β-Adrenergic- and muscarinic receptor-induced changes in cAMP activity in adult cardiac myocytes detected with FRET-based biosensor

Sunita Warrier,1 Andriy E. Belevych,1 Monica Ruse,1 Richard L. Eckert,1 Manuela Zaccolo,2 Tullio Pozzan,2 and Robert D. Harvey1

1Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio; and 2Venetian Institute of Molecular Medicine, Padua, Italy

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CAMP is an important second messenger involved in regulating a variety of activities in virtually all eukaryotic cells. In cardiac muscle, cAMP plays a key role in regulating both electrical and mechanical activity through the activation of the cAMP-dependent protein kinase (PKA). cAMP signaling pathways in the heart are commonly studied by monitoring the phosphorylation state of target proteins or functional responses associated with the phosphorylation of these target proteins. Such studies have clearly demonstrated the complexity of cAMP-dependent responses. Much attention has focused on the fact that although multiple G protein-coupled receptors can regulate cAMP accumulation, the resulting functional responses are quite diverse. For example, in cardiac myocytes, β-adrenergic receptor (βAR) stimulation typically stimulates cAMP production, whereas M2-muscarinic receptor activation can produce both inhibitory and stimulatory responses, presumably due to biphasic changes in cAMP levels (6, 15). This has led to the hypothesis that different receptors can generate unique spatial as well as temporal patterns of cAMP production.

The ability to measure cAMP accumulation has been an important tool for understanding the signaling mechanisms underlying cAMP-dependent responses. However, earlier studies have relied on techniques that limit both the spatial and temporal resolution of changes in cellular cAMP levels. More recently, a variety of biosensors have been developed that can monitor real-time changes in cAMP activity in living cells. These biological probes are based on modifications of proteins or protein complexes such as cyclic nucleotide-gated (CNG) ion channels or PKA.

The first cAMP biosensor took advantage of the change in fluorescence resonance energy transfer (FRET) that occurs when cAMP levels increase, resulting in the dissociation of catalytic and regulatory subunits of PKA tagged with appropriate fluorescent dyes (1). However, the use of this probe required it to be microinjected into cells. Goaillard et al. (5) demonstrated that it is feasible to dialyze this probe into cardiac myocytes through patch-clamp pipettes. However, difficulties with obtaining sufficient quantities of the probe and problems with diffusion of such a large protein complex remain an impediment to the routine use of this approach.

More recently, a genetically encoded version of this biosensor was created by linking the type II regulatory and catalytic subunits of PKA to cyan (CFP) and yellow fluorescent protein (YFP), respectively. Plasmids encoding each subunit can be expressed in cells using standard transfection techniques (20). This results in targeting of the cAMP biosensor to the same locations where endogenous PKA is found within the cell. This approach has been used to study cAMP responses in neonatal cardiac myocytes (11, 20). However, transfection is not an efficient means of expressing recombinant proteins in some cell types, especially adult cardiac myocytes.

The use of adenoviruses as vectors has proved to be a more reliable way of expressing recombinant proteins in cardiac myocytes (8). In fact, this approach has been used to express modified CNG channels in adult cardiac myocytes (13). These are nonselective cation channels gated by cAMP or cGMP. The mutants created have a higher sensitivity and selectivity for...
cAMP (12). Increases in cAMP activity can be detected with the use of electrophysiological techniques to monitor changes in the membrane conductance due to activation of these channels. However, because these CNG channels only sense the subsarcolemmal environment, they may be unable to provide information about what is happening to cAMP activity in other locations in the cell, such as where PKA, the primary effector responsible for functional responses, is located.

The present study describes a method utilizing multiple adenoviruses to express the genetically encoded PKA-based cAMP biosensor in adult ventricular myocytes. We then test the hypothesis that the biosensor introduced using this method is sensitive enough to respond to β-adrenergic stimulation over a physiologically relevant range of agonist concentrations and that it can detect muscarinic inhibition and as well as muscarinic facilitation of those β-adrenergic responses.

