what was found in pig airway smooth muscle. These data suggest that pGC signalling works mainly to decrease intracellular Ca²⁺ level, whereas sGC-signalling mainly decreases Ca²⁺ sensitivity.

3. METHODS TO STUDY CYCLIC NUCLEOTIDE COMPARTMENTATION IN INTACT MYOCYTES

During twenty years, most of the evidence supporting a compartmentation of cyclic nucleotide signaling in cardiac preparations was gathered using biochemical assays in fractionated dead tissues or cells. However, during the last decade, a number of sophisticated
methods have been developed which now allow to evaluate the role of cyclic nucleotide compartmentation in intact living cells.

The first such method combines a classical whole-cell patch-clamp recording of \( I_{\text{Ca,L}} \) (as a probe for cAMP/PKA activity) with a double-barreled microperfusion system (Fig. 2A).\(^{50}\) This allows to test the effect of a local application of a receptor agonist on LTCC in the part of the cell exposed to the agonist and compare it with the response of the channels located on the non-exposed part. This method provided the first evidence for a local elevation of cAMP in response to a \( \beta_2 \)-adrenergic stimulation in frog ventricular cells as compared to a uniform elevation of cAMP in response to forskolin, a direct adenylyl cyclase activator.\(^{50}\) A similar conclusion was reached using the cell-attached configuration of the patch-clamp technique in mammalian cardiomyocytes\(^{70}\) and neurons\(^{71}\) by applying a \( \beta_2 \)-adrenergic agonist either inside or outside the patch-pipette while recording single LTCC activity in the patch of membrane delimited by the pipette.

More direct methods have been developed to monitor cyclic nucleotide changes using fluorescent probes and imaging microscopy. The first such probe was FlCRhR, a fluorescent indicator for cAMP which consists of PKA in which the catalytic (C) and regulatory (R) subunits are each labeled with a different fluorescent dye, respectively fluorescein and rhodamine.\(^{72}\) Fluorescence resonance energy transfer (FRET) occurs in the holoenzyme complex R2C2 but not when cAMP binds to the R subunits and C subunits dissociate. The change in shape of the fluorescence emission spectrum allows cAMP concentrations to be visualized in real-time in single living cells, as long as it is possible to microinject the cells with the labeled holoenzyme.\(^{72}\) This in itself represents a major technical challenge, particularly in cardiomyocytes,\(^{73}\) and has prompted the search for genetically encoded probes. A cAMP probe has been generated using the same principle as FlCRhR, but by fusing a YFP and a CFP protein to R and C subunits, respectively, instead of labeling these proteins with fluorescein and rhodamine.\(^{74}\) On a similar principle, through genetic modifications of other target effectors, a number of different probes are now available for real time measurements of cAMP (Fig. 2B)\(^{75-78}\) and cGMP\(^{79,80}\) in living cells, including cardiac myocytes.\(^{53,81-84}\).

A third type of approach is based on the use of recombinant cyclic nucleotide-gated channel (CNG) channels as cyclic nucleotide biosensors (Fig. 2C). The methodology was developed in a series of elegant studies in model cell lines for the measurement of intracellular cAMP.\(^{49,85,86}\) This method uses wild-type or genetically modified \( \alpha \) subunits of rat olfactory CNG channel (CNGA2) which form a cationic channel directly opened by cyclic
nucleotides. Adult cardiac myocytes infected with an adenovirus encoding the native or modified channels elicit a non-selective cation current when, respectively, cGMP or cAMP concentration rises beneath the sarcolemmal membrane.

4. ROLE OF PDEs IN CYCLIC NUCLEOTIDE COMPARTMENTATION

4.1. PDEs and hormone specificity

Probably the first evidence for a contribution of PDEs to intracellular cyclic nucleotide compartmentation comes from a study in guinea pig perfused hearts. ISO was shown to significantly increase intracellular cAMP, cardiac contraction and relaxation, as well as phosphorylation of PLB and TnI, while the non-selective PDE inhibitor IBMX or the selective PDE3 inhibitor, milrinone, enhanced contraction and relaxation but had little or no effect on phosphorylation of PLB and TnI, despite a relatively large increase in tissue cAMP level. These results were attributed to a functional cellular compartmentation of cAMP and PKA substrates due to a different expression of PDEs at the membrane and in the cytosol.

