Sustained β₁-Adrenergic Stimulation Modulates Cardiac Contractility by Ca²⁺/Calmodulin Kinase Signaling Pathway

Wang Wang, Weizhong Zhu, Shiqiang Wang, Dongmei Yang, Michael T. Crow, Rui-Ping Xiao, Heping Cheng

Abstract—A tenet of β₁-adrenergic receptor (β₁AR) signaling is that stimulation of the receptor activates the adenylate cyclase-cAMP-protein kinase A (PKA) pathway, resulting in positive inotropic and relaxant effects in the heart. However, recent studies have suggested the involvement of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) in β₁AR-stimulated cardiac apoptosis. In this study, we determined roles of CaMKII and PKA in sustained versus short-term β₁AR modulation of excitation-contraction (E-C) coupling in cardiac myocytes. Short-term (10-minute) and sustained (24-hour) β₁AR stimulation with norepinephrine similarly enhanced cell contraction and Ca²⁺ transients, in contrast to anticipated receptor desensitization. More importantly, the sustained responses were largely PKA-independent, and were sensitive to specific CaMKII inhibitors or adenoviral expression of a dominant-negative CaMKII mutant. Biochemical assays revealed that a progressive and persistent CaMKII activation was associated with a rapid desensitization of the cAMP/PKA signaling. Concomitantly, phosphorylation of phospholamban, an SR Ca²⁺ cycling regulatory protein, was shifted from its PKA site (Ser) to CaMKII site (Thr). Thus, β₁AR stimulation activates dual signaling pathways mediated by cAMP/PKA and CaMKII, the former undergoing desensitization and the latter exhibiting sensitization. This finding may bear important etiological and therapeutical ramifications in understanding β₁AR signaling in chronic heart failure. (Circ Res. 2004;95:000-000.)

Key Words: β₁-adrenergic receptor ▪ Ca²⁺/calmodulin-dependent protein kinase II ▪ cAMP-dependent protein kinase ▪ cardiac contractility ▪ phospholamban

As a prototypal member of G protein–coupled receptor (GPCR) superfamily, β₁-adrenergic receptor (β₁AR) plays a central role in sympathetic regulation of cardiac function. Stimulation of β₁AR by catecholamines induces robust chronotropic, inotropic, and relaxant effects via the Gs-adenylate cyclase-cAMP-protein kinase A (PKA) pathway. This signaling pathway is also thought to be responsible for other functions of β₁AR, such as regulation of metabolism, gene expression, cell growth, and apoptosis. However, sustained β₁AR activation under pathological conditions such as hypertension and congestive heart failure will result in downregulation and desensitization of β₁AR attributable to the negative feedback of this pathway. Moreover, we have recently shown that sustained (24-hour) β₁AR stimulation progressively activates Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), which is obligatory to cardiac apoptosis. Therefore, in addition to the classic cAMP/PKA pathway, chronic β₁AR stimulation under certain physiological and pathophysiological circumstances may evoke pathways other than cAMP/PKA. These lines of evidence raise the question whether β₁AR modulates cardiac contractility using different sets of signaling mechanisms in short-term versus prolonged receptor stimulation.

CaMKII is a widely expressed protein kinase that modulates various functions ranging from learning and memory of the nervous system, muscle contraction, cell secretion to gene expression. In the heart, CaMKIIδ is the predominant isoform and plays a pivotal role in regulating cardiac performance and remodeling such as myocyte hypertrophy, apoptosis, and heart failure. Furthermore, CaMKII modulates an array of key proteins involved in cardiac excitation-contraction (E-C) coupling and Ca²⁺ handling, such as the sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase (SERCA)
and its regulator, phospholamban (PLB), ryanodine receptor (RyR) Ca\textsuperscript{2+} release channels, and sarcosomal L-type Ca\textsuperscript{2+} channels (LCC).\textsuperscript{18-22} However, the involvement of CaMKII in β\textsubscript{1}AR modulation of myocardial contractility remains obscure.