METHODS

Preparation of adenoviruses. Recombinant adenoviruses encoding enhanced CFP attached to the type II PKA regulatory subunit (RII-CFP) and enhanced YFP attached to the PKA catalytic subunit (C-YFP) were developed using the Clontech Adeno-X expression system (BD Biosciences), which is based on the procedure developed by Mizuguchi and Kay (9, 10). To generate an adenovirus expressing RII-CFP (Ad-RII-CFP), the insert was subcloned from a pcDNA3-RII-CFP vector (20) into the Not1-Xba1 sites of a pBluescript II (KS) vector. From this construct, the insert was then released by digestion with Not1 and Kpn1 restriction enzymes and ligated into the unique sites in the Adeno-X pShuttle vector. The expression cassette was then excised and ligated into the unique PI-SceI and I-CeuI restriction sites in the Adeno-X viral DNA. The recombinant adenoviral DNA plasmid was linearized with PacI restriction enzyme and transfected into human embryonic kidney (HEK)-293 cells to produce recombinant adenovirus. Initially the same procedure was followed to generate an adenovirus expressing C-YFP (Ad-C-YFP). However, it was found that the overexpression of the catalytic subunit prevented the formation of the adenovirus in HEK-293 cells. To circumvent this problem, the insert for C-YFP was subcloned from the pShuttle vector into the Xba1 site of the Adeno-X pShuttle vector, where its expression would be under the control of a tetracycline response element. C-YFP transcription then required a tetracycline-controlled transactivator (TA), which was expressed using a separate adenovirus (Ad-TA). Because we used an Adeno X Tet-off system, the binding of the transactivator to the tetracycline response element turned on recombinant protein expression in the absence of tetracycline. Therefore, all solutions used were tetracycline free. Viruses were amplified in HEK-293 cells, purified by CsCl gradient centrifugation, and the titers solutions used were tetracycline free. Viruses were amplified in HEK-293 cells, purified by CsCl gradient centrifugation, and the titers solutions used were tetracycline free. Viruses were amplified in HEK-293 cells, purified by CsCl gradient centrifugation, and the titers solutions used were tetracycline free. Viruses were amplified in HEK-293 cells, purified by CsCl gradient centrifugation, and the titers solutions used were tetracycline free. Viruses were amplified in HEK-293 cells, purified by CsCl gradient centrifugation, and the titers solutions used were tetracycline free. Viruses were amplified in HEK-293 cells, purified by CsCl gradient centrifugation, and the titers solutions used were tetracycline free.
RESULTS

Subcellular localization of the cAMP biosensor. Figure 1 shows an example of an adult guinea pig ventricular myocyte expressing both recombinant PKA subunits. Both RII-CFP and C-YFP were expressed in a striated pattern with some diffuse distribution throughout the rest of the cell. We characterized this striated pattern further by comparing the intensity profile of the fluorescence and transmitted light of a given region within the cell. The profile of the sarcomere light and dark bands obtained from the transmitted light image parallels the fluorescence intensity profiles of CFP and YFP. The slight shift in register between transmitted light and the fluorescence from the labeled subunits is most likely a chromatic artifact (5). The distance between the striations was calculated to be 1.84 μM (SD 0.037; n = 6), which corresponds to the resting sarcomere length in guinea pig ventricular myocytes (14).

The overlapping RII-CFP and C-YFP expression pattern is also consistent with the idea that under basal conditions, when cAMP levels are low, these two subunits are bound to one another. Because of their close proximity, excitation of CFP is expected to result in FRET, resulting in YFP fluorescence. Increasing cAMP levels leads to dissociation of PKA regulatory and catalytic subunits (4, 16), which should then result in a loss of FRET. Changes in the ratio of CFP to YFP fluorescence emission intensity are the basis for the use of this probe as a biosensor for cAMP activity. In unstimulated cells, the ratio of fluorescence at 480 and 535 nm ranged from 1.7 to 2.8 with a mean value of 2.3 (SD 0.25; n = 65). The variability most likely reflects differences in the relative level of expression of either subunit. Therefore, changes in the fluorescence ratio observed on exposure to agonists were measured relative to the baseline value in each cell, as described previously (20).

Responses to phosphodiesterase inhibition. A consequence of the balance between cAMP synthesis and degradation that exists under basal conditions is that cAMP-dependent responses can be elicited by either stimulating adenylyl cyclase activity or inhibiting phosphodiesterase activity. This is supported by that fact that nonspecific phosphodiesterase inhibitors such as IBMX are capable of producing cAMP-dependent functional responses that are similar in magnitude to those produced by β-adrenergic receptor stimulation alone (17). Consistent with this idea, we found that exposure to IBMX (Fig. 2) caused a reversible change in the FRET response. The average magnitude of the response to 100 μM IBMX was a 5.9% (SD 1.75; n = 3) increase in the CFP/YFP fluorescence ratio.

