Spatial and temporal aspects of cAMP signaling in cardiac myocytes

Radu V. Iancu, Gopalakrishnan Ramamurthy and Robert D. Harvey

Department of Physiology and Biophysics,
Case Western Reserve University, Cleveland, OH 44106-4970, USA

Summary

1. β_1-adrenergic and M_2 muscarinic receptor regulation of cAMP production plays a pivotal role in autonomic regulation of cardiac myocyte function. However, not all responses are easily explained by a uniform increase or decrease in cAMP activity throughout the entire cell.

2. Adenovirus expression of fluorescence resonance energy transfer (FRET) based biosensors can be used to monitor cAMP activity in protein kinase A (PKA) signaling domains as well as the bulk cytoplasmic domain of intact adult cardiac myocytes.

3. Data obtained using FRET-based biosensors expressed in different cellular microdomains has been used to develop a computational model of compartmentalized cAMP signaling.

4. A systems biology approach that employs quantitative computational modeling together with experimental data obtained using FRET-based biosensors has been used to provide evidence for the idea that compartmentation of cAMP signaling is necessary to explain the stimulatory responses to β_1-adrenergic receptor activation as well as the complex temporal responses to M_2 muscarinic receptor activation.

Introduction

Many different neurotransmitters and hormones are capable of regulating the electrical, mechanical, and metabolic activity of the heart by stimulating the production of cAMP. The signaling pathway typically involves receptor activation of adenylyl cyclase (AC) *via* a stimulatory G protein (G_s)-dependent mechanism. The receptor most commonly associated with activation of this signaling pathway in cardiac myocytes is the β_1-adrenergic receptor (β_1AR). However, not all receptors capable of stimulating cAMP production produce the same functional responses. The frequent explanation for such observations has been that different receptors are capable of stimulating cAMP production in distinct microdomains within the cell.[1] The classic example of this is the fact that both β_1ARs as well as $E_{2/4}$ prostaglandin receptors are capable of stimulating cAMP production, but only β_1AR activation leads to changes in acute functional responses.[2-6]

Still other types of receptors can inhibit as well as stimulate cAMP production, eliciting complex temporal responses. An example of this is the M_2 muscarinic receptor (M_2R).[7-16] In ventricular myocytes, M_2R activation alone has little or no effect on cell function. However, M_2R activation can potently inhibit the stimulatory responses associated with β_1AR activation of cAMP production by inhibiting AC activity *via* an inhibitory G protein (G_i)-dependent mechanism. This is referred to as accentuated antagonism.[17] Furthermore, in the presence of submaximal β_1AR activation, transient activation of M_2Rs actually produces a biphasic effect.[7-13] In the presence of muscarinic agonists such as acetylcholine (ACh), there is antagonism of the β-adrenergic response, but upon withdrawal of ACh, there is an exaggerated stimulatory or rebound response. This reflects the fact M_2R activation of a G_i-dependent signaling pathway produces an inhibitory effect that turns on and off rapidly, as well as a stimulatory response that turns on and off more slowly. This complex temporal response to ACh is also difficult to explain if one assumes that receptor activation produces a uniform increase or decrease in cAMP throughout the cell.

FRET-based biosensors for measuring compartmentalized cAMP responses

Until recently it has only been possible to measure cAMP activity using biochemical methods that typically involve homogenization of tissue or cell preparations.[1] While the importance of information obtained using such an approach cannot be over emphasized, obtaining evidence for compartmentation of cAMP signaling was somewhat limited by this approach. Homogenized preparations could be separated into soluble (cytosolic) and particulate (membrane) fractions by centrifugation. When this was done, β-adrenergic agonists were found to increase cAMP production and activation of protein kinase A (PKA) in both fractions, whereas prostaglandins only affected cAMP production and PKA activation in the soluble fraction.[2,6,18,19] This was consistent with the observation that most functional responses were associated with cAMP-dependent activation of type II PKA, which is found primarily in the particulate fraction.[1,20,21] These kinds of results seemed consistent with the idea that the difference in the responses to β-adrenergic agonists and prostaglandins could be explained by compartmentation of cAMP production. However, understanding how this related to what was happening in an intact cell was an open question.

