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Compartmentalized cAMP Signaling in Cardiac Ventricular Myocytes

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Abstract

Activation of different receptors that act by generating the common second messenger cyclic adenosine monophosphate (cAMP) can elicit distinct functional responses in cardiac myocytes. Selectively sequestering cAMP activity to discrete intracellular microdomains is considered essential for generating receptor-specific responses. The processes that control this aspect of compartmentalized cAMP signaling, however, are not completely clear. Over the years, technological innovations have provided critical breakthroughs in advancing our understanding of the mechanisms underlying cAMP compartmentation. Some of the factors identified include localized production of cAMP by differential distribution of receptors, localized breakdown of this second messenger by targeted distribution of phosphodiesterase enzymes, and limited diffusion of cAMP by protein kinase A (PKA)-dependent buffering or physically restricted barriers. The aim of this review is to provide a discussion of our current knowledge and highlight some of the gaps that still exist in the field of cAMP compartmentation in cardiac myocytes.

Keywords

cAMP compartmentation; cardiac myocytes; beta-adrenergic receptor; prostaglandin receptor; phosphodiesterase; protein kinase A; restricted spaces; buffering

1. INTRODUCTION

A variety of G-protein coupled receptors (GPCRs) act by stimulating cAMP production in virtually every cell in our bodies. This ubiquitous second messenger is involved in regulating a multitude of cellular functions, including metabolism, proliferation, development, and gene transcription. In cardiac myocytes, cAMP plays a particularly important role in regulating excitation-contraction coupling. Yet, despite the fact that multiple GPCRs are able to stimulate cAMP production in cardiac myocytes, not all produce the same functional responses. This can be attributed to the fact that this diffusible second messenger does not move freely throughout the cell.

The β-adrenergic receptor (βAR) is the classic example of a GPCR that elicits responses through the production of cAMP. Following agonist binding, the receptor acts via the stimulatory G protein G_s, which then elicits production of cAMP by adenylyl cyclase (AC). In cardiac myocytes, cAMP then mediates downstream functional responses by activating effector proteins that include protein kinase A (PKA), exchange protein activated by cAMP (Epac), and cyclic nucleotide gated ion channels. This sequence of events is common to a number of different GPCRs, but the resulting functional response can vary. For example, it was recognized early on that βARs as well as E-type prostaglandin receptors (EPRs) act via production of cAMP, but only βAR stimulation led to an increase in cardiac contractility [1–4]. To explain this apparent paradox, it was proposed that cAMP signaling must be compartmentalized, with different receptors producing spatially segregated pools of cAMP in discrete subcellular locations. Initial studies divided these pools into soluble and particulate fractions of cell or tissue homogenates. Consistent with this idea, βARs as well as EPRs led to generation of cAMP in soluble fractions, while only βARs stimulated cAMP production in particulate fractions. But how this related to what happens in the intact cell was unclear.

Differences in the cAMP-dependent responses produced by different subtypes of βARs is another example of compartmentalized cAMP signaling in cardiac myocytes. In ventricular myocytes, β_1AR stimulation leads to the PKA-dependent phosphorylation of multiple target proteins, including L-type Ca^{2+} channels (LTCCs) and phospholamban (PLB). However, β_2ARs produce a cAMP-dependent response that is restricted to the regulation of LTCCs [5–7]. This difference can explain how both β_1 and β_2ARs can produce an increase in contractility (a positive inotropic response). Yet, only β_1ARs consistently produce an increase in the rate of relaxation (a positive lusitropic response). The difference in responses correlates with the location of the target proteins being regulated. LTCCs are typically found in the plasma membrane of the transverse (t) tubules, where they are in close proximity to ryanodine receptors (RyRs) found in the junctional sarcoplasmic reticulum (SR) [8] (see figure 1B). This junctional membrane complex forms a restricted space called the dyadic cleft. PLB, on the other hand, is found outside of the dyadic cleft in the non-junctional SR (see figure 1C). Phosphorylation of PLB removes the inhibitory effect it has on the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), which is responsible for pumping Ca^{2+} back into the SR following contraction. By pumping more Ca^{2+} back into the SR, this further enhances the positive inotropic effect of β_1AR stimulation. It can also explain the ability of β_1ARs to produce a positive lusitropic effect.

A-kinase anchoring proteins (AKAPs) are one important means of ensuring that receptor activation of cAMP production leads to regulation of the correct target protein by PKA, especially in light of evidence that cAMP binding to and activation of PKA does not necessarily result in complete dissociation of catalytic and regulatory subunits of the holoenzyme [9]. Consistent with this idea, disrupting AKAP signaling complexes has been shown in a number of studies to prevent cAMP-dependent regulation of many different PKA-dependent responses [10, 11]. However, AKAPs alone are not sufficient to explain compartmentation of cAMP-dependent signaling. If cAMP can diffuse freely throughout the cell, any receptor capable of stimulating its production would be expected to elicit identical responses due to uniform activation of PKA everywhere, but that is not what happens.

In order to produce receptor-specific responses, there must be some way of generating localized pools of cAMP.

For many years, the ability to investigate mechanisms contributing to cAMP compartmentation was limited by the methods available for measuring cAMP activity. This problem was solved with the generation of biosensors that provide a readout of changes in cAMP activity in intact cells. The first such biosensor utilized the principle of fluorescence resonance energy transfer (FRET) [12]. This probe, called FlCRhR, was constructed using the catalytic and regulatory subunits of PKA labeled with donor (fluorescein) and acceptor (rhodamine) fluorophores, respectively. With FRET-based probes, excitation of the donor leads to transfer of energy to the acceptor when the two fluorophores are in close proximity. In this situation, the donor fluorescence is quenched and the acceptor fluorescence is sensitized. When cAMP binds the regulatory subunit, the conformational changes result in a loss of energy transfer that alters the donor-acceptor fluorescence ratio. Thus, these probes can provide a readout of changes in cAMP activity. However, the FlCRhR probe was difficult to synthesize, and it relied on microinjection or dialysis via a patch pipette in order to introduce it into cells [13], limiting its practical utility.