Many subsequent studies have examined the degree of accumulation of cAMP or activation of cAMP dependent phosphorylation in particulate and soluble fractions of cardiac myocytes. In an elegant such study performed in canine ventricular myocytes, Hohl and Li (1991) demonstrated that cytosolic and particulate pools of cAMP are differently affected by various treatments designed to raise intracellular cAMP. These authors have demonstrated that about 45% of the total cAMP is found in the particulate fraction in response to ISO but this fraction declined to <20% when IBMX was added to ISO, although total cAMP still increased approximately 3-fold. This suggests that cAMP-specific PDE activity resides predominantly in the cytosolic compartment and is responsible for the particulate cAMP microdomains generation that cause Ca\(^{2+}\) mobilization and cardiac inotropic state through particulate PKA activation and phosphorylation of membrane and contractile proteins. Even if the cell is able to generate and accumulate cAMP that exceeds what is needed for a maximal physiologic response under PDE inhibition and forskolin stimulation, only particulate cAMP content determines the physiological response. These results show that PDEs maintain the specificity of the β-adrenergic response by limiting the amount of cAMP diffusing from membrane to cytosol.

These biochemical data are in full agreement with functional studies in frog ventricular myocytes where the effect of a local application of ISO on I\(_{\text{Ca,L}}\) was tested in the
presence or absence of IBMX.\textsuperscript{50} While the \(I_{\text{Ca,L}}\) response to ISO was much higher at the side of ISO application than in the non-exposed part of the cell, complete PDE inhibition in the presence of ISO released the cAMP signal to activate LTCCs in the remote part of the cell. Thus, these results suggest that PDE activity contributes to generate cAMP microdomains involved in the β-adrenergic stimulation of \(\text{Ca}^{2+}\) channels (Fig. 3). A recent study using recombinant CNG channels demonstrates that this also applies to other \(G_{s}\)-coupled receptors (\(\beta_1\)-AR, \(\beta_2\)-AR, PGE1-R, Glu-R), with a specific pattern of PDE activity determining the specificity of the cAMP signals generated by each receptor.\textsuperscript{89} For instance, cAMP elicited by \(\beta_1\)-AR is regulated by PDE3 and by PDE4 while cAMP signal generated by Glu-R is exclusively regulated by PDE4. In mouse neonatal cardiomyocytes, PDE4D was shown to selectively impact cAMP signaling by \(\beta_2\)-AR, while having little or no effect on \(\beta_1\)-AR signaling.\textsuperscript{93} Indeed, while \(\beta_2\)-AR activation leads to an increase in cAMP production, the cAMP generated does not have access to the PKA-dependent signaling pathways by which the \(\beta_1\)-AR regulates the contraction rate, unless PDE4D is inhibited or its gene has been invalidated.\textsuperscript{93}

4.2. Specific roles of PDE isoforms in cyclic nucleotide compartmentation

The use of selective inhibitors of the dominant cardiac PDE isoforms has allowed to evaluate the contribution of four different PDE families in the compartmentation of cAMP and cGMP pathways in cardiac myocytes: PDE2, PDE3, PDE4 and PDE5. Co-immunoprecipitation experiments have further demonstrated that macromolecular complexes exist at different locations within a cardiac myocyte that include PDE3 and PDE4 isoforms, forming local signalling microdomains.

4.2.1. PDE2

Cyclic GMP stimulated PDE (PDE2) hydrolyses both cAMP and cGMP with low affinity. A single PDE2 variant, PDE2A, is expressed in cardiac tissues and in isolated cardiomyocytes of several species, including rat and human.\textsuperscript{94,95} PDE2 is found both in the cytosol and associated to functional membrane structures (plasma membrane, sarcoplasmic reticulum, Golgi, nuclear envelope).\textsuperscript{16} Although PDE2 activity is relatively small compared to other cardiac PDEs, such as PDE3 and PDE4, its presence at the plasma membrane contributes to regulate the activity of cardiac LTCCs when cGMP level is increased.\textsuperscript{4} This
was first demonstrated in frog ventricular myocytes dialyzed with cAMP and cGMP, where PDE2 is able to hydrolyze cAMP and hence reduce I_{Ca,L} upon application of cGMP, even when >5 µM cAMP is continuously dialyzed inside the cell via the patch pipette. Increased knowledge of the contribution of PDE2 to cardiac function has accumulated after the demonstration that the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) behaves as a selective PDE2 inhibitor. EHNA reverses the inhibitory effect of high concentrations of cGMP or NO-donors on I_{Ca,L} in frog ventricular and human atrial myocytes. EHNA alone stimulates basal I_{Ca,L} in isolated human atrial myocytes, indicating a possible role of basal guanylyl cyclase activity in these cells.

