Systems analysis of PKA-mediated phosphorylation gradients in live cardiac myocytes

Jeffrey J. Saucerman, Jin Zhang, Jody C. Martin, Lili X. Peng, Antine E. Stenbit, Roger Y. Tsien, and Andrew D. McCulloch

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Compartments and dynamics of cAMP and PKA signaling are important determinants of specificity among cAMP’s myriad cellular roles. Both cardiac inotropy and the progression of heart disease are affected by spatiotemporal variations in cAMP/PKA signaling, yet the dynamic patterns of PKA-mediated phosphorylation that influence differential responses to agonists have not been characterized. We performed live-cell imaging and systems modeling of PKA-mediated phosphorylation in neonatal cardiac myocytes in response to G-protein coupled receptor stimuli and UV photolysis of “caged” cAMP. cAMP accumulation was rate-limiting in PKA-mediated phosphorylation downstream of the β-adrenergic receptor. Prostaglandin E2 stimulated higher PKA activity in the cytosol than at the sarcolemma, whereas isoproterenol triggered faster sarcolemmal responses than cytosolic, likely due to restricted cAMP diffusion from submembrane compartments. Localized UV photolysis of caged cAMP triggered gradients of PKA-mediated phosphorylation, enhanced by phosphodiesterase activity and PKA-mediated buffering of cAMP. These findings indicate that combining live-cell FRET imaging and mechanistic computational models can provide quantitative understanding of spatiotemporal signaling.

Intracellular signaling through cAMP and its cAMP-dependent protein kinase (PKA) mediates hundreds of distinct cellular functions. Compartmentation and dynamics of cAMP/PKA signaling are gaining increasing acceptance as general mechanisms used to maintain signaling specificity in a context-dependent manner. In the heart, compartmentation appears to contribute to functional differences between β1- and β2-adrenergic signaling and other stimuli that increase cAMP and thus have important consequences for understanding the role of β-adrenergic signaling in the development and treatment of heart failure (1, 2). Short-term β-adrenergic signaling increases heart contractility (3), whereas prolonged exposure to β1-adrenergic agonists induces apoptosis (4). Recent live-cell imaging and electrophysiologic approaches are now providing direct measurements of compartmentation (5, 6) and cAMP signaling dynamics (7–10) in intact cells, and our increasing molecular understanding provides numerous candidate molecular mechanisms for compartmentation including caveolae (2, 11), β-arrestins (12, 13), and A-kinase anchoring proteins (AKAPs) (14, 15). A future challenge will be to understand quantitatively how these molecular signaling mechanisms orchestrate such precise context-dependent signaling in the cell.

Here, we integrate fluorescent reporters of PKA-mediated phosphorylation (16) and mechanistic computational models to characterize rate-limiting biochemical reactions in β-adrenergic signaling and identify signaling mechanisms contributing to asynchronous and spatially heterogeneous PKA-mediated phosphorylation. This combination of techniques reveals restricted diffusion, phosphodiesterase (PDE)-mediated cAMP degrada-
published as supporting information on the PNAS web site). Model predictions of β-AR and G-protein kinase responses to propranolol were much faster than the AKAR2 response to propranolol (hydrolysis of activated GsGTP with t1/2 = 2 s; Fig. 2C), showing that upstream mechanisms are unlikely to contribute. A rate-limiting role for protein phosphatases (PPs) could be probed experimentally by rapid inhibition of PKA. However, 8–8-(4-chlorophenylthio)-cAMPs [8–8-(CPT)-cAMPs], a stimulatory analog of the most membrane-permeable PKA inhibitor (Rp-8-CPT-cAMPS), exhibited a t1/2 of 6.7 min (see Fig. 7, which is published as supporting information on the PNAS web site), suggesting that sufficiently rapid PKA inhibition (seconds) is currently infeasible. Fifty percent inhibition of the model's PDE activity increased AKAR dephosphorylation t1/2 by 265%, whereas 50% inhibition of PP activity increased t1/2 by only 22%, showing that PDE activity may significantly affect AKAR2's response to propranolol. Consistent with this observation, simulations of 50 and 200 μM DMNB-cAMP uncaging (15 s UV; Fig. 2D) showed similar magnitudes of AKAR2 dephosphorylation yet different dephosphorylation rates, suggesting that the time required to degrade PKA-saturating levels of cAMP may delay AKAR2 dephosphorylation. This model-guided hypothesis was validated experimentally by comparing responses with 50 μM (t1/2 = 99 ± 4 s, n = 7) and 200 μM DMNB-cAMP (t1/2 = 162 ± 12 s, n = 5, P < 0.05; Fig. 2E).