The present study aimed at appraising roles of CaMKII and PKA in β\textsubscript{1}AR modulation of cardiac E-C coupling, with an emphasis on the signaling mechanism for the sustained β\textsubscript{1}AR stimulation. We found that both short-term and sustained β\textsubscript{1}AR stimulation are efficacious in mediating positive inotropic and relaxant effects in cardiac myocytes. Unlike the short-term β\textsubscript{1}AR stimulation, the sustained responses are mediated mainly by the CaMKII rather than the cAMP/PKA pathway. Furthermore, molecular integration of these two signaling pathways is mediated by dual site phosphorylation of key proteins involved in cardiac E-C coupling and its physiological regulation.

Materials and Methods

Isolation and Culture of Cardiac Myocytes
Cardiac myocytes were isolated from male Sprague-Dawley rat hearts using standard enzymatic technique as described previously.\textsuperscript{23} Freshly isolated myocytes were plated at a density of 0.5 to 1×10\textsuperscript{5}/cm\textsuperscript{2} in dishes precoated with 20 μg/mL laminin (Upstate Biotechnology). The culture medium (M199, SIGMA) containing (in mmol/L) creatine 5, L-carnitine 2, taurine 5, insulin-transferrin-selenium-X 0.1%, HEPES 25, and penicillin plus streptomycin 1%, was adjusted to pH 7.4 with NaOH at 37°C. Hearts using standard enzymatic technique as described previously.\textsuperscript{23} Isolation and Culture of Cardiac Myocytes

Dominant-Negative CaMKII\textsubscript{δC} Adenovirus Construction and Myocyte Infection
Dominant-negative CaMKII\textsubscript{δC} (DN-CaMKII) was generated by replacing the residue lysine43 with alanine (K43A) using the transformer site directed mutagenesis kit (Clontech). Adenoviral expression of β-gal or the HA-tagged DN-CaMKII was performed at a multiplicity of infections (MOI) of 100. Twenty four hours after adenoviral infection, norepinephrine (100 nmol/L) was added.

Measurements of Cell Shortening and Ca\textsuperscript{2+} Transients
Measurements of cell contraction and Ca\textsuperscript{2+} transients were performed 24 hours after norepinephrine exposure. Normal cultured myocytes (24 hours) were used for short-term β\textsubscript{1}AR stimulation. Myocytes were field-stimulated at 0.5 Hz in perfusion solution containing (in mmol/L) NaCl 137, KCl 4.9, CaCl\textsubscript{2} 1.0, MgSO\textsubscript{4} 1.2, NaH\textsubscript{2}PO\textsubscript{4} 1.2, glucose 15, and HEPES 20 (pH 7.4). Prazosin was added 10 minutes before short-term norepinephrine treatment. Protocols pertaining to specific experiments were given in the respective result figure.

In indicator-unloaded myocytes, cell length was monitored by an optical edge tracking method at a 3-ms time resolution.\textsuperscript{21} In myocytes loaded with the Ca\textsuperscript{2+} indicator fluo-4/AM (Molecular Probes, 20 μmol/L for 30 minutes), Ca\textsuperscript{2+} transients and cell shortening were measured with a confocal laser scanning microscope (LSM510, Carl Zeiss). Digital image analysis used customer-designed programs coded in Interactive Data Language (IDL).

Receptor Radioligand Binding Assay
Cardiac myocytes were homogenized and crude membranes were prepared by centrifuging at 35,000 g for 20 minutes at 4°C twice. β\textsubscript{1}AR radioligand binding studies were performed in membranes (25 to 100 μg/tube) using the nonselective βAR antagonist ligand \textsuperscript{125}I-lyophophindol (\textsuperscript{125}I-CYP, 1 to 300 pmol/L) as described previously.\textsuperscript{24} Nonspecific binding was determined in the presence of 10 μmol/L propranolol. The maximal numbers of binding sites (B\textsubscript{MAX}) and equilibrium dissociation constants (K\textsubscript{d}) for \textsuperscript{125}I-CYP were determined by Scatchard analysis.