The role of PDE2 in cyclic nucleotide compartmentation was first examined in frog cardiac myocytes, using the double-barreled microperfusion technique and local applications of NO-donors and/or EHNA on I_{Ca,L} stimulated by ISO. The results of that study demonstrated that local stimulation of soluble guanylyl cyclase by NO leads to a strong local depletion of cAMP near the LTCCs due to activation of PDE2, but only to a modest reduction of cAMP in the rest of the cell. This may be explained by the existence of a tight microdomain between β-ARs, LTCC and PDE2. A similar conclusion was reached recently in rat neonatal cardiomyocytes, using the FRET-based imaging technique.

PDE2 is not only involved in the control of subsarcolemmal cAMP concentration, but also controls the concentration of cGMP in that compartment. Indeed, a recent study performed in adult rat ventricular myocytes using the CNG technique compared the effects of activators of pGC (using ANP or BNP) and sGC (using NO-donors) on subsarcolemmal cGMP signals, and the contribution of PDE isoforms to these signals. The main result of that study is that the ‘particulate’ cGMP pool is readily accessible at the plasma membrane, while the ‘soluble’ pool is not, and that the ‘particulate’ pool is under the exclusive control of PDE2. Therefore, differential spatiotemporal distributions of cGMP may contribute to the specific effects of natriuretic peptides and NO-donors on cardiac function.

### 4.2.2. PDE3

Cyclic GMP inhibition of PDE3 can lead to cAMP increase and to activation of cardiac function. This mechanism accounts for the stimulatory effect of low concentrations of NO-donors or cGMP on I_{Ca,L} un human atrial myocytes. However, a recent study performed in perfused beating rabbit atria demonstrated that, depending on whether cGMP is
produced by pGC or sGC, the effects on cAMP levels, atrial dynamics, and myocyte ANP release are different, although in both cases the effects are due to PDE3 inhibition. These results suggest that cGMP-PDE3-cAMP signalling produced by pGC and sGC is compartmentalized. The role of PDE3 in cyclic nucleotide compartmentation likely depends on its intracellular distribution. PDE3 is present in both cytosolic and membrane fractions of cardiac myocytes, with important species and tissue differences. For instance, in dog heart, all PDE3 activity revealed in the membrane fraction appears to be associated with the SR membrane. Inhibition of PDE3 under such condition could lead to localized increases in cAMP and PKA pools leading to increased PLB phosphorylation.

Three isoforms of PDE3 have been identified in human myocardium. They appear to be generated from PDE3A gene and localize to different intracellular compartments: PDE3A-136 is present exclusively in microsomal fractions while PDE3A-118 and PDE3A-94 are both present in microsomal and cytosolic fractions. The presence of different PDE3A isoforms in cytosolic and microsomal fractions of cardiac myocytes is especially interesting in view of the facts that cAMP metabolism in these compartments can be regulated in an independent manner and that changes in cAMP content in these compartments correlate with changes of different physiologic parameters, such as intracellular Ca$^{2+}$ homeostasis and contractility. These observations are relevant in a physiological context since competitive inhibitors of PDE3 confer short term haemodynamic benefits but adversely affect long term survival in dilated cardiomyopathy. This biphasic response is likely to result from an increase in the phosphorylation of a large number of PKA substrates, some of which may contribute to the beneficial effects (phosphorylation of PLB) whereas others contribute to the adverse effects (phosphorylation of LTCC or RyR2). If one would suppose that different isoforms regulate different proteins in response to different signals, logically agents capable of selectively activating or inhibiting individual PDE3A isoforms may have advantages over currently available nonselective PDE3 inhibitors in therapeutic applications. For instance, an agent that selectively inhibits SR-associated PDE3A-136 might preserve intracellular Ca$^{2+}$ cycling and contractility in patients taking β-AR antagonists, without concomitant arrhythmogenic effects.