The advent of genetically encoded biosensors made it possible to express probes in a wide range of cell types with relative ease, which opened a whole new era for investigating compartmentalized cAMP signaling. One of the first such probes was actually a modified version of the cyclic nucleotide gated (CNG) ion channel [14]. This probe could be used in cells not expressing endogenous CNG channels, such as adult ventricular myocytes. Changes in cAMP near the plasma membrane could then be detected using the patch clamp technique to measure the membrane current generated when this probe was activated [15–17].

More recently, a number of genetically encoded FRET-based biosensors have been developed using different cAMP binding proteins. These include: PKA-based probes [18, 19], as well as cAMP binding domains from different isoforms of Epac (Epac1-camps and Epac2-camps) [20] and the hyperpolarization activated K^+ channel (HCN2-camps) [21]. Some of these, like the type II PKA based biosensor, are inherently targeted to specific subcellular locations through interactions with AKAPs, while others, such as Epac1-camps, Epac2-camps, and HCN-camps, are expressed uniformly throughout the cytoplasm of the cell. However, a number of Epac-based variants have been generated with targeting sequences that enable them to respond to changes in cAMP occurring in specific subcellular microdomains [22]. In addition to FRET-based biosensors that respond directly to cAMP binding, there are also A-kinase activity reporter (AKAR) probes, which respond indirectly to cAMP. They contain a consensus sequence, which when phosphorylated by PKA, undergoes a conformational change affecting FRET donor and acceptor interactions [23].

The present review focuses on how these tools, together with other newly developed approaches, have been used to investigate cAMP compartmentation in cardiac myocytes. Most previous reviews on this subject have focused on the role of localized degradation of cAMP by phosphodiesterases (PDEs). The present review includes an updated discussion of this topic, in addition to an examination of the roles played by localized production of cAMP

by different GPCRs, restricted diffusion due to PKA buffering and physically restricted spaces. Finally, we include a brief discussion of how some of these factors are affected in certain disease states.

2. LOCALIZED PRODUCTION BY GPCRs

One mechanism contributing to the generation of receptor-specific pools of cAMP in distinct intracellular microdomains involves segregating receptors into discrete subcellular locations. Some receptors are commonly associated with caveolar signaling complexes in the plasma membrane, others are specifically excluded from these membrane domains, and then there are receptors found in both domains [24–26]. Caveolae are a subset of lipid rafts enriched in cholesterol and sphingolipids and defined by the presence of caveolin, a multifunctional protein, of which there are three isoforms, with caveolin 3 (Cav3) being the predominant subtype present in cardiac myocytes [26].

β_1-Adrenergic Receptors

β_1ARs represent about 80% of the βARs in cardiac myocytes of most species [27]. These receptors are associated with both caveolar and non-caveolar signaling microdomains (see figure 1B–D) [28–32]. The widespread distribution of β_1ARs is associated with their ability to produce a global cAMP response that can be detected throughout the entire cell using FRET-based biosensors [21]. Despite producing a uniform increase in cAMP throughout the cell, there is evidence that β_1ARs found in different membrane domains produce compartmentalized responses. For instance, β_1ARs associated with caveolar/lipid raft domains appear to modulate myocyte contraction [32]. This conclusion is supported by the observation that disrupting caveolar/lipid raft domains by treatment with cholesterol-depleting agents such as methyl-β-cyclodextrin (MβCD) enhances β_1AR-mediated effects. This includes increases in LTCC activity, PLB phosphorylation, intracellular Ca^{2+} transients, and cell contraction. Consistent with these results, cholesterol depletion was also found to increase the generation of cAMP detected by the type II PKA-based biosensor, which is known to be targeted to the caveolar domains [30, 31]. In contrast, cholesterol depletion did not induce any changes in global cAMP responses detected by the non-targeted Epac2-camps probe. The reason for an increase in cAMP production within caveolar regions following cholesterol depletion is still unclear. However, Cav3 is known to have an inhibitory effect on the proteins that it interacts with [33]. Therefore, it is likely that disruption of caveolae eliminates this inhibitory effect, thereby enhancing AC activity.

Although GPCRs are most often thought to reside in the plasma membrane, recent studies have also shown that intracellular β_1ARs also play an important role in producing compartmentalized cAMP responses. This includes local production of cAMP by β_1ARs found in the Golgi. Those receptors were found to be responsible for the Epac-dependent activation of phospholipase Cε and hydrolysis of phosphatidylinositol-4-phosphate, which then contributes to the generation of hypertrophic responses [34]. β_1ARs found in the SR have also been reported to generate the local production of cAMP that leads to PKA-dependent phosphorylation of PLB and regulation of SERCA-dependent Ca^{2+} uptake (see figure 1E) [35].

β_2-Adrenergic Receptors

β_2ARs, which make up most of the remaining βARs found in cardiac myocytes, also act through the production of cAMP. However, as discussed above, the responses they produce are quite different from those elicited by β_1ARs. Some investigators attribute these differences to the fact that β_2ARs couple not only to G_s, but also to G_i signaling pathways [6, 36, 37]. The other major distinction between the βAR subtypes is in their subcellular distribution. β_2ARs are found exclusively in caveolar/lipd raft domains in cardiac myocytes (see figure 1B) [28–30, 38–42]. In addition, the βAR subtypes also vary with respect to their exact placement within a cell.