Fig. 1. Adenovirus-mediated expression of protein kinase A (PKA)-based biosensor. A: adult ventricular myocyte infected with viruses expressing PKA type II regulatory subunit tagged with cyan fluorescent protein (RII-CFP), PKA catalytic subunit tagged with yellow fluorescent protein (C-YFP), and tetracycline response element transactivator (TA). CFP (1), YFP (2), and transmitted light (3) images (see boxes under panels 1–3). B: intensity profiles for CFP emission (436 nm excitation), YFP emission (500 nm excitation), and transmitted light measured across the highlighted region (3 × 14 μm) in each panel.

Fig. 2. Changes in cAMP activity produced by phosphodiesterase inhibition. A: effect of 100 μM IBMX on the time course of the normalized CFP/YFP emission intensity ratio (ΔRRR) recorded once every 25 s. The changes in the normalized CFP and YFP emission intensities are shown below. B: pseudocolor images representing CFP/YFP emission ratios recorded under control conditions (1), during exposure to 100 μM IBMX (2), and after washout of IBMX (3).
Responses to βAR activation. Next, we tested the ability of the biosensor to detect increases in cAMP production caused by βAR stimulation of adenyl cyclase activity. As shown in Fig. 3, exposure to a maximally stimulating concentration of the β-adrenergic agonist Iso (1 μM) produced the expected change in the CFP/YFP fluorescence ratio. On average the steady-state change was 4.9% (SD 2.73; n = 7) over baseline. To determine whether the magnitude of this response was due to saturation of cAMP production or saturation of the probe, myocytes were subsequently exposed to 100 μM IBMX in the continued presence of Iso. The addition of IBMX increased the fluorescence ratio to 5.2% (SD 2.65). This small but significant (P = 0.029) increase suggests that the effect produced by maximal βAR activation might not have saturated the response of the probe, which is consistent with what was found in neonatal myocytes (20). However, the magnitude of the response to subsequent addition of IBMX was less than might have been expected (20). This suggests that whereas the change in cAMP produced by high levels of βAR stimulation may not have saturated the PKA-based biosensor, such responses are likely to be near the upper limit of detection by this probe.

The utility of biosensors such as the one being evaluated in the present study relies on their ability to detect responses to physiologically relevant levels of receptor activation. Therefore, we next examined the response to βAR stimulation over a wide range of agonist concentrations. Most responses to Iso were stable (see Fig. 4A), although the effect of 0.3 nM Iso was transient in 2 of 11 cells. The concentration dependence of the Iso response is illustrated in Fig. 4B. In these experiments, the response to various concentrations of Iso was compared with the magnitude of the response to 1 μM Iso in the same cell. It was determined that the threshold concentration for eliciting changes in cAMP activity was ~0.1 nM Iso and maximal responses were produced by concentrations >3 nM. The concentration of Iso producing a half-maximal response was 0.5 nM, and the Hill coefficient of the relationship was 1.9.

To determine how the sensitivity of the biosensor compares to the sensitivity of cAMP-dependent functional responses, we examined the effect of Iso on L-type Ca\(^{2+}\) channel activity in myocytes expressing both RII-CFP and C-YFP (Fig. 4C). These experiments were also conducted to determine whether such functional responses might be disrupted due to exogenous PKA regulatory subunits buffering cAMP or exogenous catalytic subunit activity preempting PKA-dependent responses. However, this did not appear to be the case. We found that 1 nM Iso increased the Ca\(^{2+}\) current to a level that was 75.3% (SD 35.07; n = 6) of that produced by 1 μM Iso in the same cells. This is not significantly different from the relative magnitude of change in cAMP activity produced by the same concentration of Iso. Exposure to 1 nM Iso produced a change in cAMP that was 76.6% (SD 43.52; n = 9) of that produced by 1 μM Iso in the same cells. Furthermore, 1 μM Iso increased the Ca\(^{2+}\) current by 223% (SD 115.48; n = 6) over baseline.

