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

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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 (¹⁶Ser) to CaMKII site (¹⁷Thr). 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;95:798-806.)

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

As a prototypical member of G protein–coupled receptor (GPCR) superfamily, β₁-adrenergic receptor (BAR) plays a central role in sympathetic regulation of cardiac function.¹,² Stimulation of βAR by catecholamines induces robust chronotropic, inotropic, and relaxant effects via the Gs-adenylate cyclase-cAMP-protein kinase A (PKA) pathway.³,⁴ This signaling pathway is also thought to be responsible for other functions of βAR, such as regulation of metabolism, gene expression, cell growth, and apoptosis.⁵ However, sustained βAR activation under pathological conditions such as hypertension and congestive heart failure will result in downregulation and desensitization of βAR attributable to the negative feedback of this pathway.⁵–⁷

Recent studies have revealed unanticipated complexity of βAR signal transduction. For β₂AR subtype stimulation in the heart, a parallel activation of G protein counterbalances Gs-mediated contractile response. Whereas β₁AR stimulated contractile response is thought to be mediated exclusively by the cAMP/PKA signaling pathway,⁸,⁹ βAR regulation of cell growth and remodeling involves mitogen-activated protein kinase (MAPK) cascades and phosphoinositol 3 kinase (PI3K) pathway.¹⁰,¹¹ 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.¹² Therefore, in addition to the classic cAMP/PKA pathway, chronic βAR stimulation under certain physiological and pathophysiologic circumstances may evoke pathways other than cAMP/PKA.¹³,¹⁴ 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.¹⁵ In the heart, CaMKIIδ is the predominant isoform and plays a pivotal role in regulating cardiac performance and remodeling such as myocyte hypertrophy,¹⁶ apoptosis,¹² and heart failure.¹⁷ 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\(^{2+}\) release channels, and sarcromemal L-type Ca\(^{2+}\) channels (LCC).\(^{18-22}\) However, the involvement of CaMKII in β1AR modulation of myocardial contractility remains obscure.

The present study aimed at appraising roles of CaMKII and PKA in β1AR modulation of cardiac E-C coupling, with an emphasis on the signaling mechanism for the sustained β1AR stimulation. We found that both short-term and sustained β1AR stimulation are efficacious in mediating positive inotropic and relaxant effects in cardiac myocytes. Unlike the short-term β1AR 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 (Charles River Laboratories, Wilmington, Mass) using standard enzymatic technique as described previously.\(^ {23}\) Freshly isolated myocytes were plated at a density of 0.5 to 1 × 10^6/cm\(^2\) in dishes precoated with 20 μg/mL laminin (Upstate Biotechnology). The culture medium (M199, SIGMA) containing (in mmol/L) creatine 5, L-carnitine 2, taurozine 5, insulin-transferrin-selenium-X 0.1%, HEPES 25, and penicillin plus streptomycin 1%, were adjusted to pH 7.4 with NaOH at 37°C twice. For sustained β1AR stimulation, norepinephrine (100 nmol/L with vitamin C) was added along with α1AR antagonist, prazosin (1 μmol/L), for 24 hours. All the antagonists were added at least 10 minutes before norepinephrine.

Dominant-Negative CaMKIIΔC Adenovirus Construction and Myocyte Infection

Dominant-negative CaMKIIΔC (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 the multiplicity of infections (MOI) of 100. Twenty four hours after adenoviral infection, norepinephrine (100 nmol/L) was added.

Measurements of Cell Shortening and Ca\(^{2+}\) Transients

Measurements of cell contraction and Ca\(^{2+}\) transients were performed 24 hours after norepinephrine exposure. Normal cultured myocytes (24 hours) were used for short-term β1AR stimulation. Myocytes were field-stimulated at 0.5 Hz in perfusion solution containing (in mmol/L) NaCl 137, KCl 4.9, CaCl\(_2\) 1, MgSO\(_4\) 1.2, NaH\(_2\)PO\(_4\) 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. In myocytes loaded with the Ca\(^{2+}\) indicator fluo-4/AM (Molecular Probes, 20 μmol/L for 30 minutes), Ca\(^{2+}\) 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 000g for 20 minutes at 4°C twice. β1AR radioligand binding studies were performed in membranes (25 to 100 μg/tube) using the nonselective β1AR antagonist ligand [125I]cyanoindol (125I-CYP, 1 to 300 pmol/L) as described previously.\(^ {24}\) Nonspecific binding was determined in the presence of 10 μmol/L propranolol. The maximal numbers of binding sites (B\(_{max}\)) and equilibrium dissociation constants (K\(_d\)) for 125I-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\(^{2+}\)-free medium. The precipitated proteins were evaluated with CaMKII assay kits (Upstate Biotechnology Inc) using a specific peptide substrate (KKARLRRQETVDAL) as previously described.\(^ {12}\) cAMP level was measured with the cAMP (H) assay system (Amersham Biosciences) as described previously.\(^ {24}\)