To gain insight into possible differences in PKA activity patterns at the membrane compared with the cytosol, we used a variant of AKAR2, plasma membrane-targeted AKAR2 (pmAKAR2), in which several lysines and a lipid modification domain from the small G protein Rho (KKKKSKGCLVL) were incorporated at the C-terminal end of AKAR2. pmAKAR2 expressed in neonatal cardiac myocytes was targeted to the plasma membrane with some localization nuclear localization (Fig. 3D; see also Fig. 8, which is published as supporting information on the PNAS web site). Prostaglandin E1 (PGE1) has been shown to elevate soluble cAMP in vitro yet not activate particulate PKA or have a positive inotropic effect on the heart (20). We found that in response to 1 μM PGE1, cells expressing the cytosolic AKAR2 had larger and slower emission ratio changes than cells expressing the pmAKAR2 (R/R0 = 1.22 ± 0.01, n = 3, vs. 1.10 ± 0.01, n = 4, P < 0.05; Δt1/2 = 33 ± 3 vs. 23 ± 1, P < 0.05; Fig. 3B). This effect was not due to a possible difference in local concentration of AKAR2, because 0.1 μM Iso exhibited similar magnitude responses for AKAR2 and pmAKAR2 (R/R0 = 1.10 ± 0.01, n = 9). However, the Iso-induced response of AKAR2 was delayed compared with pmAKAR2 (t1/2 = 33 ± 1 s vs. 22 ± 1 s, P < 0.05; Fig. 3C and see also Fig. 9, which is published as supporting information on the PNAS web site). This delay appeared unlikely to be mediated by PKA catalytic subunit diffusion from membrane to cytosol, because PGE1 stimulation was able to produce large increases in cytosolic phosphorylation without a corresponding increase in membrane activity. Neither response magnitude nor response time to Iso correlated with AKAR2 or pmAKAR2 expression level (Fig. 9). Thus, we hypothesized that an apparent restricted diffusion of cAMP from the membrane to the cytosol may explain the observed delay in cytosolic AKAR2 emission ratio compared with pmAKAR2. We examined this hypothesis in a simple, two-compartment extension of our above computational model (see Methods), in which CAMP is generated in a membrane compartment and can either activate membrane-bound PKA or diffuse to the cytosol to activate cytosolic PKA. With this model, an apparent diffusion coefficient DcAMP = 2 μm2/s produced a delay between membrane and cytosolic phosphorylation similar to that
seen experimentally (Fig. 3 D and E). This diffusion coefficient is much smaller than the previously measured $D_{\text{cAMP}} = 270 \, \mu m^2/s$ in the cytosol of frog olfactory cilia (21). We found that this membrane–cytosol phosphorylation delay was very sensitive to changes in the model parameter $D_{\text{cAMP}}$ (Fig. 3F; see also Fig. 10, which is published as supporting information on the PNAS web site), with apparent diffusion coefficients lower or higher than $2 \, \mu m^2/s$ producing much larger or smaller phosphorylation delays than observed experimentally. Phosphorylation delays were insensitive to varied membrane targeting of PDE except at very low diffusion rates ($D_{\text{cAMP}} = 0.1 \, \mu m^2/s$) (see Fig. 11, which is published as supporting information on the PNAS web site).

