This Review is the first in a thematic series on Novel Aspects of Cardiovascular G-Protein-Coupled Receptor Signaling: Implications for New Therapeutics, which includes the following articles:

Introduction to the Series
Biased Ligands for Better Cardiovascular Drugs: Dissecting GPCR Pharmacology
G-Protein–Dependent and –Independent Signaling Pathways and Their Impact on Cardiac Function

Compartmentalization of β-Adrenergic Signals in Cardiomyocytes

G-Protein Coupled Receptor Kinases (GRK) as Therapeutic Targets in Cardiovascular Disease
Regulators of G-Protein Signaling in the Heart and Their Potential as Therapeutic Targets

Howard Rockman, Guest Editor

Compartmentalization of β-Adrenergic Signals in Cardiomyocytes

Yang K. Xiang

Abstract: Activation of adrenergic receptors (AR) represents the primary mechanism to increase cardiac performance under stress. Activated βAR couple to Gs protein, leading to adenylyl cyclase-dependent increases in secondary-messenger cyclic adenosine monophosphate (cAMP) to activate protein kinase A. The increased protein kinase A activities promote phosphorylation of diversified substrates, ranging from the receptor and its associated partners to proteins involved in increases in contractility and heart rate. Recent progress with live-cell imaging has drastically advanced our understanding of the βAR-induced cAMP and protein kinase A activities that are precisely regulated in a spatiotemporal fashion in highly differentiated myocytes. Several features stand out: membrane location of βAR and its associated complexes dictates the cellular compartmentalization of signaling; βAR agonist dose-dependent equilibrium between cAMP production and cAMP degradation shapes persistent increases in cAMP signals for sustained cardiac contraction response; and arrestin acts as an agonist dose-dependent master switch to promote cAMP diffusion and propagation into intracellular compartments by sequestrating phosphodiesterase isoforms associated with the βAR signaling cascades. These features and the underlying molecular mechanisms of dynamic regulation of βAR complexes with adenylyl cyclase and phosphodiesterase enzymes and the implication in heart failure are discussed. (Circ Res. 2011;109:231-244.)

Key Words: adrenergic receptor □ phosphodiesterase □ protein kinase A

Cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) activation represents a key signaling mechanism on stimulation of G-protein-coupled receptors (GPCRs) for cardiac contraction and energy metabolism under stress conditions. Activation of β-adrenergic receptors (βAR), a group of prototypical GPCRs, is one of the major neurohormonal mechanisms controlling cAMP/PKA activities for physiological responses in animal hearts.1–6 β1 and β2AR are highly homologous receptors expressed in animal hearts and are responsible for enhancing cardiac performance. β3AR
plays a dominant role in increasing chronotropy and ionotropy in cardiac myocytes, whereas β2AR produces only modest chronotropic effects. In addition, a minor β3AR subtype is also expressed in myocardium and modulates myocyte function. Ligand binding induces receptor conformational changes that lead to coupling and activation of G-protein, which in turn stimulates adenylyl cyclase (AC) for cAMP production. The small-molecule cAMP functions as a second messenger that diffuses into distinct subcellular locations/compartments and activates the locally anchored/tethered PKA. Thus, the specificity of substrate phosphorylation is achieved for cellular functions such as contractile responses. One of the emerging mechanisms that safeguard the specificity of GPCR/cAMP signaling is the control of cAMP transients in space and time via degradation by cyclic nucleotide phosphodiesterase (PDE).

The concept of spatiotemporal regulation of cellular cAMP and PKA activities provides new insights into understanding how cAMP/PKA signaling is translated into physiological contraction response in highly organized muscle cells. In this paradigm, PKA is anchored to distinct subcellular structures through a family of proteins named A-kinase anchoring proteins (AKAP). In contrast, correlating to the distribution of most AC, cellular cAMP is primarily confined along the plasma membrane under neurohormonal stimulation. Despite being a diffusible small molecule, the distribution and diffusion of cAMP are rather limited because of PDE-mediated cAMP degradation. Under a specific hormonal stimulation, individual PKA anchored at different subcellular compartments will be selectively activated to phosphorylate a local pool of proteins for specific cellular processes.

A spatial distribution of cAMP/PKA signaling regulated by AC and PDE therefore is essential for selective phosphorylation of substrates important in myocyte contraction. This is critical considering that a wide range of different neurohormonal chemicals can stimulate cardiac myocytes, and many of these agonists lead to increases in intracellular cAMP, raising the point that cardiac myocytes must be able to segregate all these signals and prevent unnecessary phosphorylation under a specific stimulus. Consequently, precisely fine-tuning the βAR signaling for cardiac contractile performance is a vital mechanism to allow the body to adjust to stress. Clinically, dysfunction of the adrenergic signaling pathway contributes to cardiac arrhythmia and cardiac remodeling including myocyte apoptosis and myocyte hypertrophic growth in diseases such as myocardium infarction and heart failure.

Highly differentiated cardiac myocytes have several unique membrane structure properties. Recent studies have significantly advanced our understanding of these structures in the spatiotemporal regulation of βAR signaling in cardiac myocytes. First, myocytes contain abundant lipid rafts, specialized regions of the plasma membrane enriched in cholesterol and other lipids, and caveolae, a subset of lipid rafts that form flask-shaped invaginations of the plasma membrane enriched in particular proteins such as caveolins. Second, myocytes have an extensive t-tubular structure network that results from invagination and extension of the plasma membrane into the internal space of the cell bodies. Third, myocytes are innervated by sympathetic ganglia neurons to form adrenergic synapse, which induces highly specialized postsynaptic regions on the plasma membrane.

The relative distribution and enrichment of βAR subtypes in these specialized membrane structures facilitate recruitment and association of other signaling components in a location-dependent manner and leverage a strong impact on the production of cAMP and signaling efficiency and specificity in cardiac myocytes.

Biochemical characterization also has advanced our understanding of the organization of AC and PDE associated with βAR in cardiac myocytes. AC5/6 has been shown to be tethered by scaffold protein AKAP79 in rat brain tissues. AKAP79 is known to bind to β1 and β2AR, as well as to PKA and downstream effectors such as ion channels in various tissues. In cardiac myocytes, AC6 can be communoprecipitated with β1AR, suggesting that the receptor and AC6 could be assembled into a complex via AKAP scaffold proteins. Conversely, a group of phosphodiesterase 4D (PDE4D) selectively binding to βAR play significant roles in regulating the βAR subtype-induced neonatal myocyte contraction rate response. These receptor-associated PDE4D play critical roles in controlling cAMP and PKA activities in the vicinity of the receptors as well as in the diffusion of cAMP for cardiac responses under βAR stimulation. Overall, a balance between AC-dependent cAMP production and PDE-dependent cAMP degradation in an agonist dose-dependent manner differentially regulates cAMP/PKA signaling in cardiac myocytes.