More recently, several different approaches for measuring cAMP in intact cells have been developed. One class of cAMP biosensors utilizes the principle of fluorescence resonance energy transfer (FRET).[22-24] One of the first FRET-based biosensors for measuring cAMP activity was constructed using PKA.[25] In this case, a donor fluorophore, rhodamine, was covalently attached to the

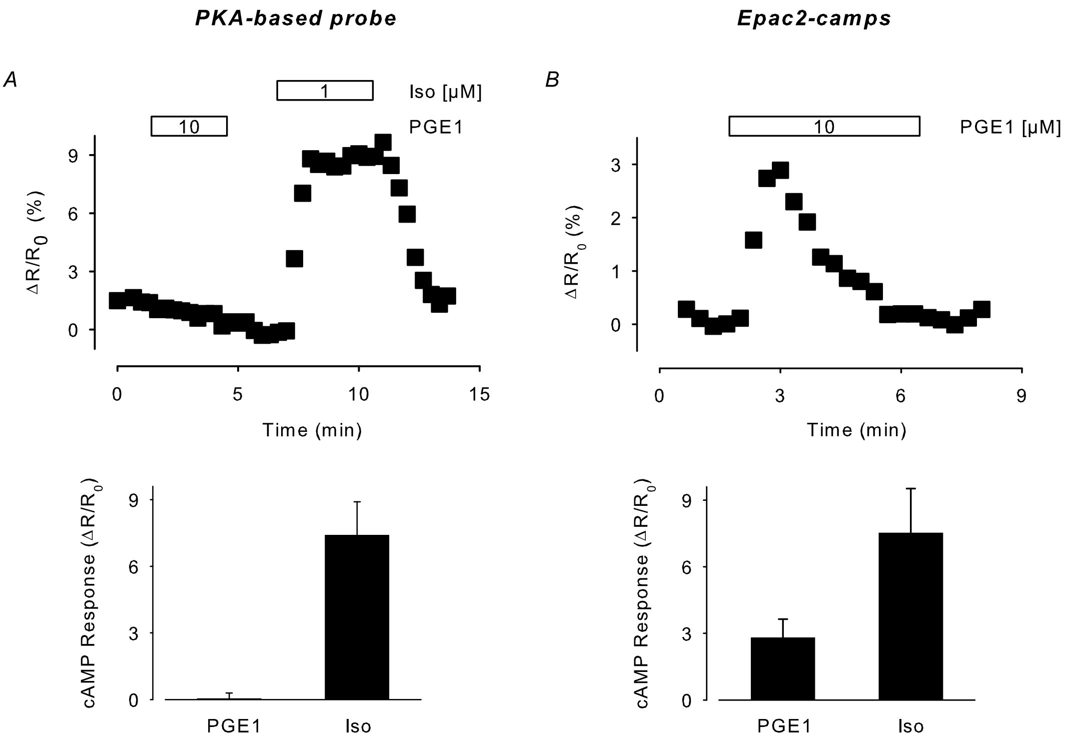

Figure 1*. A.** *PKA-based biosensor expressed in adult guinea pig ventricular myocytes.* ***Top panel: *time course of changes in the CFP/YFP fluorescence ratio (ΔR) relative to baseline (R_0) observed during exposure to PGE1 and isoproterenol (Iso).* ***Bottom panel:*** *average response to 10 μM PGE1 and subsequent exposure to 1 μM Iso (n=5).* ***B.*** *Epac2-camps expressed in adult guinea pig ventricular myocytes.* ***Top panel:*** *time course of changes in $\Delta R/R_0$ observed during exposure to 10 μM PGE1.* ***Bottom panel:*** *average response produced by 10 μM PGE1 (n=8) or 1 μM Iso (n=8). Data adapted from Warrier* et al.[34]