Responses to muscarinic receptor activation. In addition to demonstrating that the biosensor is sensitive enough to detect changes in cAMP activity produced by physiologically relevant levels of βAR activation, it would also be important to demonstrate whether or not it can be used to detect changes in cAMP activity associated with activation of signaling pathways that modulate β-adrenergic responses. To address this question, we studied the effects of ACh, a muscarinic receptor agonist, on cAMP activity in the absence and presence of various levels of βAR activation. ACh significantly decreased the fluorescence ratio observed in the presence of maximally stimulating concentrations of Iso (Fig. 5). Exposure to 200 nM Iso increased the fluorescence ratio 5.1% (SD 1.66) over baseline, and subsequent addition of 30 μM ACh decreased this value to 2.6% (SD 1.14; n = 3), which represents a 51%
Fig. 4. Concentration-dependent changes in cAMP and L-type Ca\(^{2+}\) channel activity produced by βAR stimulation. A: effect of 0.3 nM Iso, followed by 1 μM Iso on the time course of changes in ΔR/Ro recorded once every 20 s. B: changes in CFP/YFP emission intensity ratio produced by different concentrations of Iso. The cAMP response to each concentration of Iso is presented as a percentage of ΔR/Ro produced by 1 μM Iso in the same cell. Data points were fit by the following equation: cAMP response = 100/(1 + exp[(Iso - EC\(_{50}\))/slope]), where EC\(_{50}\) = 0.5 nM and slope = 1.9. The L-type Ca\(^{2+}\) current response to 1 nM Iso was normalized to the effect produced by 1 μM Iso in the same cell. The number of cells analyzed is indicated adjacent to each data point. C: time course of changes in the absolute magnitude of the peak inward Ca\(^{2+}\) current recorded during depolarizing voltage clamp steps to 0 mV once every 20 s under control conditions and after exposure to 1 nM and then 1 μM Iso. Inset, sample current traces recorded at time points indicated in C.

The present study demonstrates that a multiple adenovirus approach can be used to introduce the PKA-based cAMP biosensor into adult ventricular myocytes. It was also established that the probe expressed in this manner is able to detect changes in cAMP activity produced by physiologically relevant levels of βAR stimulation without disrupting PKA-dependent regulation of functional ion channel responses. Furthermore, this probe is capable of detecting changes in cAMP activity produced by muscarinic receptor activation. Not only was it able to detect muscarinic inhibition of β-adrenergic induced changes in cAMP activity, this biosensor was also sensitive enough to demonstrate directly for the first time that termination of muscarinic receptor stimulation causes a transient rebound increase in cAMP activity.

The subcellular expression pattern of the probe introduced using adenoviruses (see Fig. 1) is consistent with the expression pattern observed in neonatal cardiac myocytes, where RII-CFP and C-YFP were found near the Z line of the sarcomere (20). This is also consistent with the expression pattern of endogenous PKA, which is determined by the interaction of the RII subunit with A kinase anchoring proteins (19). A kinase anchoring proteins, in turn, interact with other myocyte proteins, such as L-type Ca\(^{2+}\) channels and ryanodine receptors, which are concentrated in or near the T tubule network (7). Although targeting the biosensor to specific subcellular locations limits its ability to detect changes in cAMP activity throughout the intracellular compartment, expression in the same location as endogenous PKA is likely to improve the correlation between cAMP responses detected by this probe and functional responses mediated by PKA.

The sensitivity of this biosensor to changes in cAMP is also a function of its binding affinity for cAMP. The actual affinity of the PKA RII subunit for cAMP is ~100–300 nM (11). Again, because the probe is based on PKA, the primary effector for cAMP-dependent responses in ventricular myocytes, one would expect it to respond to levels of β-adrenergic stimulation that produce functional responses. Consistent with this idea, we found that the apparent sensitivity of the probe to βAR stimulation closely correlated with the sensitivity of L-type Ca\(^{2+}\) channels in cells expressing the same probe. It is also important to note that expression of the probe did not appear to adversely affect PKA-dependent functional responses. In previous studies (2, 3), we have determined that βAR activation stimulates the L-type Ca\(^{2+}\) current in adult guinea pig ventricular myocytes with an EC\(_{50}\) of ~1 nM and that maximally stimulating concentrations of agonist increases the magnitude of the current by 200–250% over baseline. This is consistent with the sensitivity and the magnitude of the L-type Ca\(^{2+}\) channel response observed in cells expressing the exogenous PKA-based biosensor described in the present study.