Immunostaining
Fixed cells were incubated with anti-HA antibody (1:500, Covance Research Products Inc) at 4°C overnight, followed by Cy5-conjugated secondary antibody (1:1000, Jackson ImmunoResearch laboratories). Negative controls were obtained by incubating cells with only the secondary antibody.

CaMKII Activity and cAMP Accumulation
Cell lysate (500 μg protein) was first immunoprecipitated with anti-CaMKII antibody (1:100, Santa Cruz Biotechnology) in a Ca\textsuperscript{2+}-free medium. The precipitated proteins were evaluated with CaMKII assay kits (Upstate Biotechnology Inc) using a specific peptide substrate (KKALRRQETVDAL) as previously described.\textsuperscript{12} cAMP level was measured with the cAMP (H) assay system (Amersham Biosciences) as described previously.\textsuperscript{24}

Western Blotting Analysis of PLB Phosphorylation
PLB phosphorylation was detected with the phosphorylation site-specific antibodies recognizing P-Ser\textsuperscript{16} PLB or P-Thr\textsuperscript{17} PLB. Normal cultured rat ventricular myocytes were cultured in the presence of prazosin (1 μmol/L), for 24 hours. All the antagonists were added at least 10 minutes before norepinephrine.

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When compared with the response to norepinephrine stimulation (Table 2), consistent with previous in vitro reports.26 When compared with the response to short-term β,AR stimulation (10 minutes), we found that sustained β,AR stimulation was equally efficacious in mediating the positive inotropic and relaxant effects (Figure 1). These results indicate that sustained contractile response of β,AR stimulation is largely PKA-independent.

Sustained β,AR Contractile Response Is Mediated by CaMKII Signaling Pathway

Whereas short-term and sustained β,AR stimulations elicited indistinguishable responses in terms of cell contraction, there is no a priori reason that the same PKA-dependent mechanism is responsible for the contractile responses in both cases. To appraise role of PKA in mediating the sustained β,AR responses, we used two inhibitors of the cAMP/PKA signaling: PKI, a membrane permeable peptide inhibitor of PKA, and Rp-cpt-cAMPS, an inhibitory cAMP analogue. We found that PKI treatment (10 μmol/L for 30 minutes) largely blocked the effect of short-term β,AR stimulation (Figure 2A and 2B) as expected. By contrast, PKI was unable to reverse the increase in contraction amplitude in cells exposed to β,AR stimulation for 24 hours (Figure 2A and 2B). Likewise, the cAMP antagonist Rp-cpt-cAMPS (100 μmol/L for 30 minutes) significantly inhibited the effects of short-term, but not sustained, β,AR stimulation (Figure 2B). These results indicate that sustained contractile response of β,AR stimulation is largely PKA-independent.

Recently, it has been shown that sustained β,AR-stimulated cardiomyocyte apoptosis requires activation of CaMKII signaling independently of PKA.12 Previous studies have also established an important role for CaMKII modulation of phosphorylation and function of key proteins involved in cardiac E-C coupling, including LCC,21,22 SERCA,18 and its regulator PLB,19 and RyR.20,28 Next, we examined the involvement of CaMKII signaling in the contractile response to sustained β,AR stimulation. As shown in Figure 2C and 2D, the inotropic effect of sustained β,AR stimulation was reversed by KN93 (10 μmol/L for 30 minutes), a synthetic CaMKII inhibitor, whereas the effect of short-term β,AR stimulation was unaffected by KN93.

| Table 1: Effects of βAR Blockers on Sustained β,AR Contractile Responses |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|
| Culture | NE+Prop | NE+CGP | NE 24 Hours |
| TA, % | 3.57±0.70 | 2.45±0.80 | 4.16±0.41 | 10.17±1.84† | 1.84±0.56 | 3.95±1.04 |
| T90, ms | 479.1±20.7 | 504.6±35.5 | 546.1±57.2 | 321.9±11.0* | 429.0±34.0 | 393.2±14.1 |