Adult ventricular myocytes possess a complex intracellular architecture marked by the presence of an extensive t-tubular network that enables the plasma membrane to access the cell interior, facilitating excitation-contraction (EC) coupling. Nikolaev el al. employed scanning ion conductance microscopy to selectively activate β_1 and β_2ARs at specific cellular locations and measure the resulting response detected by a FRET-based biosensor [43]. With this powerful combination of techniques, the authors demonstrated that β_2ARs produced cAMP responses that were confined to t-tubules, while β_1ARs produced responses detected across all areas of the plasma membrane, not just the t-tubules. Both β_1 and β_2ARs have been reported to form signaling complexes with LTCCs [30, 31], which are primarily located in the t-tubules of ventricular myocytes, where they are associated with dyadic clefts [8]. This is consistent with the observation that βAR regulation of LTCC activity is largely limited to t-tubules [44]. It also suggests that βAR production of cAMP associated with t-tubules is most likely occurring within dyadic clefts.

The inability of β_2ARs to produce a cAMP response that extends beyond LTCC regulation has been attributed to the fact that these receptors also couple to Gi signaling pathways [6, 7, 36, 45]. Localized responses to β_2AR activity may also be regulated by phosphodiesterases (PDEs), enzymes that break down cAMP [46, 47]. Accordingly, a recent study found that activation of β_1, but not β_2ARs, was able to generate cAMP detected by a FRET-based biosensor targeted to the non-junctional SR, where PLB and SERCA are located. However, β_2AR production of cAMP was detected in those distant locations following PDE2 and PDE3 inhibition [48]. PDE4 activity has also been shown to prevent β_2AR production of cAMP from reaching RyRs just nanometers away, on the opposite side of the dyadic cleft [49]. The redistribution of PDE4 activity away from this location in cardiac hypertrophy and heart failure results in hyperphosphorylation of RyRs, causing an increase in spontaneous Ca^{2+} release from the SR and subsequent generation of ventricular arrhythmias.

β_3-Adrenergic Receptors

β_3ARs are expressed at very low levels in cardiac myocytes of some species, including humans, where they are associated with nitric oxide-dependent production of cGMP and inhibition of contractility [50]. More recently, it has been shown that β_3ARs are localized to the t-tubules, where production of cGMP is then able to regulate cAMP levels by stimulating PDE2 activity [51].

Prostaglandin Receptors

Cardiac myocytes express two EPR subtypes, EP2 and EP4, that couple to G_s and stimulate cAMP production [29, 32, 52]. However, the cAMP produced by EPRs has been found to be highly sequestered. Warrier et al. compared cAMP responses detected using a FRET-based biosensors targeted to type II PKA and one expressed globally throughout the cytosol in guinea pig ventricular myocytes [53]. In that study EPR activation produced a cAMP response that could be detected by the globally expressed probe, but not the PKA-targeted probe. They also found that EPR stimulation failed to regulate LTCC activity in these cells, which is consistent with the idea that type II PKA regulates the activity of these channels in cardiac myocytes.

Unlike the results obtained in guinea pig ventricular myocytes, EPR activation was found to elicit cAMP responses detected by the same two probes in rat myocytes, but there were still no changes in LTCC activity [32]. However, disruption of caveolar/lipid raft domains by cholesterol depletion, which enhanced β_1AR responses, had no effect on EPR-mediated responses. These results are consistent with the findings that both EP2 and EP4 receptor subtypes are excluded from caveolar membrane fractions [29, 32, 52]. Consistent with this, EPR stimulation was found to produce more pronounced cAMP responses detected by a FRET-based probe targeted specifically to non-raft domains of the plasma membrane [54]. Taken together, these results suggest that even though EPR activation elicits cAMP responses, the targeting of these receptors to non-caveolar membrane domains results in the production of cAMP in subcellular locations that does not regulate excitation or contraction (see figure 1D). Cardiac EPRs do, however, offer protection from ischemia/reperfusion injury in a cAMP-dependent manner [55, 56].

Muscarinic receptors

M_2 muscarinic receptors (M_2Rs) are also involved in regulation of cAMP production in cardiac myocytes. These receptors regulate AC activity through the inhibitory G protein G_i. However, cAMP responses elicited by M_2R exhibit a complex biphasic pattern that involves stimulation as well as inhibition of cAMP production [57]. M_2R activation produces a rapid inhibition of cAMP followed by a rebound increase in cAMP upon washout of the agonist [58, 59]. Differences in temporal properties of the stimulatory and inhibitory effects have been suggested to explain this behavior. The inhibitory response turns on and off rapidly. It is also the dominant response observed during receptor activation. However, upon agonist washout, the inhibitory effect turns off rapidly, revealing the stimulatory effect, which turns off more slowly. This type of response may elicit certain types of arrhythmogenic behaviors associated with dynamic changes in parasympathetic tone [60].

An explanation for this type of complex behavior has been attributed to cAMP compartmentation. Cardiac myocytes express different AC subtypes: AC5 and 6 as well as AC4 and 7 [61, 62]. The inhibitory effect can be explained by G_i inhibition of AC5/6 activity, while the stimulatory effect may be due to activation of AC4/7 [57, 59, 63]. While AC5/6 are localized to caveolae, AC4/7 are excluded from those membrane domains [40, 64]. Iancu et al, developed a computational model of compartmentalized cAMP signaling incorporating both M_2R and β_1AR signaling mechanisms [65, 66]. The modeling

results predicted that M_2Rs can generate complex cAMP responses by inhibiting AC5/6 in caveolar domains and stimulating AC4/7 in non-caveolar of the plasma membrane in cardiac myocytes.