Membrane Localization in Cellular Compartmentalization of Signaling

Cardiac myocytes are highly differentiated cells with several unique membrane structure properties implicated in mediat-
The PKA biosensor AKAR3 was expressed in wild-type myocytes. Cells were treated with isoproterenol at different concentrations. Changes in PKA AKAR3 FRET ratio (an indication of PKA activity) were calculated and normalized against the baseline levels. The PKA biosensor AKAR3 was expressed in wild-type myocytes. Cells were treated with isoproterenol at different concentrations. Changes in cAMP ICUE3 FRET ratio (an indication of cAMP activity) were measured. A, Time courses of changes in PKA FRET ratio were plotted. B, The initial peak increases (EC50 6.86 × 10⁻¹⁰ M) and the sustained increases (EC50 7.99 × 10⁻⁹ M) in cAMP FRET ratio were plotted. C, The initial peak increases (EC50 4.53 × 10⁻¹⁰ M) and the sustained increases (EC50 6.77 × 10⁻⁸ M) in PKA FRET ratio were plotted. Only the sustained cAMP/PKA activities promote cardiac contractile responses in neonatal and adult myocytes.

The β2-AR-induced signaling for downstream cAMP activation is sensitive to disruption of caveloe via extraction of cholesterol by detergent, whereas the β2-AR-induced effect is not altered. Thus, stimulation of β2-AR leads to activation of L-type calcium channels in a local vicinity, whereas stimulation of β1-AR leads to activation of L-type calcium channels in the distance.

Second, myocytes have an extensive t-tubular structure network that results from invagination and extension of the plasma membrane into the internal space of the cell body. Using real-time imaging in living myocytes, Nikolaev et al. observed that the cAMP induced by β2-AR is confined in the t-tubular structure in adult myocytes. In contrast, the cAMP induced by β1-AR is distributed in both plasma membrane and t-tubular structure. Moreover, a local stimulation of β1-AR at one end of elongated adult myocytes leads to a far-reaching cAMP diffusion inside of cells, whereas stimulation of β2-AR leads to confined cAMP signal at the stimulation site. Together, these studies indicate that activation of β1-AR promotes a broad distribution of intracellular cAMP signal, whereas the β2-AR actions are local. Given that the β2-AR signaling is confined in t-tubular structure and sensitive to disruption of caveolae, it would be interesting to examine whether the caveolae membrane is enriched in the t-tubular structure in cardiac myocytes.

Third, sympathetic ganglia neurons interact with cardiac myocytes to form adrenergic synapses, which induce a highly specialized zone on the plasma membrane of myocytes. Shcherbakova et al. has analyzed the distribution of βAR subtypes on myocytes relative to innervations of sympathetic ganglia neurons. Both β1 and β2-AR are highly enriched at postsynaptic regions on cardiac myocytes, which are also enriched with the scaffold proteins AKAP79 and synaptic-associated protein 97. The distribution of βAR subtypes within the innervated cardiac myocytes is further confirmed with an elegant in vitro coculture model of sympathetic ganglia neurons and cardiac myocytes. After stimulation of sympathetic ganglia neurons, the released catecholamines stimulate both β1 and β2-AR in the cocultured cardiac myocytes and induce distinct trafficking properties. Although β1-AR remain enriched at the postsynaptic region, β2-AR are redistributed from the postsynaptic membrane, presumably via receptor internalization. Together, the observed segregation of βAR plays critical roles in organizing the receptor subtype-specific signaling complexes in cardiac myocytes.

**Organization of βAR/Gs/AC Complexes for cAMP Production**

Activated βAR couple to Gs protein, which leads to activation of AC for cAMP production. The enrichment of βAR and immediate downstream components in local plasma membrane domains such as caveolae suggests that they may be preassembled into macromolecular complexes to facilitate...
signaling transduction specificity and efficiency. AC is a family of diversified genes that display different regulatory mechanisms and interaction with other signaling pathways.\textsuperscript{15,49,50} In cardiac myocytes, AC5 and AC6 represent the dominant isoforms\textsuperscript{50,51} and can be activated by \(\beta\)AR stimulation.

Accumulating evidence supports the idea that the \(\beta\)AR, G\(_i\) protein, and AC form preassembled complexes to facilitate signaling transduction. First, it is well-known that \(\beta\)AR can be preassembled with G\(_i\) protein, which is also referred to as a G-protein–precoupled receptor.\textsuperscript{52–54} This form of receptor displays higher binding affinity to ligands than that of the receptor in a G-protein–free form.\textsuperscript{55} Stripping G-proteins from membrane preparations containing AC abolishes the high-affinity binding sites.\textsuperscript{56,57} The preassembled \(\beta\)AR in complex with G\(_i\) proteins represent a receptor population that is ready for agonist stimulation and is also sensitive to low concentrations of agonist because of the high-affinity binding sites. Second, the \(\beta\)AR are known to bind a variety of scaffold proteins. \(\beta_2\)AR binds to AKAP79 and AKAP250 in various tissues.\textsuperscript{58,59} \(\beta_2\)AR also binds to Na-H exchanger regulatory factor\textsuperscript{60} and N-ethylmaleimide–sensitive factor.\textsuperscript{61,62} In contrast, \(\beta_1\)AR binds to AKAP79\textsuperscript{63} and PDZ domain-containing proteins such as synaptic-associated proteins.\textsuperscript{64} G alpha interacting protein (GAIP)-interacting protein C-terminus,\textsuperscript{65} and membrane-associated guanylate kinase inverted proteins.\textsuperscript{66} Both AKAP and synaptic-associated proteins are well-known scaffold proteins that can tether additional signaling proteins such as kinases, phosphatases, and other regulatory proteins to the adrenergic receptors.\textsuperscript{11,66} In the case of \(\beta_1\)AR, AKAP79 and synaptic-associated protein 97 form a tertiary complex that facilitates PKA phosphorylation of the activated receptor and promotes receptor recycling after internalization in HEK293 cells.\textsuperscript{67} In brain tissues, AKAP79 directly binds to AC5 and AC6,\textsuperscript{29} which serve as a coordinator to mediate PKA phosphorylation of the AC. The PKA phosphorylation inhibits AC activities, and negatively attenuate cAMP production under \(\beta\)AR stimulation.\textsuperscript{29} In cardiac myocytes, AC6 can be coimmunoprecipitated with overexpressed \(\beta_1\)AR,\textsuperscript{31} indicating that \(\beta_1\)AR could be connected to AC via AKAP79 to regulate AC activities for cAMP production.\textsuperscript{68} Finally, both \(\beta_1\)AR and \(\beta_2\)AR are enriched in the postsynaptic regions on the plasma membrane of cardiac myocytes.\textsuperscript{28} These receptors are also colocalized with AKAP79 and synaptic-associated protein 97,\textsuperscript{28} again supporting the concept that the \(\beta\)AR could exist in a preassembled complex containing G-proteins and scaffold proteins that connect to AC and PKA, and are ready to respond to catecholamine released from the nerve terminals.