TA indicates contraction amplitude; T90, time to peak to 90% relaxation of shortening; NE, norepinephrine 100 nmol/L. Prop, propranolol 10 μmol/L; CGP, CGP 20712A 0.5 μmol/L. n=11–20 cells. †P<0.001 vs control, ‡P<0.001 vs NE+propranolol.
inactivated by KN93, exerted no significant effects on either short-term or sustained β AR stimulation (data not shown). Similar to KN93, a cell-permeable peptide inhibitor of CaMKII, myristoylated autacamide-2 related inhibitory peptide (AIP, 10 μmol/L for 30 minutes), completely abolished the positive inotropic effect of sustained β AR stimulation, without affecting those of short-term β AR stimulation (Figure 2D). Thus, inhibition of cAMP/PKA and CaMKII preferentially blocked contractile modulation by short-term and sustained β AR stimulation, respectively, indicating that the initial responses depend mainly on PKA, whereas the sustained responses require CaMKII signaling.

**CaMKII-Dependent Increase of Ca²⁺ Transients in Response to Sustained β AR Stimulation**

To further investigate cellular mechanisms underlying the contractile responses in sustained versus short-term β AR stimulation, we measured Ca²⁺ transients and the corresponding cell contraction in Ca²⁺ indicator-loaded myocytes, using confocal microscopy. Figure 3A shows typical micrographs of cellular Ca²⁺ events from cells that underwent short-term and sustained β AR stimulation in the absence or presence of PKA or CaMKII inhibitor. Both short-term and sustained β AR stimulation increased Ca²⁺ transient amplitude (ΔF/Fo from 2.7±0.1 to 5.5±0.2 or 6.0±0.2 for cells with short-term or sustained β AR stimulation, respectively, n=37 to 55 cells) and contractile amplitude (from 2.5±0.3% to 12.2±0.6% or 11.1±0.6% for cells with short-term or sustained β AR stimulation, respectively, n=33 to 55; Figure 3). The short-term β AR stimulation-induced Ca²⁺ transients and contractile responses were blocked by Rp-cpt-cAMPS but not by AIP (Figure 3). In contrast, the sustained β AR stimulation–induced increases in Ca²⁺ transients and contraction were resistant to Rp-cpt-cAMPS, but were reversed by AIP (Figure 3). These data indicate that both cAMP/PKA and CaMKII signaling pathways of β AR stimulation augment cell contraction by enhancing intracellular Ca²⁺ transients.

**Effects of Dominant-Negative CaMKII on Short-Term and Sustained β AR Stimulation**

To confirm that the sustained β AR contractile effect is CaMKII-dependent, we resorted to molecular and genetic manipulation of the CaMKII signaling system. An adenovirus carrying the HA-tagged CaMKII gene with a dominant-negative mutation (DN-CaMKII, mutation K43A) that abrogates the kinase activity was constructed and infected cardiac myocytes in culture. Twenty-four hours after adenoviral infection at MOI of 100, nearly 100% cells showed intense immunostaining of DN-CaMKII as visualized by an anti-HA antibody (Figure 4A). Western Blotting analysis revealed that CaMKII-specific phosphorylation of PLB at Thr was also markedly reduced in the DN-CaMKII expressing myocytes (Figure 4B). Figure 4C and 4D show that sustained β AR stimulation in DN-CaMKII-expressing cells failed to elicit significant increases of Ca²⁺ transient amplitude or contraction amplitude. However, no significant difference between β-gal and DN-CaMKII expression groups was found in contractile and Ca²⁺ responses to short-term β AR stimulation (Figure 4C and 4D). These data corroborate that sustained β AR stimulation enhances Ca²⁺ transients and cell contraction through a CaMKII pathway, whereas the initial β AR response is largely CaMKII-independent.