In addition to PDE3A, cardiac myocytes also express a PDE3B isoform, at least in mouse. Of particular interest is the finding that this isoform forms a complex at the cardiac sarcolemmal membrane with the G protein-coupled, receptor-activated phosphoinositide 3-kinase γ (PI3Kγ). Ablation of PI3Kγ in mice (PI3Kγ−/−) induces an exacerbated heart failure
in response to aortic constriction which appears to be due to a PDE3B inhibition and to excess cAMP. But mice carrying a targeted mutation in the PI3Kγ gene causing loss of kinase activity (PI3KγKD/KD) exhibit normal cardiac contractility associated with normal cAMP levels after aortic stenosis compared to PI3Kγ-/-'. Therefore, PI3Kγ does not activate PDE3B via its kinase activity, but rather serves as an anchoring protein, which recruits PDE3B into a membrane compartment where cAMP homeostasis shapes the chronic sympathetic drive.109

4.2.3. PDE4

The PDE4 family is encoded by 4 genes (A, B, C and D) that generate approximately 20 different isoforms, each of which is characterized by a unique N-terminal region.110,111 Transcripts for PDE4A, PDE4B and PDE4D isoforms were found in rat heart.81,95,112,113 In the PDE4D family, mRNA for PDE4D1, PDE4D2, PDE4D3, PDE4D5, PDE4D7, PDE4D8 and PDE4D9 is present in rat heart112,113 but only PDE4D3, PDE4D5, PDE4D8 and PDE4D9 are expressed as proteins and active enzymes.113

An emerging theme in PDE4 action is that individual isoforms appear to be restricted to defined intracellular microenvironments thus regulating particular sets of intracellular processes (Fig. 4).111,114,115 Compartmentation of PDE4 isoforms is mediated by their unique N terminal domains which provide the ‘postcode’ for cellular localization.116 For instance, PDE4A1 contains a lipid binding domain, TAPAS, with specificity for phosphatidic acid that serves to target this PDE to specific cellular membranes.117 In the heart, PDE4D3 is targeted to sarcomeric region of cardiomyocytes through binding to an anchor protein called myomegalin,118 and to the perinuclear region through binding to muscle AKAP (mAKAP).119 This latter complex is interesting because mAKAP not only binds PKA and PDE4D3, but also Epac1 and ERK5 kinase.120 The three functionally distinct cAMP-dependent enzymes contained in this macromolecular complex (PKA, PDE4D3 and Epac1) respond to cAMP in different ranges of concentrations: PKA responds to nanomolar concentrations and would become activated early; PDE4D3 (Km 1-4 µM) and Epac1 (Kd 4 µM) would become activated once cAMP concentrations reached micromolar levels. Conversely, inactivation of PDE4D3 and Epac1 would precede PKA holoenzyme reformation as cAMP levels decline.120 Besides, phosphorylation of PDE4D3 by PKA on Ser-54 enhances its activity 111,113 and on Ser-13 increases its affinity to mAKAP,121 while phosphorylation by ERK5 on Ser 579 suppresses its activity.120 Therefore, when Epac1 is activated by cAMP, it mobilizes Rap1 which suppresses ERK5 activation and relieves the inhibition of PDE4D3. With such fine tuning, this complex
provides spatial control of PKA signaling by mAKAP anchoring and temporal control and termination of the cAMP signaling event by PDE activity in the immediate vicinity.\textsuperscript{114,119} This compartment of cAMP signaling in the perinuclear region may control the release of C subunit into the nucleus\textsuperscript{119,122} and hence gene regulation.\textsuperscript{114}