**\(\beta\)AR-Associated PDE Mediate cAMP Degradation**

Over the past decades, a series of studies has shown the dynamics of cAMP and PKA signaling in different subcellular compartments on \(\beta\)AR stimulation.\textsuperscript{16,17,33,37,38,69–71} The duration and distribution of cAMP signals are disrupted or altered on inhibition of PDE with 3-isobutyl-1-methylxanthine.\textsuperscript{37,69} These studies point out the critical roles of PDE in confining the cAMP in space and time in cardiac myocytes under adrenergic stimulation. PDE include 11 families based on their amino acid sequence homology, substrate specificities, and pharmacological properties.\textsuperscript{72} Each of the 11 PDE families has one to four distinct genes. In addition, most PDE genes encode multiple splicing variants through the usage of different promoters and alternative splicing. At least six different PDE families are expressed in animal hearts, including PDE1, PDE2, PDE3, PDE4, PDE5, and PDE8.\textsuperscript{73} PDE1, PDE2, and PDE3 can hydrolyze both cAMP and cyclic guanosine monophosphate (cGMP). PDE5 specifically hydrolyzes cGMP whereas PDE4 and PDE8 are specific for cAMP degradation. The relative expression of each PDE family varies between human and rodents, and during developmental and disease stages. In rodent hearts, PDE4 and PDE3 are the two major families, which account for \(>90\%\) of PDE activities.\textsuperscript{74} In particular, PDE4D genes have been shown associated with \(\beta\)AR subtypes and regulate the receptor-induced cAMP signaling in cardiac myocytes.\textsuperscript{74–76} Although PDE3 and PDE4 families account for a much lesser portion of overall PDE activities in human myocardium, the expression and function of PDE4 genes are well-conserved in human hearts,\textsuperscript{75} underscoring the critical roles of these genes in regulating \(\beta\)AR signaling properties in cardiac myocytes across different mammalian species. Besides PDE3 and PDE4, other PDE family such as PDE2 and PDE8 are also implicated in cAMP metabolism. PDE2 is activated by cGMP to enhance cAMP degradation, which negatively regulates the \(\beta\)AR/G\(_i\)-induced cAMP signaling.\textsuperscript{71} In contrast, deletion of PDE8A displays a greater increase in calcium signaling including L-type calcium channel and calcium spark activities, as well as calcium transients.\textsuperscript{76} The mechanism of how PDE8 is involved in the cAMP-mediated calcium handling for cardiac contraction is not clear yet.

The PDE3 family consists of PDE3A and PDE3B genes, whereas the PDE4 family contains PDE4A, PDE4B, PDE4C, and PDE4D genes.\textsuperscript{72} At resting states, both PDE3 and PDE4 activities modulate a basal intracellular concentration of cAMP by continuously hydrolyzing the cAMP synthesized by constitutively active adenylyl cyclases,\textsuperscript{70} thus maintaining a tonic PKA activity in cardiac myocytes.\textsuperscript{78} Under adrenergic stimulation, PDE play a role in controlling the duration and amplitude of cAMP signals.\textsuperscript{38,70,77} Using a cardiac contraction rate assay, the PDE4 family was found to attenuate the adrenergic stimulation of cAMP/PKA signal for enhancing contraction response.\textsuperscript{36} Further analysis with gene deficiency reveals that the PDE4D isoforms are critical for regulating the \(\beta\)AR-induced cAMP signals for contractile response in cardiac myocytes.\textsuperscript{36} Deletion of PDE4D, but not PDE4A and PDE4B, gene enhances the \(\beta\)AR-induced cAMP signals in mouse embryonic fibroblasts\textsuperscript{79} and contraction rate response in neonatal cardiac myocytes.\textsuperscript{36} Probing cAMP activities in living myocytes confirms the critical role of PDE4 family genes in the control of cAMP generated by \(\beta\)AR stimulation in both neonatal and adult cardiac myocytes.\textsuperscript{16,17,33,37,38,69–71} In comparison, PDE3 isoforms appear to be involved in regulating cAMP content in a functionally distinct pool,\textsuperscript{38,70,77} which may control the cAMP activities in the sarcoplasmic reticulum for calcium cycling,\textsuperscript{79} as well as adrenergic stimulation-induced cardiac myocyte apoptosis via inhibiting the expression of inducible cAMP early repressor.\textsuperscript{80,81} Thus,
PDE3 and PDE4 play distinct roles in modulating the cAMP signals in myocytes. In this review, the PDE4D genes are further discussed because of their direct association with βAR subtypes in cardiac myocytes and their prominent role in regulating cAMP signaling under adrenergic stimulation.