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<th>TABLE 2. βAR Density and Affinity in Response to NE Exposure</th>
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<td>Bmax, fmol/mg protein</td>
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Bmax indicates maximal no. of ICYP-binding sites; Kd, equilibrium dissociation constants. n=3.
Switch of cAMP and CaMKII Signaling During Sustained β₁AR Stimulation

To better characterize the aforementioned signaling shift during β₁AR stimulation, we directly measured cellular cAMP accumulation and CaMKII activation over a wide period of time (from 10 minutes up to 24 hours). β₁AR stimulation elicited a rapid increase of cellular cAMP production that reached its peak in 10 minutes. In the continued presence of the β₁AR agonist, however, cAMP production was reduced by 66% at 3 hours after stimulation (Figure 5A), and returned to basal level at the steady state (12 hours). The gradual decay of cAMP is consistent with the notion that cAMP/PKA signaling undergoes substantial desensitization during prolonged receptor stimulation.5–7

Parallel measurement of CaMKII activity revealed a distinctly different temporal pattern for CaMKII response during β₁AR stimulation. CaMKII activity rose exponentially (time constant τ=15 minutes) without an initial overshoot (Figure 5B). The plateau was reached after ~1 hour stimulation and was stable for at least 24 hours. Overall, the gradual sensitization of CaMKII signaling roughly mirrored the desensitization of cAMP/PKA signaling, indicating a shift from a cAMP/PKA-dominant signaling to a CaMKII-dominant signaling. The slow and nondecremental activation of CaMKII provides the basis for the sustained contractile and Ca²⁺ responses to β₁AR stimulation.

Phospholamban (PLB) as a Molecular Integrator of β₁AR-Stimulated PKA and CaMKII Signals

The SR protein PLB, in its unphosphorylated form, serves as a constitutive inhibitor of the SR Ca²⁺ ATPase. PKA and CaMKII can independently phosphorylate PLB at 16 Ser and 17 Thr, respectively, and either site phosphorylation is sufficient to reverse its inhibition on SERCA activity and subsequently elicit positive inotropic and relaxant responses.29 Thus, PLB with its dual-site phosphorylation might operate as a molecular integrator of both short-term and sustained β₁AR signaling.

To explore this possibility, we examined the site-specific phosphorylation of PLB in response to sustained and short-term β₁AR stimulation. The phosphorylation at PKA-dependent site (P-16 Ser) was increased by 7.2 ± 0.9-fold (n=4, P<0.001 versus control) at 10 minutes after exposure to norepinephrine, but was then diminished toward the basal level during sustained β₁AR stimulation (Figure 5C). Conversely, phosphorylation at the CaMKII-dependent site (P-17 Thr) was significantly increased in response to sustained, but not short-term, β₁AR stimulation (Figure 5D). These data...
support the idea that dual site phosphorylation by PKA and CaMKII in effector proteins (e.g., PLB) serves to integrate the dual signaling pathways of β₁AR stimulation.

Effect of cAMP/PKA on CaMKII Mediated β₁AR Contractile Response

Because β₁AR-stimulated cAMP/PKA signaling precedes the CaMKII signaling, it might be argued that activation of CaMKII pathway in sustained β₁AR stimulation is still dependent on the initial PKA activation. However, preinhibition of cAMP/PKA with Rp-cpt-cAMPS (100 μmol/L) did not influence the sustained β₁AR-stimulated contractile and relaxant responses or the blockade effect of AIP (Figure 6A). Conversely, direct activation of cAMP/PKA pathway by forskolin (1 μmol/L), an adenylate cyclase activator, or cpt-cAMP (100 μmol/L), an active cAMP analogue, elicited no CaMKII-dependent component in the sustained inotropic response (Figure 6B and 6C). Thus, cAMP/PKA signaling appears to be neither sufficient nor necessary for β₁AR activation of CaMKII.