**Imaging PKA-Mediated Phosphorylation Gradients.** To test whether local cAMP can generate gradients of PKA-mediated phosphorylation in a single cell, we modified our DMNB–cAMP uncaging technique by limiting UV exposure to a defined portion of the cell. Local activation of signaling complements the previous approach of genetically targeting the FRET sensor. Fig. 4A Bottom depicts a representative myocyte, where the white circle denotes the local UV illumination region and the ends of the cell proximal (p) and distal (d) to this activation site are labeled. In each cell, a global 5-s UV exposure was given to demonstrate functional response to DMNB–cAMP throughout the cell [Fig. 4A Right Top (image 2)]. Once the AKAR2 emission ratio returned to baseline, a local 5-s UV exposure was given only to the proximal end of the cell. Local uncaging of DMNB–cAMP produced a gradient of PKA-mediated phosphorylation with a delay in peak response at the distal compared with the proximal end [Fig. 4A Right Middle (image 4)]. The approximate time course of these responses and results of subsequent exposure to 0.1 $\mu M$ Iso are shown in Fig. 4B. We quantified the phosphorylation propagation time as $\Delta t_{\text{MAX}} = t_{\text{MAX}} - t_{\text{MIN}}$ and the magnitude of the phosphorylation gradients as $\Delta R_p/\Delta R_d = (R_{\text{MAX}} - R_{\text{MIN}})/(R_{\text{MAX}} - R_{\text{DMIN}})$. Quantitation of phosphorylation gradient magnitude as $\Delta R_p/\Delta R_d$, similar to that used in ref. 6, facilitates comparison of compartmentation with other experiments and computational models. We observed significantly larger propagation delays [$\Delta t_{\text{MAX}} = 20 \pm 1 \, s$, $n = 5$ (local uncaging)] vs. $1 \pm 0.3 \, s$, $n = 5$ (global uncaging), $P < 0.05$] and phosphorylation gradients due to local uncaging compared with global uncaging [$\Delta R_p/\Delta R_d = 4.5 \pm 0.4$, $n = 8$ (local uncaging)] vs. $1.3 \pm 0.1$, $n = 8$ (global uncaging), $P < 0.05$; Fig. 4C].

**Modeling PKA-Mediated Phosphorylation Gradients.** To examine signaling mechanisms contributing to such PKA-mediated phosphorylation gradients, we created a 2D version of our model of cAMP signaling by using geometry from individual myocytes (see Methods). By using an apparent diffusion coefficient $D_{\text{cAMP}} = 200 \, \mu m^2/s$, approximately that measured in the cytosol of simple cells (21), we simulated a local 5-s UV exposure as done in the imaging experiments above. As seen from the phosphorylation images (Fig. 5A), time courses (Fig. 5B), and quantitative comparison of phosphorylation propagation time [$\Delta t_{\text{MAX}} = 17 \, s$]) and gradient magnitude [$\Delta R_p/\Delta R_d = 5.0$].
PKA-mediated phosphorylation gradients in a 2D computational model of local DMNB–cAMP uncaging ($D_{\text{CAMP}} = 200 \, \mu m^2/s$). (A) AKAR phosphorylation images after global uncaging and then local uncaging (5-s UV activation restricted to red circle, timepoints indicated in B), p, proximal; d, distal. (B) Emission ratio time course from regions proximal and distal to the local uncaging site. (C) Model validation of propagation time ($\Delta t_{\text{MAX}}$) and gradient magnitude ($\Delta R_p/\Delta R_D$) for local DMNB–cAMP uncaging.

5C), our model predicted similar dynamics and phosphorylation gradients to those seen experimentally. These similarities between model and experiment suggested that the model may capture many of the essential mechanisms responsible for the observed PKA-mediated phosphorylation gradients.