**Agonist Dose-Dependent Association and Sequestration Between PDE4D Isoforms and βAR**

The function of individual PDE in adrenergic signal transduction is also dependent on their distribution within the three-dimensional matrix of the cell. Studies show that both PDE3 and PDE4 are highly enriched in the membrane fraction and are resistant to detergent extraction. Thus, PDE are usually tightly anchored/ethered to the membrane or protein complexes in respect to other regulatory and effector elements. Subsequently, two groups have independently characterized the selective association of PDE4D8 and PDE4D9. Richter et al. have shown that PDE4D8 directly binds to β1AR via the C-terminal of receptor, although the receptor binding sites on PDE4D8 are yet to be identified. Conversely, β1AR is shown to coimmunoprecipitate together with the N-terminal region of PD4D8 in neonatal cardiac myocytes, indicating that the variable N-terminal region contains the information for selective binding to the receptor. Activation of β1AR preferentially enhances the activity of PDE4D8 and PDE4D9 in HEK293 cells, indicating that β1AR can potentially associate with other PDE4 isoforms besides PDE4D8. Moreover, inhibition of PDE4D8 activities with overexpression of the catalytically inactive form of PDE4D8 or the unique N-terminal domain is sufficient to enhance both cAMP production and myocyte contraction rate response on β1AR stimulation.

In comparison, the association between β2AR and PDE4D isoforms are much more complex. In neonatal cardiac myocytes, β2AR displays a broad binding to different isoforms in PDE4D family, including PDE4D3, PDE4D5, PDE4D7, PDE4D8, and PDE4D9, with a preferential binding to PDE4D9 and, to a lesser extent, PDE4D8. Interestingly, the basal levels of binding between β2AR and PDE4D are reduced in cells lacking both arrestin 2 and arrestin 3 genes, indicating a possible role of arrestin in organizing the β2AR/PDE4D complexes. Supporting this notion, arrestin 3 is shown to organize a stable complex between PDE4D3 and relaxin family peptide receptor 1. Selective inhibition of individual PDE4D isoforms shows that PDE4D9, as well as PDE4D5 and PDE4D8, can affect either the basal contraction rate or the β2AR signaling-induced contraction rate response in neonatal cardiac myocytes. The effect of different PDE4D isoforms on β2AR signaling is dependent on dynamic association between individual isoforms with the activated receptors.

**Stimulation With Subnanomolar of Agonist: A Transient cAMP Is Dominated by PDE4D Activities Within the Preassembled βAR Complexes**

With the advancement of live-cell imaging approaches, recent characterizations of cardiac adrenergic signaling have generated evidence to not only solidify the concept of spatial distribution and regulation of cAMP signals in subcellular organelles but also provide evidence for mechanisms to address the sustained and agonist dose-dependent contractile responses in cardiac myocytes. These studies suggest that well-orchestrated receptor association with AC and PDE as well as activation of these enzymes play a regulatory role in fine-tuning cardiac contractile responses. In this new paradigm, the AC-dependent cAMP production and the PDE-dependent cAMP degradation dictate an agonist dose-dependent equilibrium of cAMP activities, which produces a transient cAMP signal at minimal concentrations of agonist or a sustained increase at the different levels over the baseline at higher concentrations of agonist stimulation.

At subnanomolar concentrations of isoproterenol stimulation, the cAMP production is rapidly accumulated. This high potency of isoproterenol suggests that the cAMP response is induced by preassembled βAR/G/AC complexes with high-affinity binding for ligands. As a result, the cAMP production is rapidly saturated at 10^-8 M of isoproterenol, a concentration that is well above the binding constant at the high-affinity sites. Accordingly, the activation of receptor and G protein occurs almost simultaneously within 50 ms, whereas the cAMP production is produced within 2 seconds because of rapid activation of AC in the complex. In addition, the expression of AC is the rate-limiting factor for the βAR/G/AC–induced cAMP production in cardiac myocytes. In agreement, overexpression of AC6, but not the β2AR, significantly enhances the maximal cAMP synthesis in cardiac myocytes. These observations challenge the traditional view that cAMP production can be further enhanced at higher concentrations of βAR agonist because of recruitment of G proteins to available βAR on the cell surface.

However, at this concentration range, the βAR-induced cAMP signals are very transient in cardiac myocytes. One of the major reasons is that the cAMP signals lead to activation of PKA in the same complexes, which phosphorylates and activates at least two distinct negative feedback mechanisms to attenuate signaling. One mechanism is to phosphorylate and inhibit AC activity for cAMP synthesis, and another is to phosphorylate and activate PDE to enhance cAMP degradation. Consequently, inhibition of PKA via a PKA inhibitor, knocking down the expression of AKAP79, or displacing PKA holoenzymes from AKAP79 significantly prolongs cAMP signals under βAR stimulation. Between these two negative feedback mechanisms, the PDE4-mediated cAMP degradation seems to play a dominant role in modulating the cAMP signals. Although overexpression of AC6 significantly enhances the peak level induced by adrenergic stimulation, the overall cAMP signals still display a transient response with a rapid attenuation. In contrast, overexpression of PDE4D8 is sufficient to completely inhibit cAMP signaling induced by saturated doses of agonist in neonatal cardiac myocytes. Moreover, inhibition of PDE4 is sufficient to convert a transient cAMP response to a sustained signal. All this evidence argues that the PDE4D-mediated cAMP degradation serves as the major mechanism to attenuate the receptor stimulated cAMP signals.
also argue the limited impact of PKA phosphorylation of AC on the cAMP equilibrium when compared to the cAMP degradation by PDE. Together, the balance between syntheses vs degradation of cAMP dictates both peak levels and duration. The PDE4-mediated cAMP degradation is so overwhelmingly dominant that when overexpressed, it completely degrades any cAMP produced from βAR stimulation in cardiac myocytes.

More importantly, the transient cAMP signals are confined in the local domain surrounding the βAR on the plasma membrane (Figure 2). Using PKA-based cAMP biosensors, Zaccolo et al. have shown that cAMP accumulation is confined along t-tubular structures in rat neonatal cardiac myocytes. Inhibition of PDE with 3-isobutyl-1-methylxanthine produces a much broader distribution of cAMP signals. This is also consistent with early biochemical evidence showing that activation of βAR selectively stimulates type II PKA in cardiac tissues and the majority of type II PKA is membrane-bound in cardiac myocytes. Therefore, the cAMP produced by βAR activation is highly localized and targeted to type II PKA for substrate phosphorylation.

The transient and local cAMP signals lead to phosphorylation of the receptors and their associated proteins but have limited access to distant substrates, such as PKA targets on the sarcoplasmic reticulum membrane and targets associated with myofibrils. The opposing actions on cAMP signals by both AC and PDE4 in local compartments argue that both enzymes are localized in proximity to, or potentially in direct association with, the same βAR/G protein complex. Evidence for a direct βAR/AC/PDE complex is missing in cardiac myocytes. However, a recent study has already provided the first example of coexistence of AC and PDE in the same relaxin family peptide receptor 1 complex for sustained tonic cAMP/PKA activities under subpicomolar concentration of agonist stimulation.