Discussion

Time-Dependent Shift of β₁AR Dual Signaling Pathways

We have systematically examined the signaling mechanisms underlying cardiac contractile modulation by short-term and sustained β₁AR stimulation (24 hours), using myocyte culture combined with genetic manipulation, confocal imaging, and biochemical measurements. In contrast to the anticipated receptor desensitization,⁵⁻⁷⁻⁷⁷ we demonstrated that short-term and sustained β₁AR stimulation similarly enhance Ca²⁺ transients and contraction and accelerate relaxation (Figures 1 and 3). Despite phenomenological similarities, inhibition of CaMKII by specific inhibitors or adenoviral expression of DN-CaMKII exerts profound inhibitory effects on the sustained, but not short-term, β₁AR responses, whereas inhibition of the cAMP/PKA pathway preferentially blocks the responses to short-term β₁AR stimulation (Figures 2 to 4). By tracking cAMP production and CaMKII activation over an...
extended time course (Figure 5), we have uncovered that, CaMKII activity rose to a plateau that does not show any noticeable decay, whereas the cAMP/PKA signaling subsides in the continued presence of β1AR agonist. These results indicate that β1AR signaling undergoes a time-dependent switch from the PKA-dominant pathway to the CaMKII-dominant pathway after receptor stimulation.

Because inhibition of the cAMP/PKA pathway did not alter the responses to sustained receptor stimulation and receptor-independent cAMP/PKA signal failed to elicit CaMKII-dependent response (Figure 6), activation of the CaMKII pathway may not be consequential to the transient cAMP/PKA activation. In other words, different pathways initiated from the same GPCR may manifest desensitization or sensitization independently.6,12,30 As compared with β1AR-stimulated CaMKII activation in β2AR double knockout mouse cardiac myocytes overexpressing β1AR (≈3-fold, τ ≈60 minutes), the elevation of CaMKII activity by native β1AR in heart cells is modest (35% over baseline) yet exhibits faster kinetics (τ ≈15 minutes) (Figure 5B). These quantitative discrepancies might reflect differences in the receptor density or the coupling efficiency of β1AR to downstream signaling pathways in these two systems.

Molecular Integration of β1AR-Stimulated PKA and CaMKII Signals

That short-term and sustained β1AR contractile and Ca2+ responses are virtually indistinguishable indicates a seamless integration of β1AR-stimulated PKA and CaMKII signals at the molecular and cellular levels. Indeed, we detected that a shift of PLB phosphorylation from the PKA-dependent site (16Ser) in short-term β1AR stimulation to the CaMKII-dependent site (17Thr), which correlates well with the time-dependent changes in cAMP production and CaMKII activation in sustained β1AR stimulation. Because either site phosphorylation of PLB is sufficient to release its inhibition on the SERCA,28,31,32 PLB serves as a key molecular integrator of the dual signaling pathways of β1AR stimulation. Disinhibition of SERCA activity by PLB phosphorylation will enhance SR Ca2+ recycling, accelerate relaxation of Ca2+ transients and cell contraction, and subsequently increase the SR Ca2+ load,12 contributing to both the positive inotropic and relaxant effects of β1AR stimulation. It is noteworthy that the relaxant effect of sustained norepinephrine exposure was not significantly influenced by CaMKII inhibitors (Figure 2C), suggesting the possibility for a differential regulation of peak contraction and relaxation by sustained β1AR stimulation.

In addition to PLB, previous studies have also shown that LCC Ca2+ currents are regulated by both PKA and CaMKII.21,33 Our preliminary data suggested the enhancement of LCC Ca2+ currents during initial (10 to 30 minutes) and sustained (3 to 6 hours) β1AR stimulation is mediated by PKA- and CaMKII-dominant mechanisms, respectively, suggesting LCC serves as another molecular integrator of the PKA and CaMKII signals of β1AR stimulation. In addition, it has been documented that either PKA or CaMKII can phosphorylate RyRs,20,34 although functional consequence of such phosphorylation remains controversial.34–36