3. LOCALIZED DEGRADATION BY PHOSPHODIESTERASES

If the production of cAMP is localized, it stands to reason there must be some mechanism to prevent it from freely diffusing throughout the cell in order to preserve targeted functional responses. PDE enzymes, which breakdown cAMP, are believed to play a central role in this process (see figure 1) [67, 68]. At least 4 different PDE families: PDE1, PDE2, PDE3, and PDE4, are found in cardiac myocytes [69]. PDE1 and PDE2 can hydrolyze both cAMP and cGMP, while PDE3 preferentially hydrolyzes cAMP and PDE4 is specific for cAMP. These PDEs also vary in how they are regulated. PDE2 can be allosterically stimulated by cGMP, PDE3 can be competitively inhibited by cGMP, and both PDE3 and PDE4 can be activated by PKA-dependent phosphorylation Selective inhibitors have been used to identify the contribution of the different PDE isozymes to cAMP compartmentation. The exception to this is PDE1, for which the availability of selective inhibitors is limited [70, 71].

PDE2

PDE2 activity is mainly associated with the membrane fractions of cardiac myocytes [72, 73]. Accordingly, the greatest changes in cAMP activity observed following inhibition of PDE2 have been detected by FRET-based biosensors targeted specifically to different regions of the plasma membrane [54]. Regulation of PDE2 by cGMP has also been shown to play an important role in affecting localized cAMP production associated with LTCCs as well as RyRs [74, 75]. In addition, local cAMP signaling involving PDE2 has been shown to regulate mitochondrial function in cardiac myocytes [76]. Furthermore, PDE2-induced cAMP-dependent responses play a significant role in regulating cardiac hypertrophy [77]. Consistent with this hypothesis, PDE2 activity was found to be enhanced in cardiac hypertrophy [78, 79], and the overexpression of PDE2 offered protection against norepinephrine-induced hypertrophy [79]. These studies suggest that the localized control of cAMP microdomains due to PDE2 activity may have therapeutic potential [77, 80]. Additionally, a recent report found evidence suggesting that PDE2 inhibition may enhance cAMP activity associated with PLB/SERCA, which may have a clinical application in augmenting cardiac relaxation in certain types of heart failure [48].

PDE3

Depending on the animal species, PDE3 exhibits a more diverse distribution pattern with activity found in both membrane and soluble fractions [81–83]. Accordingly, inhibition of PDE3 activity led to detection of cAMP responses by FRET-based biosensors that were targeted to sarcolemma as well as bulk cytosol in cardiac myocytes [54]. In neonatal rat ventricular myocytes, PDE3 activity was more prominently confined to cytosolic regions that are occupied by the type I regulatory subunits of PKA [75]. The authors also found that cGMP-mediated inhibition of PDE3 activity modulates localized cAMP responses in these domains.

PDE3 can be divided into two subfamilies, PDE3A and PDE3B. While PDE3A has been shown to be the major subtype found in hearts from most species, both PDE3A and PDE3B are expressed in murine cardiac myocytes [17, 84–86]. In human heart, alternative splicing and post-transcriptional processing generate 3 isoforms of PDE3A [84, 87]. These isoforms exhibit differences in the N-terminus sequence, which has been shown to determine not only the subcellular localization, but also the feedback regulation of these enzymes by PKA or PKB phosphorylation [84, 87].

In mouse heart, knockdown of PI3Kγ was found to increase cAMP levels and enhance contractility [88]. Consistent with these observations, a later study found that PI3Kγ stimulates the activity of PDE3B in a kinase-independent manner [86]. This study found that PI3Kγ acts as an anchoring protein that recruits PDE3B, but not PDE3A, to specific locations in myocytes. In a subsequent study, the authors found that PI3Kγ functions as an AKAP that forms a signaling complex with type II regulatory subunit of PKA and PDE3B [89]. This study demonstrated that localized cAMP levels were regulated by the PKA-dependent activation of PDE3B in cardiac myocytes. However, an ensuing study found that the knockdown of PDE3B did not alter cardiac contractility in the mouse [90]. The authors of that study showed an increase in intracellular Ca^{2+} transient amplitude as well as SR Ca^{2} content in ventricular myocytes from PDE3A-deficient mice. The authors' finding that PDE3A forms a signaling complex with PLB and SERCA2a could explain these results. No change in LTCC currents was apparent in myocytes from PDE3A knockout mice suggesting that the PDE3A may represent the predominant isoform in most species and its activity is preferentially located close to SERCA2a and PLB within non-junctional SR domain.

While PDE3 may selectively regulate cAMP around PLB and SERCA under basal conditions, others have found evidence for PDE3-dependent regulation of LTCC activity in ventricular myocytes following βAR stimulation [91]. Notably, studies that monitored changes in βAR stimulation of cAMP responses within regions associated with the type II subunit of PKA or bulk cytosol found that PDE3 activity may not be as important as other PDE isoforms, at least in adult mouse and neonatal rat myocytes [21, 92].

PDE4

Several studies have shown that inhibition of basal PDE4 activity leads to an increase in cAMP, especially in membrane and type II PKA-associated microdomains [54, 92, 93]. However, inhibition of PDE4 alone has not been found to alter downstream functional effects such as LTCC activity in resting cells [16, 93, 94]. Following βAR stimulation, on the other hand, the role of PDE4 in mediating cAMP dependent responses becomes markedly more prominent, suggesting that PDE4 is involved in regulating receptor mediated cAMP-dependent effects, including LTCC activity [16, 21].