Because of its limited access to distant substrates, such as PKA targets on the sarcoplasmic reticulum membrane or associated with myofibrils, the transient cAMP signals have minimal impact on the contractility of ventricular myocytes. However, these local cAMP/PKA signals should be accessible to other local effectors such as ion channels on the plasma membrane in cardiac myocytes. Both the PDZ proteins...
(synaptic-associated proteins and Na-H exchanger regulatory factors) and the AKAP proteins are shown to organize the βAR complexes with downstream channels, including L-type calcium channels, cystic fibrosis transmembrane conductance regulator, and Na-H exchanger in different tissues and cells. Physical associations between the βAR and these ion channels enable the local cAMP signals to be effective in modulating channel activities in the local vicinity. For example, in sinoatrial node cells, these cAMP activities may be able to stimulate contractile responses via activating cyclic nucleotide-gated ion channels in the vicinity. In neonatal ventricular cardiac myocytes, the same signaling machinery probably regulates both cyclic nucleotide-gated ion channels and L-type calcium channels for enhancing contraction rate. However, in adult cardiac myocytes, the stimulation of local cAMP signaling has minimal effect on contractile shortening.

**Stimulation With Micromolar of Agonist: A Dose-Dependent Sustained Increase of cAMP Signaling Is Shaped by Sequestration of PDE4 From the βAR/Gs/AC Complexes**

On further increases in agonist concentration (from $10^{-9}$ M to $10^{-5}$ M), the cAMP signaling is gradually shifted from transient responses to saturated and sustained responses (Figure 1). This shifting is accompanied with an agonist dose-dependent dissociation of PDE4D8 from the β1AR. This dissociation of PDE4 from the activated βBAR leads to a shift in balance between cAMP production and cAMP degradation. As a result, the cAMP signals are maintained at incremental levels above the baselines in an agonist dose-dependent manner. Using another biosensor to directly measure PKA activities, De Arcangelis et al have shown that the activation of downstream PKA also displays similar sustained responses at high concentrations of agonist (Figure 1). These data show that stimulation of βAR induces sustained cAMP and PKA signals, a feature correlated to the physiological contractile responses that are sustained under the very same stimulation. The prominent role of PDE4 in shaping the duration of cAMP response is also supported by a series studies with pulse stimulation of βAR agonists. In these studies, a pulse stimulation of βAR is introduced to cardiac myocytes with a short perfusion of agonists. Such a short stimulation induces a transient response of cAMP signaling, and the decreases in cAMP signals are significantly attenuated by inhibition of PDE4 or by inhibition of PKA. Meanwhile, as discussed previously, overexpression of PDE4D8, a β1AR-associated isoform, is sufficient to abolish cAMP response even under stimulation with saturated concentrations of isoproterenol.

This elegant cAMP equilibrium under adrenergic stimulation also displays several interesting features. First, the system is self-adjustable to establish a balance between cAMP production and cAMP degradation maintained at different levels. Although the dissociation of PDE appears to be the most critical factor in determining the equilibrium levels, other factors can weigh in. These include receptor phosphorylation for desensitization and internalization, G-protein inactivation through GTP hydrolysis, and PKA phosphorylation and inactivation of AC. Second, continuous cAMP production is obligatory to maintain an equilibrium, which is attributable to continuous presence of agonist within the extracellular space. Removal of agonist or addition of a βAR antagonist leads to rapid attenuation of cAMP signals to the baseline levels. Therefore, a sustained cAMP response is dependent on continuous activation of the βAR/Gs/AC system to maintain the cAMP production. Third, the sustained cAMP activities are able to diffuse out of the confinement of the βAR microdomains, which permits access to the PKA enzymes anchored on different subcellular structures, including the sarcoplasmic reticulum and myofibrils in cardiac myocytes. These PKA activities lead to persistent phosphorylation of substrates such as phospholamban (PLB) on the sarcoplasmic reticulum membrane and troponin I (TnI) associated with myofibrils for sustained contractile responses in neonatal and adult cardiac myocytes. Last, the increases in sustained cAMP levels display a close correlation with the increases in sustained contraction rate responses in neonatal myocytes in an agonist dose-dependent fashion. Together, these data have, for the first time to our knowledge, demonstrated specific pools of cAMP activities capable of modulating the βAR agonist dose-dependent contractile response in cardiac myocytes.

Biochemically, to maintain a sustained cAMP production, a pool of βAR/G-protein complexes needs to be occupied by ligands constantly to stimulate the associated AC. Because the β1AR does not undergo agonist-induced internalization in cardiac myocytes, the observations suggest a rapid uncoupling/recoupling cycle to maintain a pool of the receptor in a complex form associated with Gs, and AC. Alternatively, it could simply be because of a stable β1AR/Gs/AC complex under stimulation. In agreement, the association of AC6 and the β1AR is not altered on stimulation of either $10^{-9}$ M or $10^{-5}$ M of isoproterenol. In comparison, the PDE4D8 selectively dissociates from the β1AR at much higher doses of agonist stimulation.

**Arrestin Mediates Sequestration of PDE4D for cAMP Diffusion and Propagation**

As of today, the mechanisms for transportation of PDE4D isoforms in cardiac myocytes are not clear. In one possible scenario, the desensitized and internalized β1AR serve as the initiator for complex formation between the receptor, arrestin, and different PDE4D isoforms. The β1AR could keep shuttling between the G-protein complexes and the arrestin complexes via rapid internalization and recycling to maintain the equilibrium. Alternatively, because G-protein receptor kinase phosphorylation happens on the receptor at high concentration of agonist stimulation, it indicates that these receptors do not form complexes with G-protein and have low-affinity binding sites for agonists (Figure 2B). In this scenario, two different pools of βAR are presented, a G-protein–precoupled pool and a G-protein–free pool. At low concentrations, only the precoupled receptor/G-protein complexes are activated because of their high-affinity binding to ligands and the PDE4D isoforms associated with the same complexes confine the cAMP signals within the local vicin-
ties (Figure 2A). However, at high concentrations, the G-protein-free receptors are also activated via low-affinity binding to ligands and are selectively phosphorylated by G-protein receptor kinases for internalization, which serve as the sites to sequestrate PDE4D via arrestin binding (Figure 2B). As a result, the cAMP production machinery (G-protein–pre-coupled receptors) is segregated from the cAMP degradation machinery (PDE4D). In this scenario, arrestins function as a master regulator to switch off cAMP degradation, which permits accumulation and diffusion of cAMP in an agonist dose-dependent fashion. Only the accumulated and diffusible cAMP is sufficient in promoting PKA phosphorylation of proteins such as TnI and PLB for cardiac contraction.