We next used our spatial model to test the role of individual signaling mechanisms toward the generation of PKA-mediated phosphorylation gradients. We began by examining the sensitivity of variation in $D_{\text{CAMP}}$ on phosphorylation gradient magnitude $\Delta R_p/\Delta R_D$ (Fig. 64) and propagation time $\Delta t_{\text{MAX}}$ (Fig. 6b) after local uncaging. Although a wide range of diffusion coefficients could produce phosphorylation gradients and propagation delays, only $D_{\text{CAMP}}$ near 200 $\mu m^2/s$ could produce both propagation delays and gradients consistent with the experimental measurements. Increasing $D_{\text{CAMP}}$ to an unrealistically high 100 $\mu m^2/s$ in the model completely eliminated compartmentation (Fig. 6b), verifying a critical role for nonrapid diffusion in creating signaling gradients.

Consistent with many other studies of cAMP compartmentation (5–7), our model simulations predicted inhibition of PDEs to greatly decrease PKA-mediated phosphorylation gradients ($\Delta R_p/\Delta R_D$ from 5.0 to 1.0; Fig. 6b). Model analysis suggested two distinct roles for PDEs in phosphorylation gradients: (1) restricting basal cAMP and (2) directly enhancing signaling gradients by providing a “sink” for cAMP separated from the “source” of cAMP. PDE inhibition with 1 mM 3-isobutyl-methylxanthine (IBMX) raised basal cAMP considerably in both model and experiment due to high basal adenyl cyclase (AC) activity, such that a direct role for PDEs in enhancing signaling gradients could not be observed. However, we found that simultaneous inhibition of AC and PDE in the model produced an intermediate compartmentation magnitude ($\Delta R_p/\Delta R_D = 3.2$), which could be attributed to the direct role for PDEs in compartmentation independent of basal cAMP. Consistent with previous studies using P-site AC inhibitors in cardiac myocytes (22), the P-site AC inhibitor MDL-12,330A was unable to inhibit basal AC activity (data not shown), so we have not yet validated this hypothesis experimentally.

Buffered cAMP (PKA-bound cAMP protected from PDE catalysis) comprises a significant proportion of total basal cAMP (23, 24) and has been predicted to stabilize basal cAMP near a region of maximal PKA sensitivity (25). In our simulations, eliminating cAMP buffering reduced PKA-mediated phosphorylation gradients by 46% (Fig. 6b), suggesting that cAMP buffering may contribute significantly to cAMP/PKA compartmentation. We had assumed that the relevant signaling proteins diffused much more slowly than cAMP in the 2D model. By relaxing this assumption for PKA and PDE, we tested the contributions of PKA anchoring and PDE scaffolding, respectively, to PKA-mediated phosphorylation gradients. An apparent diffusion coefficient $D_{\text{PKA}} = 30 \, \mu m^2/s$ reduced gradients somewhat ($\Delta R_p/\Delta R_D = 3.8$), whereas $D_{\text{PDE}} = 30 \, \mu m^2/s$ or $D_{\text{AKAR}} = 30 \, \mu m^2/s$ did not appreciably reduce phosphorylation gradient magnitude ($\Delta R_p/\Delta R_D = 5.1$ or 4.6).

Discussion

Integrating live-cell imaging and mechanistic computational models may facilitate quantitative understanding of how dynamics and compartmentation contribute to the function of cell-signaling networks. Here, we used a FRET reporter of PKA activity, AKAR2, and computational models to investigate mechanisms underlying PKA signaling dynamics and compartmentation in the neonatal cardiac myocyte. Combining experimental perturbations and model analysis allowed estimation of kinetics for G activation, cAMP accumulation, and PKA-mediated phosphorylation, revealing cAMP accumulation near PKA as a rate-limiting step in response to β-AR agonists in this system. AKAR2 dephosphorylation rates, consistent with measured dephosphorylation rates of troponin I and C-protein (26), revealed that saturation of PKA with CAMP can delay substrate dephosphorylation, indicating a capacity for cAMP synthesis greatly exceeding the requirements for PKA activation. This hypothesis is consistent with observed CAMP
“spill-over” from the particulate to the soluble fraction of cardiac myocyte homogenates (24, 27). However, Iso-stimulated phosphatase inhibition may also contribute to decreased dephosphorylation rates (25).