In the absence of βAR stimulation, exposure to ACh had no obvious effect on basal cAMP activity. There was no perceptible change in the fluorescence ratio recorded on exposure to and after subsequent washout of 10 μM ACh alone (n = 4; Fig. 6A). However, exposure to and subsequent washout of 10 μM ACh in the presence of a threshold concentration of Iso (0.1 nM) produced a transient increase in cAMP activity that peaked within the first minute of washing out ACh and then subsided back to baseline (Fig. 6B). The peak fluorescence ratio measured in the presence of 0.1 nM Iso after ACh washout was 1.9% (SD 1.63; n = 3) above that measured in the presence of 0.1 nM Iso just before exposure to ACh in the same cells.

**DISCUSSION**

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CNG ion channels have also been used to detect cAMP changes in adult rat ventricular myocytes. However, they appear to exhibit a distinctly different sensitivity to agonist stimulation. Rochais et al. (13) demonstrated that the CNG channel expressed in cardiac myocytes responds only to concentrations of Iso $10 \text{nM}$. This is 100-fold less sensitive than the PKA-based probe used in the present study (see Fig. 4). Furthermore, the CNG channel was unable to detect changes in cAMP activity in myocytes exposed to concentrations of IBMX ($100 \text{nM}$) expected to maximally inhibit PDE activity (13). Again, this is distinctly different from the response we observed, where the same concentration of IBMX produced a robust change in the fluorescence ratio due to cAMP activation of the PKA-based probe (see Fig. 2). The most likely explanation for the difference in the apparent sensitivity of the two biosensors has to do with the location within the cell where they are expressed and/or their affinity for binding cAMP. Other than being in the plasma membrane, the exact location that CNG channels are found when expressed in cardiac myocytes is not known. However, their low sensitivity to agonist induced responses suggests that they may be located in a domain that is separated from the source of cAMP generation. Furthermore, these modified CNG channels have a significantly lower affinity ($1–10 \text{ nM}$) for cAMP (12). Together, these factors are likely to affect the ability of CNG channels to detect small changes in cytosolic cAMP activity in cardiac ventricular myocytes.

The fact that the cAMP biosensor used in the present study produced reversible responses indicates that reassociation of the recombinant PKA subunits takes place when cAMP levels return to baseline and that mixing of endogenous and recombinant subunits does not occur to any significant degree. More recent studies (18) suggest that the consistent reversibility of these responses might be because cAMP binding does not necessarily result in complete dissociation of regulatory and catalytic subunits, especially with type II PKA. The reversibility of the biosensor’s response to cAMP made it feasible to use this probe to detect the more complex temporal responses to muscarinic receptor activation.

In the present study, we found that exposure to ACh produced a decrease in cAMP activity but only after cAMP levels had first been elevated by βAR activation (see Fig. 5). Furthermore, washout of ACh produced a transient increase in cAMP activity but only in the presence of a submaximally stimulating concentration of Iso (see Fig. 6B). There was no rebound increase in cAMP on washout of ACh in the absence of Iso (Fig. 6A) or in the presence of a maximally stimulating concentration of Iso (see Fig. 5). These results are consistent with the idea that the effect that muscarinic receptor activation has on cAMP-dependent functional responses in cardiac myocytes is actually much more complex than simply inhibition of...
βAR-induced cAMP production. The net response is a balance between inhibitory and stimulatory mechanisms (6). During muscarinic receptor activation, the dominant effect is inhibition of cAMP responses due to inhibition of adenyl cyclase type 5 and/or 6 by the α-subunit of Gi. However, muscarinic receptor activation can produce a stimulatory response that is consistent with Gίβγ-subunits activating adenyl cyclase type 4 and/or 7 (3). This stimulatory effect can be observed as a transient rebound response on washout of ACh. The fact that this type of rebound response in ventricular myocytes can only be observed in the presence of a submaximally stimulating AR agonist supports the idea that such effects involve the stimulation of cAMP production. However, because of the transient nature of the response, it has not been possible to prove this hypothesis by directly measuring changes in cAMP activity until now.

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