In agreement with this hypothesis, on stimulation with catecholamines, PDE4D8 dissociates from the activated β2AR in both HEK293 cells and neonatal cardiac myocytes in an agonist concentration–dependent manner.33,34 At a minimal of 10−7 M of isoproterenol, PDE4D8 displays dissociation from the receptor in HEK293 cells, which reaches the peak level at 10 minutes of stimulation.33 Conversely, β2AR displays a much more complex association with different PDE4D isoforms. Although β2AR binds to primarily PDE4D9 and PDE4D8 at a resting state, PDE4D9 dissociates from the receptor and PDE4D8 is recruited to the receptor after a transient dissociation after agonist stimulation.35 In addition, PDE4D3 and PDE4D5 are also recruited to the activated β2AR.35,83,84 The recruitment/sequestration of different PDE4D isoforms is dependent on the formation of β2AR and arrestin complexes. In a series of recent studies, the arrestin binding sites have been mapped on the C-terminal region, which is conserved throughout the PDE4D family, and the unique N-terminal domain of PDE4D5.103 Under adrenergic stimulation, PDE4D5 can be ubiquitinated by the E3-ubiquitin ligase, Mdm2, which is scaffolded by arrestin. Ubiquitination of PDE4D5 elicits an increase in the fraction of PDE4D5 sequestration by arrestin in cells, thus potentially decreasing the fraction of PDE4D5 associated with βARs.104 Meanwhile, PDE4D5 also can be modified by sumoylation, which enhances PKA-mediated activation but attenuates the ERK-mediated inhibition of the enzyme activities.105 Together, these data argue a critical role of arrestin in switching off the PDE4D isoform-dependent cAMP degradation under adrenergic stimulation in an agonist dose-dependent fashion. It is thus critical to further explore molecular and cellular mechanisms of how arrestin affects the selective association of PDE4D isoforms with different receptor complexes and shuttling these complexes in distinct cellular organelles in cardiac myocytes.

**PDE4 Controls cAMP Access to AKAP-Localized PKA in Distinct Cellular Organelles**

The spatiotemporal regulation of βAR signaling in cardiac myocytes is also dependent on the distribution of PKA in distinct subcellular compartments. PKA is consisted of two regulatory subunits and two catalytic subunits. On cAMP binding, the catalytic subunits are released from the regulatory subunits and activated to phosphorylate downstream targets. PKA is tethered to subcellular organelles as well as the cytoskeletal system via binding AKAP. AKAP are a large family of structurally divergent genes that share a common region in binding to the regulatory subunits of PKA. Because of the ability of AKAP to bind different cellular proteins/structures in distinct subcellular compartments, PKA therefore is anchored to these locations.10 This is critical to facilitate the proximity between PKA and its targets, leading to preferential phosphorylation of a local pool of substrates for specific cellular function such as myocyte contraction.

In addition to the aforementioned βAR-associated AKAP, a growing list of AKAP is expressed in cardiac tissues, and these AKAP regulate both βAR signaling and myocyte contraction.10 For example, AKAP18 isoforms are differentially associated with L-type calcium channels and sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA) in cardiac myocytes.106–108 Disruption of PKA anchoring to the L-type calcium channel by AKAP18α significantly inhibits the βAR-induced regulation of the channel activities.106 In comparison, AKAP18β scaffolds PKA together with PLB and SERCA to conduct PKA phosphorylation of PLB under adrenergic stimulation,109 thus regulating SERCA-mediated calcium uptake into the sarcoplasmic reticulum in cardiac myocytes.

Another cardiac AKAP, mAKAP, interacts directly with ryanodine receptor 2 on the sarcoplasmic reticulum. mAKAP also scaffolds PKA and phosphatase 2A, PDE4D3, which forms a signaling module to tightly regulate phosphorylation and activity of ryanodine receptor for calcium release from the sarcoplasmic reticulum.19,110 mAKAP is also implicated in cross-regulating protein kinase G signaling in cardiac myocytes for phosphorylation of TnI and myocyte contraction.111,112 In addition, mAKAP is shown to localize at the nuclear envelope of cardiac myocytes to coordinate transmission of cAMP/PKA signals and other signaling into nucleus for cardiac remodeling via phosphorylation of histone deacetylase 5.114,115

Upon dissociation and sequestration of PDE, the βAR-induced cAMP is capable of diffusing into cardiac myocytes from the plasma membrane to reach the PKA anchored onto the different cellular organelles. Because most PDE4 and PDE3 are membrane-bound,72,116 it is plausible that the diffusion of cAMP through the intracellular space is relatively easy and with limited restriction. Supporting this notion, a local release of caged cAMP leads to PKA activation at a distance.117 In another study using local perfusion to activate βAR, the cAMP is detected almost throughout the body of myocytes.57 However, this scenario can be complicated by a few other factors. First, myocytes have an extensive t-tubular structure throughout the cell body, and both βAR are presented on the t-tubular membrane. Therefore, agonists can theoretically diffuse through the t-tubular structure to activate the signaling machinery for cAMP production in different locations, rendering the necessity for long-distance intracellular cAMP diffusion. Supporting this notion, AC5 is anchored by mAKAP on the sarcoplasmic reticulum membrane.118 Whereas the architecture of the complex formation remains to be characterized, these physical associations raise a possibility that cAMP can be produced at the vicinity of the sarcoplasmic reticulum. Meanwhile, membrane t-tubular structures and myofibrils form extensive physical “barriers” throughout the cell body of
cardiac myocytes. These barriers limit the cAMP diffusion induced by a local stimulation of βAR.47 The development of t-tubular structures also underscores the difference between neonatal myocytes and adult myocytes, indicating a much more tightly controlled cAMP diffusion in adult cardiac myocytes.38 In the future, direct measurement of cAMP and PKA activities with locally tethered biosensors in a subcellular compartment like the sarcoplasmic reticulum119 will be crucial to analyze the spatiotemporal regulation of cAMP for cardiac contractile responses under adrenergic stimulation.