While there are four subfamilies of PDE4, only three: PDE4A, PDE4B, and PDE4D are believed to be expressed in cardiac tissue [94–96]. PDE4D and PDE4B together have been shown to provide about 90% of the total PDE activity in neonatal rat cardiac myocytes [92]. Alternative splicing and the use of different promoters results in an even greater number of isoforms [97–99]. Diversity in the N-terminal domains is responsible for targeted

expression of the PDE4 variants to different microdomains [97, 100]. However, due to the high sequence homology of the catalytic site among the different PDE4 isoforms, subtype-selective pharmacological inhibitors are not readily available. To overcome this limitation, transgenic mice have been used to examine the role of specific PDE4 isoforms.

In order to distinguish between the roles of PDE4B and PDE4D in regulating compartmentalized cAMP signaling, Leroy et al. generated PDE4B or PDE4D knockout mice [101]. The authors found that both are part of a signaling complex that includes the LTCC. However, only knockdown of PDE4B, but not PDE4D, increased the Ca^{2+} current following βAR stimulation. Moreover, PDE4B knockdown also augmented the susceptibility to arrhythmias in mice.

The role of PDE4B in regulating receptor-mediated compartmentalized cAMP responses was further characterized in a study using FRET-based biosensors and biochemical methods [102]. Upon β_1AR stimulation, cAMP responses were found to be enhanced specifically in plasma membrane domains in cardiac myocytes obtained from PDE4B-deficient neonatal cardiac myocytes. No change in cAMP responses were seen in the bulk cytosolic compartment. It is likely that the rise in membrane-associated cAMP was localized to the dyadic cleft, since the only proteins resident within this region, LTCCs and RyRs, were phosphorylated. Phosphorylation of TnI and PLB remained unchanged. These effects appear to be specific for β_1AR, since the authors found no difference in cAMP responses detected in bulk cytosolic or membrane domains following activation of β_2AR or EPR between the wildtype and PDE4B knockdown myocytes.

PDE4D3 has been shown to be a component of a macromolecular signaling complex that includes the RyR in mouse heart [103]. This correlates with the finding that, following βAR stimulation, cAMP levels were significantly higher only in the localized regions associated with the Z lines, but not bulk cytosol, in PDE4D3 knockout mice. Consistent with this finding, RyR2s were found to be hyper-phosphorylated, which in turn reduced their interaction with calstabin2, increasing the leakage of Ca^{2+} and the frequency of arrhythmias. These findings may provide a mechanistic explanation to the observation that there is a down regulation of PDE4D3 and an increased incidence of arrhythmias and sudden cardiac death in patients with heart failure [103].

PDE4D5 has been shown to be involved in regulating hypertrophic responses induced by chronic βAR stimulation in neonatal mouse cardiac myocytes [104]. In that study, PDE4D5 was reported to directly interact with HSP20, a small heat shock protein known to have cardioprotective properties. This close association afforded the regulation of HSP20 by cAMP/PKA-mediated phosphorylation. Using targeted FRET-based biosensors, pharmacologic inhibition of PDE4 was found to elicit a greater rise in cAMP levels in domains associated with HSP20 as compared to the bulk cytosol.

PDE4D5 also plays a role in regulating β_2AR signaling. Following activation, these receptors can undergo phosphorylation by G-protein coupled receptor kinases (GRKs). This results in the binding of β-arrestin and the recruitment of PDE4D5 [46, 47], which can downregulate cAMP activity near the receptor. This is supported by the fact that inhibition

of PDE4D5 activity selectively enhances PKA phosphorylation of the receptor, facilitating its ability to couple to G_i inhibition of AC as well as activation of ERK1/2 signaling [105]. Inhibition of GRK2 phosphorylation of the β_2AR has also been shown to block PDE4D5 (and PDE4D3) association with the receptor, facilitating its ability to regulate myocyte contraction [106]. This is different from the β_1AR, which is associated with PDE4D8 under baseline conditions, but then dissociates following receptor activation [107].

PDE activity plays an essential role in regulating the compartmentalized cAMP signaling in cardiac myocytes. PDEs are often viewed as functional barriers that prevent cAMP from moving between microdomains [4]. However, experimental evidence indicates that cAMP concentrations may be quite high throughout most of the cell, even under baseline conditions [54, 108, 109]. Under these circumstance, PDEs may actually act as “sinks” that deplete cAMP in localized regions [65, 110, 111]. However, PDE activity by itself may not be sufficient to explain cAMP compartmentation [112]. Because the turnover rate for PDE hydrolysis of cAMP is slow, assuming that cAMP moves at a rate equal to free diffusion, the amount of PDE activity found in a typical myocyte is not sufficient to prevent cAMP from moving unimpeded throughout the cell [110]. These results suggest that PDEs can only generate compartments if the rate of cAMP diffusion is sufficiently restricted by some other mechanism(s).

4. LIMITED DIFFUSION

PKA Buffering

The actual diffusion coefficient for cAMP in cardiac myocyte has been estimated using raster image correlation spectroscopy (RICS). RICS is a technique based on the principles of fluorescence correlation spectroscopy, which calculates diffusion coefficients from the fluctuations in fluorescence intensity within small confocal volumes [113]. Using the fluorescently-labeled cAMP derivative, 8-[Pharos-450]-cAMP (φ450-cAMP) [114], it was determined that the diffusion coefficient is approximately 10 $\mu m^2/s$. This is markedly slower than the rate of free diffusion predicted for a molecule that size, which is 300 $\mu m^2/s$. The slow rate of diffusion could not be ascribed to an effect of PDE activity, since φ450-cAMP is resistant to hydrolysis [115]. However, addition of the fluorophore did not alter its affinity for PKA binding.