regulatory subunit of PKA and an acceptor fluorophore, fluorescein, was attached to the catalytic subunit. This probe was introduced into cells by microinjection or dialysis *via* a patch pipette.[25-27] In the resting state, the two fluorophores are in close proximity (<100 Å) to one another. Under these conditions, excitation of the donor leads to direct transfer of energy to the acceptor resulting in its fluorescence. When cAMP levels increased, binding of the nucleotide to the regulatory subunit results in reorientation of the catalytic and regulatory subunits and a loss of FRET. By measuring the change in the donor/acceptor fluorescence ratio, it is possible to obtain a readout of changes in cAMP activity in an intact cell. More recently, genetically encoded versions of this type of biosensor have been developed,[28,29] including one in which cyan (CFP) and yellow fluorescent protein (YFP) were used as the donor and acceptor fluorophores, respectively. Such probes have made it possible to introduce the biosensor in a variety of cell types using standard transfection techniques. By using the type II regulatory subunit of PKA, which contains peptide sequences that bind to A kinase anchoring proteins (AKAPs), this type of probe can then be expressed in the same pattern as endogenous type II PKA.[28,30] As a result, this sensor is expected to respond to cAMP specifically in type II PKA signaling domains.

Other FRET based cAMP biosensors have been developed using the exchange protein activated by cAMP (Epac).[31-33] The principle involved is the same as that for the PKA-based probe. An increase in cAMP activity results in a loss of FRET that can be used as an indicator of changes in cAMP activity. However, with the Epac-based probes, CFP and YFP are attached to the amino and carboxy termini of the same protein. There are various versions of Epac-based biosensors, but one created by Nikolaev *et al.*[31] was constructed using only the cAMP binding domain of type 2 Epac. This Epac2 cAMP sensor (Epac2-camps) lacks any anchoring sequences that might target it to specific locations within the cell. The result is a biosensor that is able to diffuse freely throughout the cytoplasm.

In our initial studies, we tested the proof of concept that because the PKA-based probe and Epac2-camps exhibit different expression patterns, when expressed in adult ventricular myocytes, they would be able to differentiate between responses to different agonists. Type II PKA is found primarily in the particulate fraction of homogenized cardiac preparations. Therefore, we predicted that the type II PKA-based biosensor would detect responses to agonists that stimulate cAMP production in the particulate fraction of cells but not the soluble fraction. On the other hand, if the Epac2-based probe is freely diffusible, we predicted that it would respond to agonists that stimulate