Because PDE are also present at different cellular organelles, and in the light of the observation that mAKAP directly tethers PDE4D3 in the same complex with PKA,120 arrival of cAMP signals to these locations/organelles would be short-lived unless the local PDE are inhibited or sequestrated. Interestingly, PDE4D3 displays increased binding to βAR/arrrestin complexes under receptor agonist stimulation in cardiac myocytes.39 These observations suggest that the βAR stimulation could also sequester the PDE away from the mAKAP-anchored PKA on the sarcoplasmic reticulum membrane (Figure 2B) and from other different organelles, such as myofibrils. The sequestration allows accumulation of cAMP which promotes sustained PKA activities and phosphorylation of ryanodine receptor 2 and SERCA-associated PLB to maintain elevated sarcoplasmic reticulum calcium cycling for contractile response. In agreement with this notion, both forskolin and isoproterenol induce similar cAMP and PKA contractile response. In agreement with this notion, both forskolin and isoproterenol induce similar cAMP and PKA activities in cytosol,41,62,101,130,131 whereas the βAR-induced cAMP activities in the sarcoplasmic reticulum are much higher than those induced by forskolin.119 This suggests that activation of βAR machinery preferentially promotes cAMP accumulation on the sarcoplasmic reticulum for PKA activation. Moreover, sustained PKA activities on the sarcoplasmic reticulum are also dependent on continuous occupation of the receptor with ligands.119 Therefore, these data indicate that despite a close proximity between the βAR/Gi/AC machinery on the t-tubular membrane and the PKA substrates on the sarcoplasmic reticulum, the βAR-induced cAMP appears to undergo at least two independent steps to activate PKA on the sarcoplasmic reticulum: sequestration of PDE from βAR/Gi/AC complexes to open the gate for cAMP diffusion and removal or inhibition of PDE from the sarcoplasmic reticulum to allow accumulation of cAMP for sustained PKA activation and phosphorylation of targeted proteins (Figure 2B).

In addition, AKAP also can scaffold phosphatases in the complex with PKA, which serves as another negative feedback loop to counterbalance the PKA activities. Recent studies show that phosphatases 2A are transported away from myofibrils under βAR stimulation.121 This observation supports the idea that the negative-feedback phosphatases, like PDE, are sequestrated from the targeted organelles to maintain sustained PKA phosphorylation for cardiac contractile response. However, phosphatases also can be recruited to the PKA/AKAP complexes77 and activated by PKA122 to counter the PKA activities. This negative feedback may be critical when the cAMP activities are extremely high in cardiac myocytes, such as after PDE are artificially inhibited.77 Under these conditions, phosphatases serve as a downstream protective mechanism to prevent hyperphosphorylation of substrates, such as PLB and TnI by PKA, and prevent myocyte from overstimulation.77 Clinically, both hyperphosphorylation123 and decreased phosphorylation124 have been observed in many proteins in failing hearts, which underscores the important role of PDE and phosphatases in maintaining normal cardiac function.125

**Divergent β1 and β2AR Signaling in Cardiac Myocytes**

Although both β1AR and β2AR are significantly expressed in cardiac myocytes, activation of individual subtypes in myocardium exerts divergent and sometimes even opposing effects on cardiac function. Activation of the β2AR leads to increased contractile response and rate in vitro,38,126 and deletion of the β1AR in myocardium completely abolished the contractile responses on perfusion of isoproterenol,127,128 In contrast, activation of the β2AR has minimal effects on both contraction rate and contractility in vitro,38,126 and deletion of the β2AR does not affect the contractile responses to perfusion of isoproterenol.128,129 These observations suggest that β1AR and β2AR induce distinct cellular signaling for specific function in cardiac myocytes. The β1AR-induced and β2AR-induced signals are probed in the myocytes lacking individual β1AR and β2AR genes. In myocytes lacking the β1AR, stimulation of β1AR induces sustained cAMP and PKA signals that are able to promote PKA phosphorylation of PLB and TnI, and contraction responses in both neonatal and adult cardiac myocytes.38 In myocytes lacking the β2AR, stimulation of β2AR induces transient cAMP and PKA signals, which can promote PKA phosphorylation of the receptor, but has limited access to PLB and TnI in neonatal cardiac myocytes for small increases in contraction rate response.38 The duration of cAMP and PKA activities in adult myocytes under β2AR stimulation are even shorter,38 indicating a tight segregation of cAMP signals. As a consequence, the β2AR stimulation fails to promote the cardiac myocyte contractile shortening response.38

In addition, the cAMP signal induced by β2AR can be further shaped by the receptor coupling to Gi,38,101 Interestingly, the β2AR/Gi coupling is dependent on both PKA and G-protein receptor kinase phosphorylation and requires trans- portation of activated receptor via internalization and recycling.41,62,101,130,131 Therefore, inhibition of Gi does not alter the cAMP response at 10^{-9} M of isoproterenol stimulation, a concentration not sufficient to promote G-protein receptor kinase phosphorylation.101 At saturated concentrations of isoproterenol (10^{-5} M), inhibition of Gi promotes duration of cAMP signal and PKA phosphorylation on the sarcoplasmic reticulum and on the myofibrils.101 Consequently, inhibition of Gi also enables the β2AR stimulation to promote calcium signaling132–134 and myocyte contractile responses.101

The observations of dissociation of PDE4D8 from the β1AR and association of PDE4D8 to the β2AR/arrrestin complex under high concentrations of agonist stimulation raise a possibility for synergistic effects on cAMP accumulation and diffusion when both receptors are coactivated in cardiac myocytes. In this model (Figure 2B), the β1AR/arrestin serves as the sequestration mechanism not only for PDE4D8 dissociated from the β1AR but also for PDE4D5.
from the cytosol and PDE4D3 from the sarcoplasmic reticulum membrane. Thus, the cAMP produced from β1AR activation has a clear path to travel from the plasma membrane to the sarcoplasmic reticulum. Several lines of evidence support this hypothesis. First, β1AR is the major subtype to promote cardiac contractile responses, whereas β2AR has a minimal role in promoting contractile responses. Second, β1AR displays limited internalization, whereas β2AR undergoes robust internalization on agonist stimulation. The sequestration of PDE4 by β1AR/arrestin complex on the endosome facilitates segregation of the cAMP production by β1AR/Gs/AC machinery from the cAMP degradation enzymes. Therefore, costimulation of β1AR and β2AR at the postsynapse could lead to transportation of PDE4D8 from the β1AR/Gs/AC complex at the postsynapse to the β2AR/arrestin complex on the endosome, opening the “gate” for the receptor-induced cAMP diffusion and propaga
tion into the organelles such as the sarcoplasmic reticulum in cardiac myocytes.