PKA is believed to be the primary effector for the actions of cAMP in cardiac myocytes. This kinase exists a heterotetrameric complex with two catalytic and two regulatory subunits [116]. Binding of two cAMP molecules to each regulatory subunit leads to activation of the catalytic subunits, which then regulate the activity of downstream proteins through phosphorylation. The fact that the regulatory subunits of PKA bind cAMP raises the possibility that they may also function as a buffer for cAMP, slowing its diffusion throughout the cell (see figure 1). In fact, previous computational modeling studies have predicted such a scenario [14, 112].

The idea that buffering by PKA could explain the slow rate of diffusion measured by RICS was supported by the images of cells loaded with φ450-cAMP. It displayed a nonuniform, highly localized fluorescence pattern, suggesting that φ450-cAMP was not diffusing freely

throughout the cell, but instead was binding to something. The fluorescence pattern was actually consistent with the pattern for distribution of mitochondria, which occupy ≥30% of the volume of a cardiac myocyte, and φ450-cAMP co-localized with a fluorescent label for mitochondria. Labeled cAMP also co-localized with a fluorescently labeled type II regulatory subunit of PKA, and this pattern of fluorescence was disrupted by treating the cells with Ht31, a peptide that interferes with the interaction between the type II regulatory subunit of PKA and A kinase anchoring proteins (AKAPs). The Ht31 peptide also increased the φ450-cAMP diffusion coefficient measured using RICS. This suggest the rate of cAMP diffusion in cardiac myocytes is slowed due to the buffering effect of binding and unbinding to PKA regulatory subunits anchored to the outer membrane of mitochondria. It has recently been determined that there are at least three different mitochondrial AKAPs involved, D-AKAP1, D-AKAP2, and ACBD3 [117]. Similar diffusion coefficients were also found in morphologically simpler HEK293 cells, suggesting that this behavior is not unique to cardiac myocytes [114]. However, Bock et al. [118] have argued that the slow movement of cAMP in HEK293 cells may not involve PKA buffering.

Consistent with the idea that AKAPs and PKA buffering contribute to cAMP compartmentation, treatment of cardiac myocytes with the Ht31 peptide has also been reported to allow β_2AR production of cAMP to propagate throughout the cell [43]. The cAMP buffering capacity of PKA in cardiac myocytes has been estimated at about 1.2 μM [119, 120]. This is similar to estimates of the basal concentration of cAMP present in these cells [108], and would be consistent with previous studies suggesting that most cAMP is bound to PKA under basal conditions [119, 121]. It is also interesting to note that a recent study reported that the ratio of regulatory to catalytic subunits is 6:1 in cardiac myocytes [122]. This suggests the possibility that the excess regulatory subunits may be acting as a buffer to limit the free diffusion of cAMP throughout the cell.

Physically Restricted Spaces

Electron microscopy reveals that intracellular structures, including myofilament proteins, SR, and mitochondria, occupy as much as 90% or more of the volume of an adult ventricular myocyte [123]. This implies that there is very little free space for the movement of solutes such as cAMP. This is particularly true in certain locations such as the dyadic cleft (figure 1). The question then is whether or not these restricted spaces contribute to cAMP compartmentation. A recent *in silico* study took advantage of cryo-transmission electron microscopy images to generate a realistic 3D framework in order to explore this question as it relates specifically to the dyadic cleft [110]. Besides its critical role in EC coupling, the dyadic cleft is also a locus of cAMP synthesis by βARs. In this model, structures such as t-tubules, the SR, and mitochondria acted as impenetrable barriers to cAMP, limiting its free space for diffusion. When physiologically relevant concentrations of PDE activity were used to create a “barrier” to cAMP movement into and out of the dyadic cleft, the model predicted that cAMP gradients could be generated within this confined space, but only when the diffusion coefficient was slowed due to values previously attributed to the effects of PKA buffering (10 $\mu m^2/s$).

The complexity of the arrangement of various structures such as mitochondria, t-tubules, myofilaments and the SR, allows formation of other restricted spaces within cardiac myocytes as well. Similarly, it is likely that the same principles that limit the movement of cAMP within the dyadic cleft also apply to other areas of the cell. In a recent study the mobility of cAMP was measured in cardiac myocytes expressing FRET-based cAMP biosensor [124]. Use of a micro-perfusion system allowed the authors to apply a βAR agonist to one-half of the cell, while measuring the time course of changes in cAMP activity in the other half. They fit the results with a diffusion-reaction equation taking into account the predicted rates of cAMP synthesis and degradation. From this, they estimated the diffusion coefficient of cAMP to be about 35 μm^2/sec. Consistent with the study by Agarwal et al. [114], this value is much slower than the predicted rate of free diffusion. However, the reason for slower movement was ascribed to the intracellular tortuosity created by the complex internal structure in cardiac myocytes rather than the buffering due to PKA binding. Using fluorescence recovery after photobleaching (FRAP) technique, this study found that the movement of fluorescein-conjugated cAMP, a fluorescent analog of cAMP, was 3–4 times higher in neonatal as compared to adult cardiac myocytes. The authors argued that the reason is due to lower structural tortuosity/complexity because of fewer mitochondria in neonatal cells. Direct experimental evidence in support of this conclusion awaits further research.