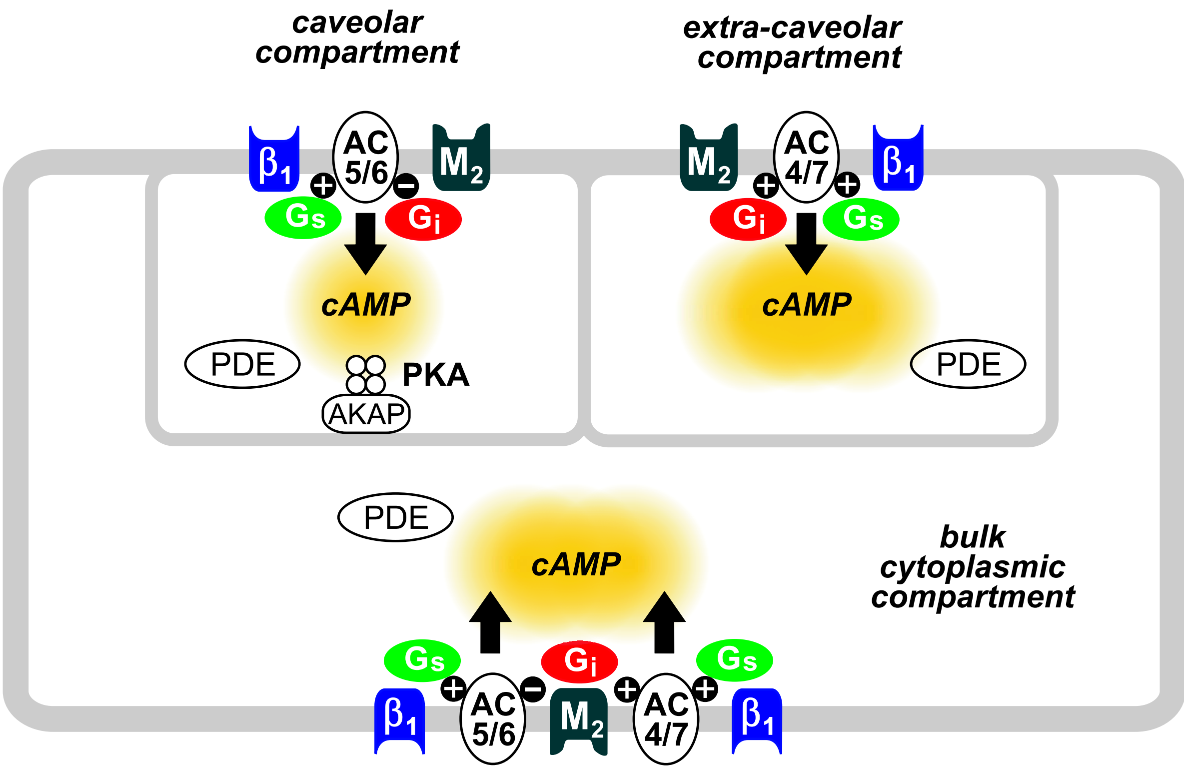

Figure 2. Model of compartmentalized cAMP signaling pathways in a cardiac ventricular myocyte. *β_1-adrenergic receptor (β_1), M_2 muscarinic receptor (M_2), stimulatory (G_s) and inhibitory (G_i) G-proteins, adenylyl cyclase types 5 and 6 (AC5/6), adenylyl cyclase types 4 and 7 (AC4/7), phosphodiesterase (PDE).*

cAMP production in the soluble fraction.

We expressed these probes in adult guinea pig ventricular myocytes using an adenovirus based approach.[34] In myocytes expressing the PKA-based probe, exposure to the prostaglandin receptor agonist PGE1 failed to elicit a detectable change in FRET, even though subsequent exposure to the β-adrenergic agonist isoproterenol (Iso) resulted in a significant response (Figure 1A). However, PGE1 was clearly able to stimulate cAMP production, because exposure of myocytes expressing the Epac2-based probe to PGE1 resulted in a significant, albeit transient response (Figure 1B). These results support the conclusion that FRET based biosensors expressed in different microdomains can be used to detect compartmentalized responses in intact cardiac myocytes. These results are also consistent with the idea that the PKA-based biosensor responds specifically to cAMP in a particulate or membrane domain associated with type II PKA that is not accessible to the soluble or bulk cytoplasmic domain through free diffusion.

Can cAMP compartmentation explain complex temporal responses?

The stimulatory and inhibitory effects of M_2R activation can be explained in part by the different types of AC that are expressed in cardiac muscle and how they are regulated by M_2R activation of G_i.[8] Cardiac myocytes express AC types 5 and 6 (AC5/6) as well as AC types 4 and 7 (AC4/7).[35] However, while the activated α subunit of G_i ($G_i\alpha$) inhibits AC5/6, it has no affect on AC4/7.[36-38] In fact, AC4/7 activity is actually stimulated by G_i βγ subunits.[39,40] In this way, activation of the M_2R can both inhibit as well as stimulate cAMP production (see Figure 2). However, this still does not readily explain the difference in the speed of the stimulatory and inhibitory responses.

A clue as to the potential explanation for the complex temporal response has come from studies demonstrating that AC5/6 is expressed in cholesterol rich fractions of the plasma membrane specifically associated with caveolin-3, a scaffolding protein that is involved in forming caveolae.[41-43] On the other hand, AC4/7 appears to be found primarily in cholesterol rich lipid rafts that do not include caveolin-3.[43,44] This suggests that M_2R inhibition and stimulation of cAMP may be occurring in different subcellular locations. Inhibition of cAMP production occurs in a caveolar domain. This is also where type II PKA is found,[42,45] and again, activation of type II PKA correlates closely with regulation of functional responses.[1,20,21] Stimulation of cAMP production occurs in an extracaveolar membrane compartment. Based on this, we hypothesized that the rebound stimulatory effect that is associated with functional responses is due to the time-dependent flux of cAMP from an extracaveolar to a caveolar compartment.