Compared with cells expressing the cytosolic PKA activity sensor AKAR2, response to PGE1 in cells expressing the membrane-tagged PKA activity sensor pmAKAR2 was markedly reduced, suggesting that PGE1 receptors are not well coupled functionally to PKA substrates at the plasma membrane. This finding is consistent with both functional data and molecular evidence suggesting that prostaglandin receptors may be excluded from caveolae of cardiac myocytes, where β-ARs and their downstream targets appear concentrated (2, 29). Iso caused similar increases in yellow/cyan emission ratio for both cytosolic and membrane-tagged PKA sensors, yet cytosolic responses to both PGE1 and Iso were delayed. Model analysis suggested that such phosphorylation asynchrony may arise from restricted cAMP diffusion between the plasma membrane and cytosol (9), a hypothesis consistent with faster cAMP accumulation at the plasma membrane than cytosol as reported in cell lines (9, 10). Simulated phosphorylation delays were much more sensitive to $D_{AMP}$ than to AC, PDE, or PKA expression levels. However, subcellular mechanisms responsible for such restricted diffusion are unclear. Although physical submembrane microdomains abound in cardiac myocytes, calcium diffuses from these microdomains to the cytosol of cardiac myocytes on a millisecond timescale (3). These data show that PKA signaling may generate both spatially heterogeneous and asynchronous phosphorylation signals.

Although we did not find striated patterns of PKA-mediated phosphorylation with AKAR2 or pmAKAR2 in response to uniform β-AR stimulation as reported for cAMP (5), we did observe gradients of PKA-mediated phosphorylation in response to local uncaging of DMNB–cAMP. This result confirms that cAMP compartmentation can facilitate gradients in PKA-mediated phosphorylation, a necessary step toward mediating functional diversity of PKA signaling. We probed the mechanistic requirements for the observed PKA-mediated phosphorylation gradients by using a computational model with 2D geometry from individual cardiac myocytes. Unrestricted phosphorylation with AKAR2 or pmAKAR2 in response to uniform AR stimulus. Cardiac myocytes produced gradients of PKA-mediated phosphorylation in response to local cAMP generation, direct evidence of compartmentation at the level of protein phosphorylation. These phosphorylation gradients were produced predominantly by restricted cAMP diffusion, PDE-mediated cAMP degradation, and PKA-mediated cAMP buffering.

Methods

Cell Culture. Neonatal cardiac myocytes were isolated from the ventricles of 1- to 2-day-old Sprague–Dawley rats by using the Neonmys isolation kit (Cellutron, Highland Park, NJ) and cultured on 35-mm glass-bottom dishes (MatTek Corp., Ashland, MA). Myocytes were transfected with AKAR2 (16, 17) 1 day after isolation by using FuGene6 transfection reagent (Roche Diagnostics, Indianapolis, IN) and imaged 2 days after transfection (~50% transfection efficiency). AKAR2 is a recombinant protein containing two β-AR stimulus. Cardiac myocytes produced gradients of PKA-mediated phosphorylation in response to local cAMP generation, direct evidence of compartmentation at the level of protein phosphorylation. These phosphorylation gradients were produced predominantly by restricted cAMP diffusion, PDE-mediated cAMP degradation, and PKA-mediated cAMP buffering.
imaging and kept at 35°C with a stage heater (Carel, Manheim, PA) during imaging. Myocytes were imaged on an Eclipse TE300 inverted microscope (Nikon, Tokyo, Japan) with a 60× PlanApo-chromatic objective. Cascade 512F CCD camera (Photometrics, Tucson, AZ), and MetaFluor software (Version 6.2; Universal Imaging Corporation, Downingtown, PA). Imaging was performed by using a 430/25-nm excitation filter (for CFP) and simultaneously recording CFP (470/30 nm) and YFP (535/30 nm) emissions with a DualView emission splitter (Chroma filters; Optical Insights, Santa Fe, NM). Images were acquired with 1-s exposure every 5 s.