5. DISEASE INDUCED CHANGES IN cAMP COMPARTMENTATION

A number of changes in compartmentalized cAMP responses have been associated with disease states such as cardiac hypertrophy and heart failure. This includes factors affecting the localized production of cAMP. Nikolaev et al [43] found that β_2AR production of cAMP changed from being specific to t-tubules regions under normal conditions, to producing changes in cAMP that could be detected in all areas of the cell. In addition, changes in AC activity have been associated with certain disease states. Although AC5/6 are the predominant isoforms expressed in the heart, there is also evidence that AC9 plays an important role in local regulation of certain cAMP-dependent responses. For example, genetic deletion of AC9 in mice leads to the development of bradycardia and heart failure [125]. AC9 is also part of a signaling complex with KCNQ1 K^+ channels [126, 127], and disruption of this interaction leads to arrhythmogenesis [128].

Hypertrophy and heart failure have also been associated with many changes affecting localized degradation of cAMP by PDEs. Loss of PDE4D activity was shown to cause a localized increase in cAMP activity that leads to hyper-phosphorylation of RyRs, resulting in altered SR Ca^{2+} release and arrhythmogenesis associated with heart failure [103]. Similarly, the loss of PDE4 activity associated with heart failure resulted in an increase in the local production of cAMP by β_2ARs, leading to PKA-dependent phosphorylation of RyRs and an increase in arrhythmogenesis [49]. Regulation of a local pool of cAMP by PDE2 activity has also been implicated in the development of hypertrophy [77, 79]. In addition, changes in PDE2- and PDE3-dependent regulation of local cAMP responses have been reported to contribute to altered βAR responses associated with cardiac hypertrophy [79, 80]. Changes in β_3AR production of cGMP have also been reported to affect local regulation of cAMP activity by PDE2 [51].

Heart failure is also associated with changes that might be expected to affect PKA buffering and physically restricted spaces. These include the loss of mitochondrial AKAPs [129], loss of mitochondrial organization [130], and disruption of dyadic clefts [131]. Although one might predict that these changes could affect cAMP compartmentation by altering diffusion, proof awaits further investigation.

6. CONCLUSIONS AND FUTURE DIRECTIONS

This review focuses on four general mechanisms believed to play a role in ensuring the specificity of the receptor-mediated functional responses by compartmentalizing cAMP activity in cardiac myocytes. These include localized production of cAMP by different receptors, localized degradation of cAMP by PDEs, and limited diffusion due to the effects of PKA buffering and physically restricted spaces.. Most previous work has focused on demonstrating the functional significance of either localized production or localized degradation of cAMP. However, our understanding of the role that limited diffusion of cAMP plays in compartmentation and its contribution to functional responses is largely undeveloped. We can directly measure the diffusion of cAMP, so we know that it does not move freely throughout the cell. We also have direct evidence that this is due, at least in part to the effects of PKA buffering. Yet, the details of how this works are not fully understood. For example, why does cAMP appear to preferentially associate with PKA associated with mitochondria? Is it because PKA is concentrated there, or is there something different about the PKA located there? What is the role of type I vs type II PKA in buffering the movement of cAMP? What about the role of excess regulatory subunits? Free subunits bind cAMP with an affinity in the nM range, while the cAMP affinity of the holoenzyme is in the μM range [132]. Could it be that the free regulatory subunits function primarily as a buffer for cAMP? Furthermore, evidence that limited diffusion, due to PKA buffering or physically restricted spaces, contributes to cAMP compartmentation is largely based on theoretical predictions. While computational studies suggest that slow diffusion can contribute to the generation of cAMP gradients in spaces as small as the dyadic cleft (see figure 1), experimental evidence supporting these predictions is lacking. A clearer picture of the role that PKA buffering and/or physically restricted spaces play in affecting functional responses awaits a better understanding of the factors involved.It is also important to recognize that while the majority of studies looking at the mechanisms of cAMP compartmentation in the heart have been conducted using ventricular myocytes, the same basic principles are likely to apply in other cell types, such as atrial myocytes, where there are a number of structural and organizational differences [133]. For example, atrial myocytes are enriched in caveolae, which could provide a basis for segregation of receptors similar to what is seen in ventricular myocytes [134]. However, atrial myocytes have also been reported to exhibit a wide variation in the organization of t-tubules [134]. This could affect the location of cAMP production by different βARs. Atrial preparations have also been reported to vary in the relative expression and distribution of PDE isoforms [135]. This too is likely to influence subcellular cAMP gradients. However, atrial myocytes also exhibit a lower density of mitochondria [136], which together with a less well developed t-tubule system might affect factors limiting cAMP diffusion. It is unclear how all of these differences collectively might be predicted to

affect cAMP compartmentation in atrial myocytes. The answer to this question awaits future investigation.

A key to ensuring that the receptor-specific functional effects are elicited is by isolating appropriate downstream signaling partners together into subcellular signaling complexes in proximity to the pools of cAMP produced by that receptor. Technological advances have significantly advanced our understanding of the fundamental mechanisms that control spatiotemporal dynamics of cAMP activity. A major breakthrough was provided by the development of genetically-encoded biosensors that can be expressed in specific subcellular microdomains. Furthermore, computational modeling has provided a means to test the feasibility of various hypotheses and generate new predictions. Advanced imaging techniques are continuously being tested to gain better understanding of the role that the localized cAMP responses play in health and diseases. Novel strategies are likely to continue to evolve from the use of these tools and provide insights directed at developing new therapeutic interventions to combat cardiac disease.