Western Blotting Analysis of PLB Phosphorylation

PLB phosphorylation was detected with the phosphorylation site-specific antibodies recognizing P-16Ser PLB or P-17Thr PLB (24 hours) elicited a 3.3-fold increase of the contraction (Figure 1C), which was accompanied by a 47% decrease of the 90% relaxation time (Figure 1D). Notably, the effects of sustained β1AR stimulation on cell contraction and relaxation were fully reversible on washout of the agonist, as was the case with short-term β1AR stimulation (Figure 1). That sustained contractile responses are fully and rapidly reversible excludes the possibility that they were due simply to a long-term “memory” formed during prolonged receptor stimulation.\(^ {25}\)

As is the case with short-term β1AR stimulation, sustained contractile response of β1AR was sensitive to a nonselective β1AR blocker, propranolol (10 μmol/L), or a specific β1AR blocker, CGP 20712A (0.5 μmol/L). Pretreatment with propranolol or CGP 20712A prevents the increase of contractility by subsequent 24-hour norepinephrine stimulation (Table 1AR antagonist, ligand 1AR stimulation were performed in membranes (25 to 100 μg/tube) using the nonselective β1AR antagonist ligand [125I]cyanoindol (125I-CYP, 1 to 300 pmol/L) as described previously.\(^ {24}\) Nonspecific binding was determined in the presence of 10 μmol/L propranolol. The maximal numbers of binding sites (B\(_{max}\)) and equilibrium dissociation constants (K\(_d\)) for 125I-CYP were determined by Scatchard analysis.

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ICI 118,551 (0.5 μmol/L), a β_AR specific antagonist, did not affect the 24-hour norepinephrine-induced response (data not shown). Radioligand binding assay of receptor density in crude membrane fraction revealed a trend of decrease (though statistically insignificant) in β_AR density after 24-hour norepinephrine stimulation (Table 2), consistent with previous in vitro reports. 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). This observation is somewhat unexpected because it has been well established that cAMP/PKA signaling desensitizes within hours during prolonged β_AR stimulation.5,27

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. KN92 (10 μmol/L), an

| Table 1. Effects of βAR Blockers on Sustained βAR Contractile Responses |
|-----------------------------|---------------------|---------------------|---------------------|---------------------|
| Culture 24 Hours | NE + Prop 24 Hours | NE + CGP 24 Hours | 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 from peak to 90% relaxation; NE, norepinephrine 100 nmol/L; Prop, propranolol 10 μmol/L; CGP, CGP 20712A 0.5 μmol/L. n=11–20 cells. *P<0.05; †P<0.001 vs other groups.
and sustained preferentially blocked contractile modulation by short-term (Figure 2D). Thus, inhibition of cAMP/PKA and CaMKII-induced Ca\textsuperscript{2+} peptide (AIP, 10\textsuperscript{-6} mol/L) for 30 minutes), completely abolished the positive inotropic effect of sustained \(\beta_{1}\)AR stimulation, without affecting those of short-term \(\beta_{1}\)AR stimulation (Figure 2D). Thus, inhibition of cAMP/PKA and CaMKII preferentially blocked contractile modulation by short-term and sustained \(\beta_{1}\)AR stimulation, respectively, indicating that the initial responses depend mainly on PKA, whereas the sustained responses require CaMKII signaling.

### CaMKII-Dependent Increase of Ca\textsuperscript{2+} Transients in Response to Sustained \(\beta_{1}\)AR Stimulation

To further investigate cellular mechanisms underlying the contractile responses in sustained versus short-term \(\beta_{1}\)AR stimulation, we measured Ca\textsuperscript{2+} transients and the corresponding cell contraction in Ca\textsuperscript{2+} indicator-loaded myocytes, using confocal microscopy. Figure 3A shows typical micrographs of cellular Ca\textsuperscript{2+} transients and shortenings from cells that underwent short-term and sustained \(\beta_{1}\)AR stimulation in the absence or presence of PKA or CaMKII inhibitor. Both short-term and sustained \(\beta_{1}\)AR stimulation increased Ca\textsuperscript{2+} transient amplitude (\(\Delta F/F_0\)) from 2.7±0.1 to 5.5±0.2 or 6.0±0.2 for cells with short-term or sustained \(\beta_{1}\)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 \(\beta_{1}\)AR stimulation, respectively, \(n=33\) to 55; Figure 3). The short-term \(\beta_{1}\)AR stimulation-induced Ca\textsuperscript{2+} and contractile responses were blocked by Rp-cpt-cAMPS but not by AIP (Figure 3). In contrast, the sustained \(\beta_{1}\)AR stimulation-induced increases in Ca\textsuperscript{2+} 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 \(\beta_{1}\)AR stimulation augment cell contraction by enhancing intracellular Ca\textsuperscript{2+} transients.