The same PDE4D3 was also found recently to be an integral component of the RyR2/Ca\textsuperscript{2+}-release channel complex at the SR membrane.\textsuperscript{123} In addition to RyR2 and PDE4D3, this complex is composed of mAKAP, PKA, FKBP12.6 (calstabin2, a negative modulator or channel-stabilizing subunit of RyR2), and the protein phosphatases PP1 and PP2A.\textsuperscript{124,125} PKA phosphorylation of Ser2809 on RyR2 increases the open probability of the Ca\textsuperscript{2+}-release channel and decreases the binding affinity for the channel-stabilizing subunit calstabin2, contributing to SR Ca\textsuperscript{2+} store depletion.\textsuperscript{125} Of particular interest is the observation that heart failure in patients and animal models causes PKA hyperphosphorylation of RyR2 and “leaky” RyR2 channels that promote cardiac dysfunction and arrhythmias.\textsuperscript{125} Two sets of evidence indicate that this is due to a reduction in PDE4D3 activity in the RyR2 complex:\textsuperscript{123} first, although total PDE4 activity may be enhanced in pressure-induced congestive heart failure\textsuperscript{126}, PDE4D3 levels in the RyR2 complex appear reduced in failing human hearts;\textsuperscript{123} second, genetic inactivation of PDE4D in mice is associated with a cardiac phenotype comprised of a progressive, age-related cardiomyopathy and exercise-induced arrhythmias, despite normal global cAMP signaling.\textsuperscript{123} These results emphasize the importance of cAMP signaling microdomains and point to the intriguing possibility that deregulation of specific compartments may lead to a disease state.

A final example of a complex around a PDE4 isoform in heart is the one formed by PDE4D5 and β-arrestins.\textsuperscript{127} β-arrestins are scaffold proteins that initiate desensitization of β2-AR (as well as several other G-protein-coupled receptors) by translocating from the cytosol to bind activated receptors at the plasma membrane. Recent studies have shown that β-arrestins can form stable complexes with all four PDE4 subfamilies in cytosol\textsuperscript{127} but that PDE4D5 possesses a unique amino-terminal region that confers preferential interaction with β-arrestins\textsuperscript{115,116,128,129}. The specific role of this PD4D5/β-arrestin interaction in the β2-AR signaling cascade comes from a unique feature of this particular receptor which can couple to both Gs and Gi.\textsuperscript{130} Upon agonist challenge, β2-AR couples to Gs that activates AC, thereby elevating local cAMP concentration and activating membrane PKA anchored to AKAP-79.\textsuperscript{129} PKA in turn phosphorylates the β2-AR which triggers a shift in its coupling from Gs to Gi, hence activating ERK through a Src-regulated pathway.\textsuperscript{131} Therefore, recruitment by the
activated β2-AR of the PD4D5/β-arrestin puts a brake in the PKA phosphorylation of the receptor, and prevents its shift to G\textsubscript{i}-signaling cascade; conversely, disruption of this complex enhances PKA phosphorylation of the β2-AR, leading to a dramatic change in its function.\textsuperscript{131,132}

4.2.4. PDE5

PDE5 is highly expressed in vascular smooth muscle, and its inhibition is a primary target for the treatment of erectile dysfunction and pulmonary hypertension.\textsuperscript{133,134} Although the contribution of PDE5 to the regulation of cardiac function is a matter of debate,\textsuperscript{135-137} there is evidence for PDE5 expression in cardiac myocytes, both at the mRNA\textsuperscript{138} and protein level.\textsuperscript{83,139} Recently, PDE5 inhibition using sildenafil (Viagra) was shown to decrease the β-adrenergic-stimulation of cardiac systolic and diastolic function in dog,\textsuperscript{139} mouse,\textsuperscript{83} and human\textsuperscript{137,140} as well as the β-stimulation of I\textsubscript{Ca,L} in guinea pig ventricular myocytes.\textsuperscript{141} In mouse ventricular myocytes sildenafil was shown to inhibit apoptosis\textsuperscript{142} and to reduce infarct size following ischemia/reperfusion in the myocardium.\textsuperscript{143} Moreover, chronic exposure to sildenafil was found to prevent and reverse cardiac hypertrophy in mouse hearts exposed to sustained pressure overload.\textsuperscript{144} Most recently, PDE5 was also shown to contribute to intracellular cGMP compartmentation in cardiac myocytes.\textsuperscript{87} Indeed, using the recombinant CNG channel approach to measure subsarcolemmal cGMP concentration in adult rat ventricular myocytes, sildenafil produced a dose-dependent increase of the CNG current activated by NO-donors but had no effect on the current elicited by ANP. Therefore, PDE5 exerts a specific spatiotemporal control on the pool of intracellular cGMP synthesized by sGC, but not that generated by pGC which, as discussed above, is under the exclusive control of PDE2.\textsuperscript{87} This could be either because PDE5 is more closely compartmentalized with sGC than pGC, or because PKG, which activates PDE5,\textsuperscript{133} is compartmentalized with sGC but not pGC. Differential spatiotemporal distributions of cGMP may therefore contribute to the specific effects of natriuretic peptides and NO-donors on cardiac function.\textsuperscript{66-69,145}