Using computational modeling to investigate compartmentalized cAMP signaling

In order to test our hypothesis for the complex response to M_2R activation, we developed a computational model of compartmentalized cAMP signaling in a cardiac myocyte.[46] This model was created using the wealth of quantitative kinetic data existing in the literature. The compartmentalized nature of the model was made possible because of more recent information describing the relative distribution of the various proteins involved in cAMP signaling between cytosolic and membrane fractions as well as among different membrane fractions of cardiac myocytes. The key element in this case is the inclusion of AC5/6 activity in the caveolar domain and AC4/7 activity in the extracaveolar domain. The model assumes that there are three compartments (Figure 2). The first compartment is a caveolar domain that includes 10% of the plasma membrane and makes up 1% of the total cell volume. The second compartment is an extracaveolar domain that includes 20% of the plasma membrane and makes up 2% of the cell volume. The third compartment is the bulk cytoplasmic domain that is associated with the remainder of the plasma membrane and makes up 97% of the cell volume.

We then took advantage of results obtained using the PKA-based biosensor to validate the model. This probe appears to selectively respond to cAMP in a type II PKA signaling domain (see Figure 1). Furthermore, type II PKA is associated with the membrane fraction of cardiac myocytes in general and the caveolar membrane fraction in particular.[42,45] Therefore, we compared the responses detected by the PKA-based probe to those predicted in the caveolar domain of the model. We were able to demonstrate that the model is able to accurately describe the concentration dependence of responses to β_1AR activation. It is also able to reproduce both the inhibitory and stimulatory responses produced by M_2R activation in the presence of β_1AR stimulation.[46]

One advantage of using a computational approach is that it makes it possible to see what is happening in different microdomains in order to get a better understanding of the model's ability to produce different behaviours. If we look specifically at AC5/6 and AC4/7 activity as well as cAMP levels in the caveolar and extracaveolar domains (Figure 3), we can see that exposure to a submaximally stimulating concentration of Iso results in a slight increase in cAMP production by all AC isoforms, which is associated with a slight increase in cAMP levels in both the caveolar and extracaveolar domains. However, subsequent exposure to ACh results in an inhibition of cAMP production by AC5/6 and cAMP levels in the caveolar domain, while there is a significant stimulation of cAMP production by AC4/7 and cAMP levels in the extracaveolar domain. This creates a significant gradient in cAMP concentrations, which results in a flux of cAMP from the extracaveolar to caveolar compartments. Although this causes a slight increase in cAMP in the caveolar domain, the actual concentration of cAMP is assumed to be below the threshold for producing functional responses. Upon withdrawal of ACh and termination of M_2R activation, there is a rapid reversal of AC5/6 inhibition as well as AC4/7 stimulation. However, the cAMP gradient does not dissipate immediately. The result is that extracaveolar cAMP continues to diffuse into the caveolar domain, resulting in cAMP levels transiently increasing to a level much higher than that observed prior to M_2R activation. Eventually, phosphodiesterase (PDE) activity breaks down the excess cAMP, explaining the transient nature of the stimulatory response.

Conclusions and future directions

The development of cAMP biosensors provides a means for directly measuring cAMP activity in different subcellular locations within live cells. This can be helpful in demonstrating that different receptors regulate cAMP responses in distinct microdomains. These biosensors have also been helpful in developing a quantitative computational approach to understanding more complex behaviours.

One outcome of this type of systems biology approach is the generation of non-intuitive predictions that can then be tested experimentally. For example, results obtained from the original version of our model suggest that the basal level of cAMP in the bulk cytoplasmic compartment of cardiac ventricular myocytes should be ~1 μM. This is significantly higher than the ~100 nM levels suggested to exist in the caveolar domain by measurements obtained using the PKA-based biosensor.[46] This implication is important because it suggests that compartmentation plays an important role even under basal conditions by maintaining microdomains where cAMP levels are significantly below that found throughout most of the cell. This allows receptor signaling to regulate cAMP concentration over a range that modulates the activity of high affinity effectors such as type II PKA, which has a Kd of 300 nM.[47]

A second unexpected prediction of the model has to do with the role of PDE activity in creating the microdomains involved in compartmentation of β_1AR and M_2R responses. It is often suggested that PDEs act as functional barriers responsible for creating compartments.[1,48] Consistent with this idea, different PDE isoforms have been shown to be targeted to specific subcellular locations.[47,49] Furthermore, the non-uniform distribution of PDE activity between compartments of our model is essential for determining the concentration of cAMP within each compartment, and therefore the gradients between compartments. However, PDE activity alone is not sufficient to maintain those gradients.[46] The model predicts that there must be some other factor contributing to the limited diffusion of cAMP between these compartments.