Uncaging of DMNB–cAMP was performed by illumination with a 175-W xenon lamp (Sutter, Novato, CA) and a UV (360/40 nm) excitation filter and dichroic for the time specified. In some experiments, local uncaging was performed by adjusting the field diaphragm to a 40-μm-diameter spot. This technique was validated by using DMNB-caged fluorescein dried onto glass slides. UV exposure uncaged dried DMNB–fluorescein with apparent τ = 1.8 ± 0.1 s, n = 4, and DMNB-cAMP in cardiac myocytes with apparent τ = 3.1 ± 0.1 s, n = 6, as measured by AKAR2 emission ratio. Characterization of DMNB–cAMP membrane transport and uncaging kinetics is shown in Figs. 12 and 13, which are published as supporting information on the PNAS web site.

To obtain emission ratio time courses, YFP or CFP emission intensities for each image were averaged over a region of interest and background substracted, and the yellow/cyan emission ratio was calculated and normalized by the ratio before reagent application. For emission ratio images, a similar approach was used on a pixel-by-pixel basis with a 5 × 5-pixel median filter using MetaMorph software (Universal Imaging Corporation).

Statistical significance was determined at P < 0.05 by using Student’s t test. Data are expressed as mean ± SEM.

**Computational Models.** We have previously described and validated a mechanistic computational model of β-AR signaling in adult rat cardiac myocytes (25) and methods for model development (33). This model was used here with the following modifications and additions. Rate constants for AC catalysis (kAC = 0.025 s−1) and PDE concentration ([PDE Tot] = 0.014 μM) were adjusted to obtain kinetics more consistent with our measured response to Iso

Uncaging of DMNB–cAMP was incorporated by estimating effective DMNB–cAMP concentrations (6%) of total concentration, tested response to 5–200 μM DMNB–cAMP normalized by the subsequent response to 1 mM 3-isobutyl-methylxanthine) and cAMP uncaging rates (kPhot = 0.1 s−1 by varying UV exposure time) by using least-squares fits between experiments and the model (Figs. 12 and 13). AKAR phosphorylation rate constants were estimated from Kemptide (34), the substrate on which AKAR is based.

For some studies, a simple two-compartment version of this model was used, containing both membrane and cytosolic species for cAMP, PDE, PKA, phosphatase, and AKAR. Total concentrations of PDE, PKA, phosphatase, and AKAR were assumed similar in membrane and cytosolic compartments. The diffusive flux of cAMP was modeled as $J_{cAMP} = \frac{D_{cAMP}}{L} \cdot \frac{SA}{V_{CELL}} \cdot \left[L_{cAMP} - L_{cAMP,mem}\right]$. Geometric parameters were estimated from neonatal rat cardiac myocytes as follows: $SA = 4,000 \mu m^2$, $L = 2.5 \mu m$, $V_{CELL} = 12,500 \mu m^3$, and $V_{MEM} = 0.02 \cdot V_{CELL}$. (For model parameters, see Table 1.)

For simulation of local DMNB–cAMP uncaging, we developed a 2D version of our single-compartment model described above. To achieve geometrical variability, images of several neonatal myocytes were segmented to create grid-like meshes. Our base model contained 2,542 of the 1.5 × 1.5-μm elements, and we also confirmed numerical convergence by comparing with a higher resolution mesh containing 10,168 of the 0.75 × 0.75-μm elements. The numerical solution of the 2D model used a finite volume approach as described in ref. 35, whereas the compartmental models were solved by using LSODA. All models were implemented by using Virtual Cell software (36) and are freely available in the Virtual Cell environment for download or online use at www.nrcc.mb.uchc.edu/applications/published%.20.models.html.

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