4.2.5. Cooperative role of PDE isoforms

In many examples, more than one PDE isoform is involved in controlling the cAMP or cGMP concentration at any given intracellular location inside a cardiomyocyte. For instance, in the case of cGMP, both PDE2 and PDE5 were found to control the
subsarcolemmal concentration of cGMP upon activation of sGC by NO-donors in rat cardiomyocytes as demonstrated by selective inhibition of each PDE isoform. Indeed, EHNA or sildenafil used alone raised subsarcolemmal cGMP to a lower level as when the two inhibitors were applied together or when both PDEs were blocked by IBMX.\(^8\) Similarly, the activity of cardiac LTCCs or the force of contraction is affected by the hydrolytic activity of several PDEs, since inhibition of a single PDE isoform is insufficient to raise cAMP level enough to activate these parameters.\(^9\) Real time measurements of cAMP in isolated cardiomyocytes using either the FRET-based or the recombinant CNG channel method have shown that PDE4 and to a lesser extent PDE3 regulate the amplitude of cAMP response upon a β-adrenergic stimulation.\(^8\) The more prominent role of PDE4 vs. PDE3 families may partly result from a larger stimulatory effect of PKA phosphorylation on the former providing a faster negative feedback regulation on cAMP concentration.\(^9\) Indeed, blockade of PKA strongly increased the cAMP signal at the membrane upon β-AR stimulation of adult rat ventricular myocytes.\(^8\)

5. PATHOPHYSIOLOGICAL ROLE OF cAMP COMPARTMENTATION?

Cyclic AMP microdomains may be important not only for the hormonal specificity but also to prevent a global rise in cAMP which is known to be deleterious.\(^1\) This was illustrated in functional studies performed on the transgenic mouse line AC8TG, in which the human neuronal Ca\(^{2+}\)/calmodulin activated type 8 adenylyl cyclase (AC8) protein is specifically expressed in cardiomyocytes.\(^1\) In this animal model, AC and PKA activity are increased, respectively, 7- and 4-fold,\(^1\) although the animals show no sign of hypertrophy or cardiomyopathy at up to 3-months of age.\(^1\) Isolated perfused hearts from AC8TG mice show an increased heart rate, larger amplitude of contraction, faster kinetics of contraction and relaxation, and no response to ISO as compared to non-transgenic (NTG) mice.\(^1\) At the single cell level, myocytes from AC8TG hearts contract faster and stronger, develop larger and faster Ca\(^{2+}\)-transients, which represent the hallmarks of an improved SR function.\(^1\) However, most surprisingly, \(I_{Ca,L}\) amplitude was identical in AC8TG and NTG hearts.\(^1\) Therefore, a compensatory mechanism must take place in AC8TG mice to prevent a continuous cAMP/PKA stimulation of cardiac LTCCs and the deleterious consequence of a Ca\(^{2+}\) overload. That mechanism appears to be an increase in cAMP-PDE activity and a rearrangement of PDE isoforms.\(^1\) Hence, at the single cell level, the response of \(I_{Ca,L}\) to an application of IBMX is twice larger in AC8TG vs. NTG hearts, indicating that cardiac
expression of AC8 is accompanied by a strong compartmentation of the cAMP signal that
shields LTCCs and protects the cardiomyocytes from Ca\(^{2+}\) overload.\(^{152}\). Therefore, through
enhanced PDE activity and compartmentation, the AC8TG mouse model provides a nice example where cAMP only makes the good, not the evil.