It is important to note that our computational model is a working hypothesis. It can provide a theoretical framework for testing the feasibility of complex hypotheses. Yet, the model itself is only as good as the data

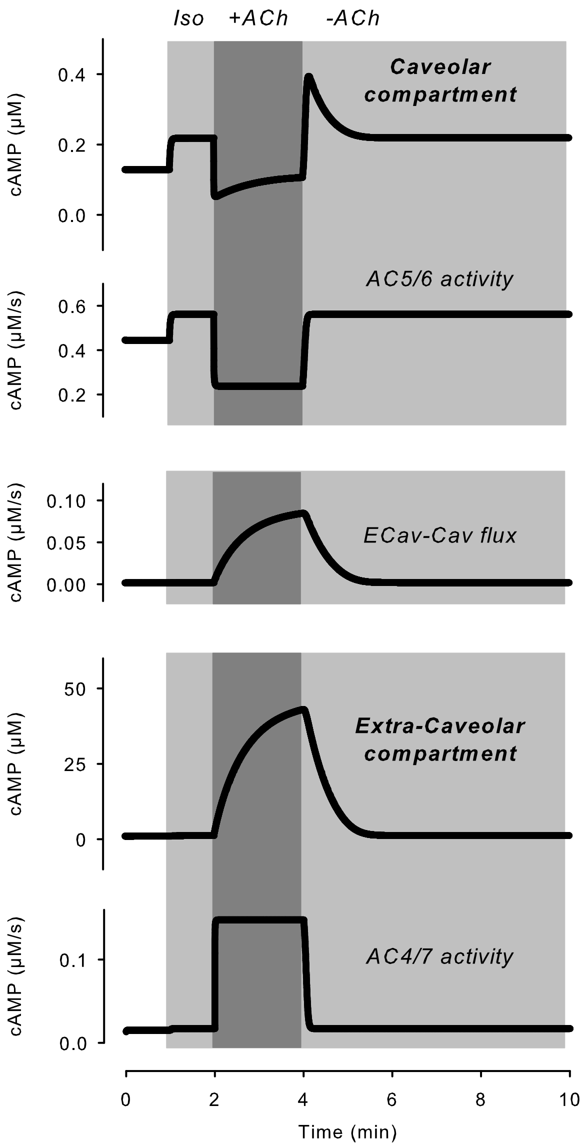

Figure 3. Kinetics of changes in cAMP activity associated with muscarinic stimulatory response. *Changes in caveolar and extracaveolar cAMP production and concentration caused by transient exposure to acetylcholine (ACh) in the presence of a submaximally stimulating concentration of isoproterenol (Iso). Adapted from Iancu* et al.[46]

on which it is based. Therefore, it is imperative to continually evaluate the model using new experimental data. In this respect, the use of different cAMP biosensors should only improve our ability to generate more quantitative means of studying cell signaling. This should also facilitate our ability to expand the model to include other signaling pathways that regulate cAMP activity. For example, the current version of the model only contains the β_1 subtype of β-adrenergic receptor. This allows direct comparison of the model's output with experimental results obtained using guinea pig ventricular myocytes, which only express this subtype of βAR.[50,51] However, cardiac myocytes from most mammals also express a significant subpopulation (10-20%) of β_2ARs.[1] Furthermore, β_2ARs are able to produce compartmentalized cAMP-dependent responses because of their ability to couple to G_i as well as G_s signaling pathways.[1,52] Therefore, a systems biology approach to studying cAMP signaling may provide a useful means of investigating the diversity of response produced by many different types of receptors.

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Author for correspondence:
Robert D. Harvey
Department of Physiology and Biophysics
Case Western Reserve University
10900 Euclid Avenue
Cleveland, OH 44106-4970
USA

Tel: +1 216 368 5521
Fax: +1 216 368 3952
E-mail: rdh3@case.edu