### Effects of Dominant-Negative CaMKII on Short-Term and Sustained \(\beta_{1}\)AR Stimulation

To confirm that the sustained \(\beta_{1}\)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\textsuperscript{17} was also markedly reduced in the DN-CaMKII expressing myocytes (Figure 4B). Figure 4C and 4D show that sustained \(\beta_{1}\)AR stimulation in DN-CaMKII-expressing cells failed to elicit significant increases of Ca\textsuperscript{2+} transient amplitude or contraction amplitude. However, no significant difference between \(\beta_{1}\)-gal and DN-CaMKII expression groups was found in contractile and Ca\textsuperscript{2+} responses to short-term \(\beta_{1}\)AR stimulation (Figure 4C and 4D). These data corroborate that sustained \(\beta_{1}\)AR stimulation enhances Ca\textsuperscript{2+} transients and cell contraction through a CaMKII pathway, whereas the initial \(\beta_{1}\)AR response is largely CaMKII-independent.

### Table 2. \(\beta_{1}\)AR Density and Affinity in Response to NE Exposure

| Culture 24 Hours | Fresh Cells | NE 10 Minutes | NE 24 Hours |
|------------------|-------------|---------------|-------------|
| \(B_{\text{max}}\), fmol/mg protein | 16.00±2.12 | 15.93±0.26 | 12.26±1.93 |
| \(K_d\), pmol/L | 26.82±4.60 | 16.46±2.75 | 16.28±4.26 |

\(B_{\text{max}}\) indicates maximal no. of ICYP-binding sites; \(K_d\), equilibrium dissociation constants. \(n=3\).

### Figure 2. Effects of PKA and CaMKII inhibition on short-term and sustained \(\beta_{1}\)AR contractile responses.

A. Typical examples of the effect of a membrane permeable peptide inhibitor of PKA, PKI (10\textsuperscript{-6} mol/L), on contraction amplitude of cells subjected to short-term or sustained \(\beta_{1}\)AR stimulation by norepinephrine (NE 100 nmol/L plus prazosin 1 \textsuperscript{-6} mol/L). B. Average effects of PKI and Rp-cpt-cAMPS (100 \textsuperscript{-6} mol/L), \(n=20\) to 35 cells from 3 to 4 hearts for each data point. \(\dagger P<0.001\) vs baseline, NE+PKI and NE+Rp-cpt-cAMPS groups; \(\ddagger P<0.001\) vs baseline. C. Typical examples of the effect of the CaMKII inhibitor, KN93 (10 \textsuperscript{-6} mol/L). D. Average effects of KN93 and AIP (10 \textsuperscript{-6} mol/L), \(n=20\) to 35 cells from 3 to 4 hearts for each data point. \(\ddagger P<0.001\) vs baseline, NE+KN93, and NE+AIP groups.
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 16Ser and 17Thr, 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-16Ser) 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-17Thr) was significantly increased in response to sustained, but not short-term, β1AR stimulation (Figure 5D). These data

![Figure 3](image3.png)

**Figure 3.** CaMKII-dependent increase of Ca2+ transients in response to sustained β1AR stimulation. A, Line-scan confocal images of Ca2+ transients in cells subjected to short-term (NE, 10 minutes) or sustained (NE, 24 hours) β1AR stimulation with or without AIP (10 μmol/L) or Rp-cpt-cAMPS (100 μmol/L). B and C, Average results. Ca2+ transient amplitude was indexed by ΔF/F0. n=30 to 40 cells from 3 to 4 hearts for each data point; *P<0.05 vs basal, †P<0.001 vs basal.