Functional Significance of β1AR Signaling Switch

The finding of time-dependent switch between two signaling pathways of β1AR stimulation bears important implications in understanding physiological modulation of cardiac function as well as the etiology and pathophysiology of cardiac diseases associated with chronically exaggerated βAR signaling. First, the present result reassures that short-term β1AR stimulation, as in fight-or-flight response or during exercise, is largely mediated by the cAMP/PKA pathway, rapid activation of which enables a beat-to-beat regulation of cardiac performance. However, the cAMP/PKA signaling desensitizes nearly completely (Figure 5A) and is unlikely to play any major role for more enduring responses. In contrast, the CaMKII signaling does not undergo any appreciable desensitization over the period of observation (up to 24 hours). If this can be extrapolated to chronic β1AR stimulation in vivo, a corollary is that long-term β1AR effects, beneficial or toxic, must be due primarily to CaMKII-dependent signal transduction. This concept resonates with several lines of evidence found in previous studies. First, βAR-stimulated cardiac cell hypertrophy is largely PKA-independent but requires CaMKII activation.37,38 Second, CaMKII, but not PKA activation is obligatory to β1AR-mediated cardiac apoptosis.12 Third, whole-animal and clinic data hint on that cardiac toxic effects of chronically enhanced βAR stimulation are likely PKA-independent.39 Emerging evidence also suggests that increased CaMKII activity in human heart failure might play a compensatory role for the decreased cardiac contractility in failing hearts.40,41

Possible Mechanisms Underlying CaMKII Activation

The exact mechanisms underlying CaMKII activation and the switch of signaling pathways remain unknown. Preliminary observations showed that the β1AR CaMKII-dependent response is insensitive to G i/G o inhibition by pertussis toxin (1.5 μg/mL, added 3 hours before norepinephrine). Results in Figure 6 further suggest that direct and sustained activation of the cAMP/PKA signaling failed to activate CaMKII, and that blockage of cAMP/PKA does not prevent CaMKII from activation after β1AR stimulation; thus, the β1AR-cAMP signaling is neither necessary nor sufficient for the activation of β1AR-induced CaMKII signaling. Apart from the cAMP/PKA pathway, some evidences support a direct coupling between LCC and GPCRs including β1AR.42,43 Overexpression of G sλ has also been shown to activate LCC currents via PKA-independent mechanisms.44 If LCC could be activated in a PKA-independent manner, LCC Ca2+ entry might be responsible for the gradual CaMKII activation during β1AR stimulation. In this scenario, the cAMP-dependent activation of LCC may differ from β1AR-dependent, but cAMP-independent, LCC activation because sustained cAMP-dependent activation of LCC by forskolin or active cAMP analogue does not express the CaMKII signaling even after 24 hours. Recent studies have also hinted on a G protein–independent GPCR signaling. For instance, the carboxyl terminus of β1AR can directly interact with PDZ-motif containing proteins such as PSD-95 and Ras exchanger regulatory factor.45,46 By analogy, β1AR might directly acti-
vate element(s) of the CaMKII pathway through G protein–

independent mechanisms. Future investigations are warranted to explore these possibilities.

In summary, \( \beta \text{AR} \) modulation of cardiac E-C coupling invokes dual signaling pathways mediated by cAMP/PKA and CaMKII, respectively. The cAMP/PKA signaling is biphasic with a prominent early peak, whereas the CaMKII signaling is slow but persistent. During the signaling switch, the inotropic and relaxant responses are maintained at the steady state because of the convergence of PKA and CaMKII signals onto common effector proteins involved in E-C coupling and intracellular Ca\(^{2+} \) regulation. As a result, the effects of sustained \( \beta \text{AR} \) stimulation (eg, inotropy, cell growth, and cell death) are due primarily to CaMKII, rather than PKA signaling. These findings provide mechanistic insights into \( \beta \text{AR} \) modulation of cardiac function and GPCR signaling, and suggest new concepts for mechanistic understanding and therapeutic treatment of cardiac conditions such as hypertension and chronic heart failure that are associated with sustained elevation of endogenous catecholamines.

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