Cross-Talk Among βAR and Other GPCR for cAMP/PKA Signaling Transduction

Although the expression of β1AR is relative low in myocardium, the expression is detected in different mammalian species, from human to rodents, and regulates cardiac function. Activation of β1AR induces different signaling pathway in cell lines or primary tissues, including Gs, AMP-activated protein kinase, and endothelial nitric oxide synthase. Early studies with functional characterization have shown that activation of β1AR leads to contradictory observations, ranging from significantly enhanced contractile response to minimal or even reduced contractile responses. Using myocytes lacking both β1AR and β2AR, Devic et al. have shown that stimulation with βAR-specific agonist isoproterenol or β1AR-specific agonist CL-316243 induced a small decrease in rate in spontaneously contracting mouse neonatal myocytes. This observation indicates that β1AR directly exerts a negative effect on contraction responses in murine cardiac myocytes. Recent studies have revealed that cross-talk between β1AR-induced cAMP and other βAR-induced cAMP in mouse cardiac myocytes. The β2AR-induced cAMP activates PDE2 to enhance its catalytic activity for cAMP, which negatively modulates the cAMP induced by Gs signaling. In this case, inhibition of β1AR significantly enhances the maximal cAMP accumulation after stimulation of wild-type myocyte with norepinephrine. Meanwhile, inhibition of PDE2 also promotes cAMP accumulation induced by norepinephrine. Interestingly, a third generation of cardiac-specific and β1-selective blocker nebivolol also stimulates endothelial nitric oxide synthase, which can enhance nitric oxide/cGMP activities to attenuate the cAMP signaling for myocytes contractile function. Further examination of this signaling cross-talk may help us to better understand how drugs like nebivolol work in heart failure patients.

The fact that many GPCR expressed in myocytes display relative segregation indicates that they have distinct function
alities. However, many receptors are also localized/enriched in lipid rafts/caveolae, raising the opportunities for signaling cross-talk between other GPCR and adrenergic signaling. For example, muscarinic stimulation can attenuate cAMP signaling induced by βAR. This is dependent on the ligand occupation of muscarinic receptor; the inhibition was rapidly reversed when the agonist was removed. The mechanism underlying the cross-talk for the observed response in cAMP signals is not entirely clear. The coupling of muscarinic receptor to Gs is involved in the cross-talk. However, the rapid reverse of the inhibitory effect suggests that additional modulation of other regulators such as PDE could play a role in the process. Further analysis with live-cell imaging will help to understand how other neuro
dhormonal stimulation affects the βAR signaling cascades under various physiological and clinic cardiac conditions.

Clinical Implication of Spatiotemporal Regulation of Adrenergic Signaling in Cardiac Myocytes

Downregulation of the β1AR and adrenergic response are hallmarks of human heart failure as a result of chronic stimulation under elevated circulating catecholamines in plasma and increased sympathetic tone. Recently, evidence has emerged that the adrenergic receptor-induced cAMP/PKA signaling is also altered in cardiac myocytes under chronic conditions. These alterations probably occur before downregulation of the β1AR, an indicator of the ending stage in heart failure. Using a sophisticated imaging technique, Nikolaev observed that β2AR is redistributed from t-tubular structure to the plasma membrane in failing cardiac myocytes, thus broadening the cAMP distribution under agonist stimulation. In hypertrophic or failing hearts, the expression of PDE3A, PDE4A, PDE4B, PDE4D, and PDE5A are downregulated, although the increase in expression of PDE5A is also reported in different studies. In comparison, the PDE1A and PD2A expressions are increased in hypertrophic heart. The contradictory reports on expression levels of PDE genes in these studies are likely attributable to the detection of the proteins at different stage of diseases as well as to using different animal models. For example, a recent report shows that the expressions of PDE4 and other PDE, such as PDE1, PDE2, PDE5, are broadly increased in the early stage of cardiac hypertrophy induced by chronic angiotensin II perfusion. Of these observed changes, the decreased expression of PDE4D3 and its association with ryanodine receptor 2 is particularly interesting because it causes elevated cAMP/PKA activities in the local domain for hyperphosphorylation of ryanodine receptor 2. Such a hyperphosphorylation leads to an increase of channel activities, contributing to leaking of calcium from the sarcoplasmic reticulum, as well as arrhythmia and sudden death in heart failure patients. Together, these data underscore the evolving expression of PDE isofoms in compensating the alteration of adrenergic signaling during the development of heart failure, thus offering potential targets for clinical therapy.

Conclusion

Recent advances in biochemical characterization of βAR complexes and the development of live-cell imaging of spatiotemporal regulation of βAR signaling in cardiac myocytes offer new paradigms to understand how signaling transduction is translated into physiological contractile response. The discussion on separation of cAMP production vs
cAMP accumulation and diffusion, as well as the equilibrium between cAMP production and degradation, offers new concepts to dissect how the βAR/cAMP signaling is transduced in the highly differentiated and structurally rigid cardiac myocytes in an agonist dose-dependent manner. This information also offers new directions to analyze subtle alterations during development of pathological conditions. Several key questions remain to be addressed. The compositions of the βAR complexes need to be further characterized, particularly whether PDE and AC are associated with the same receptor complexes and whether different pools of βAR exist in a single cell. The mechanim of PDE dissociation from the activated receptors and the master role of arrestin as a switch for cAMP accumulation and diffusion at high concentration of agonist stimulation remain to be further characterized. These are critical steps for accumulation and propagation of cAMP signals for physiological cardiac contraction. Moreover, the role of PDE3 in adrenergic signaling and cardiac contractile regulation remains to be investigated. Last, and most importantly, an understanding of how the precisely controlled spatiotemporal cAMP signals are altered is essential during early adaption in myocardium in chronic conditions, such as diabetes and chronic inflammation, and during development of heart failure. Any insights in understanding these processes will potentially impact on the research direction and clinical practice.

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Yang K. Xiang

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