![Figure 4](image4.png)

**Figure 4.** Effects of DN-CaMKII expression on short-term and sustained β1AR responses. A, Confocal imaging of HA immunofluorescence in rat cardiac myocytes expressing either β-gal or HA-tagged DN-CaMKII. B, Expression of DN-CaMKII reduced PLB phosphorylation at 17Thr (P-17Thr). C and D, Ca2+ and contractile responses to short-term and sustained β1AR stimulation in cells expressing β-gal or DN-CaMKII. n=33 to 43 cells from 4 to 5 hearts for each data point; *P<0.05, †P<0.001 between groups.
support the idea that dual site phosphorylation by PKA and CaMKII in effector proteins (eg, 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, 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

![Figure 5](image5.png)

**Figure 5.** cAMP formation, CaMKII activity, and site-specific PLB phosphorylation during prolonged β₁AR stimulation. A, Time course of cAMP response to norepinephrine (NE, 100 nmol/L plus prazosin 1 μmol/L), n=5. B, Time course of CaMKII activation in response to norepinephrine (NE, 100 nmol/L plus prazosin 1 μmol/L); n=4. C and D, Western blotting of PLB phosphorylation at the PKA-dependent site (P-16Ser, C) and the CaMKII-dependent site (P-17Thr, D). n=4, *P<0.05 vs control, †P<0.001 vs control.

![Figure 6](image6.png)

**Figure 6.** Role of cAMP/PKA in β₁AR activation of CaMKII signaling. A, Preinhibition of cAMP/PKA did not alter the CaMKII-dependent contractile response at 24 hours β₁AR stimulation. n=20 to 35 cells from 3 rats. †P<0.001 between groups. B and C, Contractile responses to forskolin (1 μmol/L, 1 or 24 hours) or cpt-cAMP (100 μmol/L, 1 or 24 hours) were insensitive to CaMKII blockers. n=15 to 20 cells from 3 rats. †P<0.001 vs other groups.
noticeable decay, whereas the cAMP/PKA signaling subsides in the continued presence of β AR agonist. These results indicate that β AR 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 β AR-stimulated CaMKII activation in β AR/AR double knockout mouse cardiac myocytes overexpressing β AR (≈3-fold, t ≈60 minutes), the elevation of CaMKII activity by native β AR in heart cells is modest (35% over baseline) yet exhibits faster kinetics (t ≈15 minutes) (Figure 5B). These quantitative discrepancies might reflect differences in the receptor density or the coupling efficiency of β AR to downstream signaling pathways in these two systems.

Molecular Integration of β AR-Stimulated PKA and CaMKII Signals

That short-term and sustained β AR contractile and Ca2+ responses are virtually indistinguishable indicates a seamless integration of β AR-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 (18Ser) in short-term β AR stimulation to the CaMKIIDependent site (17Thr), which correlates well with the time-dependent changes in cAMP production and CaMKII activation in sustained β AR stimulation. Because either site phosphorylation of PLB is sufficient to release its inhibition on the SERCA,38,31,32 PLB serves as a key molecular integrator of the dual signaling pathways of β AR stimulation. Disinhibition of SERCA activity by PLB phosphorylation will enhance SR Ca2+ cycling, 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 β AR 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 β AR 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) β AR stimulation is mediated by PKA- and CaMKII-dominant mechanisms, respectively, suggesting LCC serves as another molecular integrator of the PKA and CaMKII signals of β AR stimulation. In addition, it has been documented that either PKA or CaMKII can phosphorylate RyR2,20,34 although functional consequence of such phosphorylation remains controversial.34–36

Functional Significance of β AR Signaling Switch

The finding of time-dependent switch between two signaling pathways of β AR 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 β AR signaling. First, the present result reassures that short-term β AR 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 β AR stimulation in vivo, a corollary is that long-term β AR 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, β AR-stimulated cardiac cell hypertrophy is largely PKA-independent but requires CaMKII activation.37,38 Second, CaMKII, but not PKA activation is obligatory to β AR-mediated cardiac apoptosis.12 Third, whole-animal and clinic data hint on that cardiac toxic effects of chronically enhanced β AR 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 β AR CaMKII-dependent response is insensitive to G/Gi 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 β AR stimulation; thus, the β AR-cAMP signaling is neither necessary nor sufficient for the activation of β AR-induced CaMKII signaling. Apart from the cAMP/ PKA pathway, some evidences support a direct coupling between LCC and GPCRs including β AR.42,43 Overexpression of Gsα 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 β AR stimulation. In this scenario, the cAMP-dependent activation of LCC may differ from β AR-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 β AR can directly interact with PDZ-motif containing proteins such as PSD-95 and Ras exchanger regulatory factor.45,46 By analogy, β AR might directly activate element(s) of the CaMKII pathway through G protein-independent mechanisms. Future investigations are warranted to explore these possibilities.
In summary, βAR modulation of cardiac E-C coupling involves dual signaling pathways mediated by cAMP/PKA and CaMKII, respectively. The cAMP/PKA signaling is biphastic 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 βAR stimulation (eg, inotropy, cell growth, and cell death) are due primarily to CaMKII, rather than PKA signaling. These findings provide mechanistic insights into βAR 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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