The concept that cardiac cAMP signaling can produce both ‘good’ and ‘bad’ effects depending on where within the cell it is being activated is certainly relevant to all forms of heart failure (HF), where major alterations in cAMP signaling occur. The evidence reviewed here demonstrate that physiologic cAMP signaling is confined in specific subcellular domains due to local activities of specific PDEs. We believe that the ‘good’ outcomes require a strict localized control of the cAMP signaling, leading to activation of only a limited number of substrates; the ‘bad’ outcomes occur when compartments are disorganized, a situation likely to exist during the morphological rearrangements that accompany hypertrophy and HF. Therefore, an in-depth analysis of cAMP signaling in pathologic hypertrophy and heart failure may provide new treatments of HF acting on localized cAMP signaling to improve heart function and clinical outcomes.

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Figure 1. Regulation of cardiac ECC by cAMP pathways. Upon liberation of noradrenaline by sympathetic nerve terminals, β1-ARs activate cardiac AC isoforms (AC5 and AC6) via the stimulatory G protein (Gs). Cyclic AMP is being synthesized and activates PKA which phosphorylates different intracellular targets: LTCC phosphorylation leads to an increased Ca\(^{2+}\) influx and RyR2 phosphorylation at the SR membrane to an increased Ca\(^{2+}\)-induced Ca\(^{2+}\) release, both acting to enhance the force of contraction; PLB phosphorylation leads to an increased SR Ca\(^{2+}\) uptake by SERCA and TnI phosphorylation to a reduction in Ca\(^{2+}\)-sensitivity of the myofilaments, both acting to accelerate contractile relaxation; on the long term, CREB phosphorylation in the nucleus activates transcription. These effects are antagonized by a liberation of acetylcholine from parasympathetic nerve terminals, through activation of muscarinic M2 receptors and inhibition of AC activity via inhibitory G proteins (Gi). For further details, see Text.

Figure 2. Methods used to examine intracellular cyclic nucleotide (CN) compartmentation in intact cardiomyocytes. A) Double-barreled microperfusion coupled with whole-cell patch-clamp technique. A cardiomyocyte is positioned transversally at the mouth of two adjacent capillaries separated by an intermediate septum \(\approx\)5 µm thick. The cell can thus be exposed to two different solutions, for instance to ISO on one side and control (Ctrl) solution on the other side, and the activity of LTCCs can be followed separately on each side of the cell by removing Ca\(^{2+}\) ions from the other side.\(^50\) B) FRET-based imaging method. YFP and CFP proteins are fused to a CN binding protein (for instance Epac\(^{75,76}\) or a catalytic-inactive PKG\(^{79}\)). CN binding reduces FRET between CFP and YFP and the change in shape of the fluorescence emission spectrum allows CN concentrations to be visualized in real-time.
C) Recombinant CNG channels. Wild-type or genetically modified α subunits of rat olfactory CNG channel (CNGA2) form a cationic channel directly opened by CN. Cardiomyocytes infected with an adenovirus encoding the native or modified channels elicit a non-selective cation current (I_{CNG}) only when CN concentration rises beneath the sarcolemmal membrane. For further details, see Text.

Figure 3. PDE-dependent cAMP compartmentation. Activation of a Gs-coupled receptor (R) leads to AC activation and cAMP production in a compartment delimited by PDE activity. Different subsets of PDE isoforms may contribute to hormonal specificity by confining PKA phosphorylation to a limited number of substrates (LTCCs in the example shown). For further details, see Text.

Figure 4. cAMP signaling microdomains around anchored PDE4D. PDE4D-AKAP interaction provides a molecular basis for a compartmentation of cAMP signaling. AKAP proteins bind RII subunits of PKA as well as various PKA effectors involved in cardiac function (e.g. RyR2 or LTCC). The presence of PDE4D in the same compartment limits the spread of cAMP to other compartments and controls the kinetics of the functional response. For further details, see Text.
Figure 1
A) Double-barreled microperfusion

B) FRET-based imaging

C) Recombinant CNG channels

Figure 2
Figure 3
Figure 4