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HIGHLIGHTS

- Localized production of cAMP by different GPCRs is often involved.
- Localized degradation of cAMP by phosphodiesterases alone may not be sufficient.
- Restricted diffusion of cAMP due buffering by protein kinase A is a likely factor.
- Diffusion of cAMP limited by physical barriers may also contribute.
- Multiple factors affecting cAMP compartmentation are altered by disease.

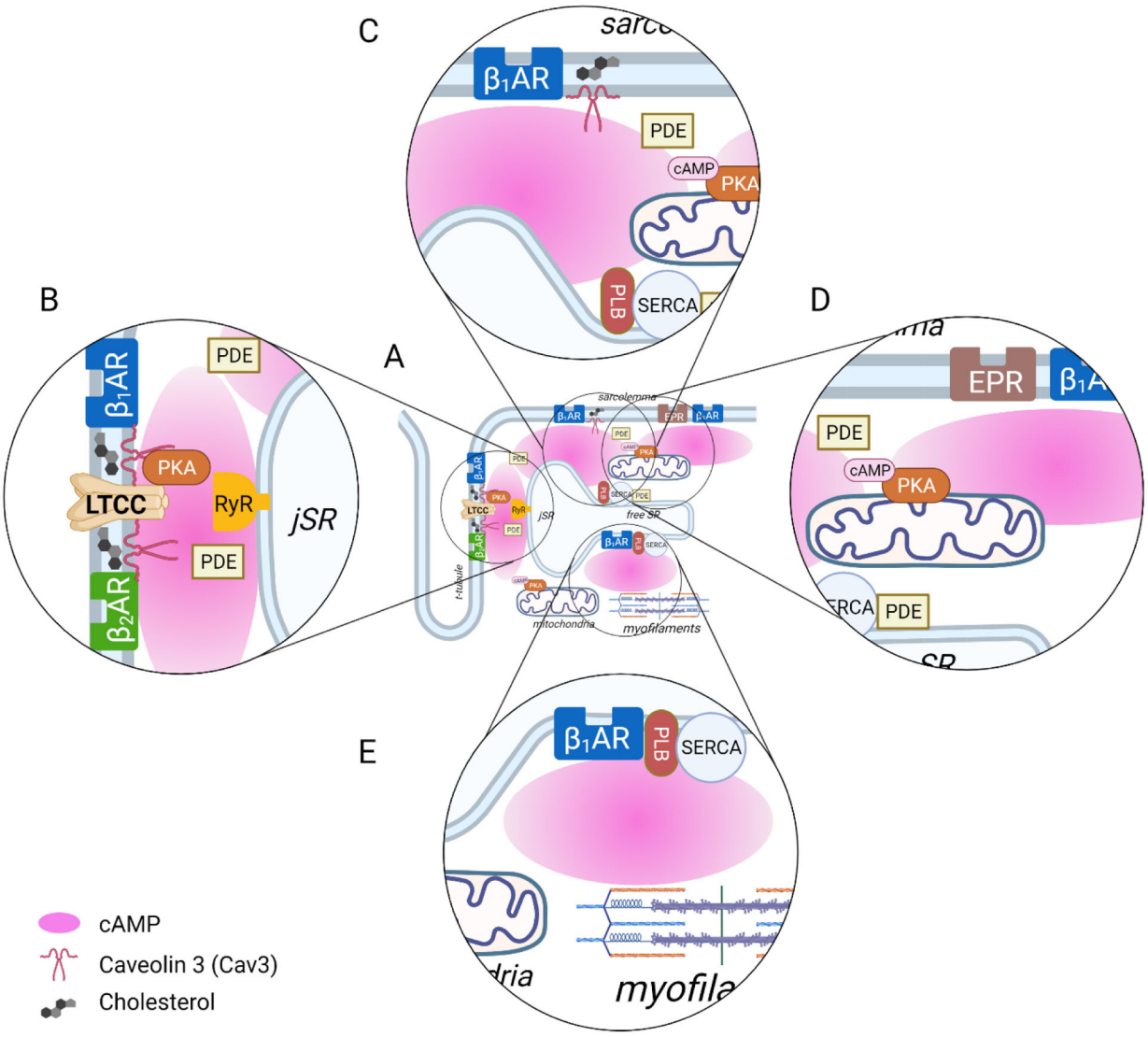

Figure 1.

Compartmentalized cAMP signaling by different receptors in adult ventricular myocytes. (A) Distribution of receptors to specific locations, along with targeted PDE activity, buffering of cAMP by protein kinase A (PKA), and physical barriers to diffusion allows for generation of local pools of cAMP. While β_1ARs are expressed throughout the cell, β_2ARs are confined to the plasma membrane lining the transverse tubules. β_2ARs are also found primarily in caveolar lipid raft domains of the plasma membrane; β_1ARs can be found in caveolar as well as non-caveolar membrane domains; and EPRs are found primarily in non-caveolar membrane domains. (B) β_1ARs and β_2ARs form signaling complexes with L-type Ca^{2+} channels (LTCCs), which are found in dyadic clefts. Both of these receptors produce cAMP that can regulate LTCCs as well as ryanodine receptors (RyRs) within this restricted space. (C) β_1ARs are also able to contribute to a distinct pool of cAMP that regulates the activity of the sarco/endoplasmic reticulum ATPase (SERCA) via PKA-dependent phosphorylation of phospholamban (PLB) (D) E type prostaglandin receptors (EPRs) contribute to a pool of cAMP that does not affect PKA regulation of LTCCs

or PLB, which are involved in excitation-contraction coupling, although it can produce cardioprotective effects. (E) An intracellular pool of β_1ARs found in the SR have also been reported to produce a localized response that leads to phosphorylation of PLB and regulation of SERCA.