Regulation of PKA Binding to AKAPs in the Heart
Alterations in Human Heart Failure

Daniel R. Zakhary, PhD; Christine S. Moravec, PhD; Meredith Bond, PhD

Background—cAMP-dependent protein kinase (PKA) regulates a broad range of cellular responses in the cardiac myocyte. Downstream regulation of the PKA pathway is mediated by a class of scaffolding proteins called A-kinase anchoring proteins (AKAPs), which sequester PKA to specific subcellular locations through binding to its regulatory subunit (R). However, the effect of RII autophosphorylation on AKAP binding and the degree of RII autophosphorylation in failing and nonfailing human hearts remains unknown.

Methods and Results—We investigated AKAP-RII binding by overlay analysis and surface plasmon resonance spectroscopy and measured RII autophosphorylation in human hearts by backphosphorylation. Binding of Ht31 peptide (representing the RII-binding region of AKAPs) to cardiac RII was increased 145% (P<0.01) for autophosphorylated RII relative to unphosphorylated control. By surface plasmon resonance, RII autophosphorylation significantly increased binding affinity to Ht31 by 200% (P<0.01). Baseline PKA-dependent phosphorylation of RII was significantly decreased 30% (P<0.05) in human hearts with dilated cardiomyopathy compared with nonfailing controls.

Conclusions—These results suggest that AKAP binding of PKA in the heart is regulated by RII autophosphorylation. Therefore AKAP targeting of PKA may be reduced in patients with end-stage heart failure. This mechanism may be responsible for the decreased cAMP-dependent phosphorylation of proteins in dilated cardiomyopathy that we and others have previously observed. (Circulation. 2000;101:1459-1464.)

Key Words: cardiomyopathy ■ enzymes ■ proteins ■ spectroscopy

CAMP-dependent protein kinase (PKA) is a tetrameric holoenzyme consisting of 2 catalytic (C) and 2 regulatory (R) subunits.1 By substrate phosphorylation, PKA regulates a broad range of responses in the cardiomyocyte. Downstream regulation of the PKA pathway is mediated by A-kinase anchoring proteins (AKAPs), which sequester PKA to specific subcellular sites through binding to R.2 Although β-adrenoceptor density is reduced in failing human myocardium,3 evidence for defects distal to the receptor/adenylyl cyclase complex is rapidly accumulating. For example, even after stimulation by positive inotropic agents that act beyond the receptor, such as dibutryl-cAMP and cAMP-phosphodiesterases inhibitors, a decreased positive inotropic response in failing hearts is still observed.4 Furthermore, protein phosphorylation mediated by β-adrenergic activation is decreased in the failing human heart5,6 as well as in animal models of cardiac hypertrophy.7 We previously found decreased baseline troponin-I (TnI) phosphorylation and decreased protein levels of the regulatory subunits of types I and II PKA (RI and RII) in myocardial samples from patients with end-stage dilated cardiomyopathy (DCM).5 Therefore the PKA signaling pathway appears to be downregulated at several levels beyond the β-receptor in human heart failure.

Type II regulatory subunits have an autophosphorylation site, serine 95, in the primary sequence, which is the only residue phosphorylated by C,8 whereas type I regulatory subunits have a pseudophosphorylation site.4 In vitro studies on cardiac RII indicate that autophosphorylation of RII by C at serine 95 controls activation of the kinase by decreasing affinity for C 10-fold.9,10 However, the degree of RII autophosphorylation in normal and failing human hearts has not been examined. Our aims were 2-fold: (1) to determine whether RII autophosphorylation affects RII binding to AKAPs and (2) to measure PKA-dependent RII phosphorylation in human hearts with DCM and in nonfailing (NF) human hearts.

We previously showed colocalization of RII with AKAP100 in ventricular myocytes.11 Interestingly, there were pockets of RII that did not colocalize with AKAP and regions of unoccupied AKAPs. In addition, previous studies

Received June 18, 1999; revision received September 16, 1999; accepted October 11, 1999.
From the Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, Ohio (D.R.Z., C.S.M., M.B.); and the Department of Molecular Cardiology (D.R.Z., M.B.), Lerner Research Institute, and Center for Anesthesiology Research (C.S.M.), Cleveland Clinic Foundation, Cleveland, Ohio.
Correspondence to Meredith Bond, PhD, Department of Molecular Cardiology, NB50, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44195. E-mail bondm@ccf.org
© 2000 American Heart Association, Inc.
Circulation is available at http://www.circulationaha.org
showed that the $K_d$ for AKAP-RII interaction ($\approx 10$ nmol/L) is within physiological concentrations of most AKAPs. Together this suggests that a mechanism exists for regulating this interaction. However, the role of RII autophosphorylation in regulating AKAP binding of PKA has not yet been examined. This is the first study to determine the role of RII phosphorylation in regulating AKAP anchoring in the heart. We used the Ht31 peptide, which represents the RII binding domain of AKAPs, to measure binding affinities of dephosphorylated and autophosphorylated cardiac RII (RII-P) to Ht31 by both peptide overlay analysis and surface plasmon resonance (SPR) spectroscopy.

Results

Characterization of RII Recombinant Protein

We generated purified recombinant RII subunits by cAMP-agarose affinity chromatography. The eluate from the cAMP-agarose column was separated by 10% SDS-PAGE followed by Coomassie blue staining. As shown in Figure 1, bands of $\approx 57$ and $\approx 55$ kDa were identified on the Coomassie blue–stained gel for and RII-P and RII (lanes 4 and 5), respectively. The identity of RII was confirmed by $32P$ phosphorylation and immunoblot analysis as previously described (data not shown). Consistent with previous reports, we find that autophosphorylation of cardiac RII results in a decreased mobility in SDS gels. Because the RII autophosphorylation site is at a proteolytically sensitive hinge region, we first determined whether PKA phosphorylation affected proteolysis of RII. Phosphorylation by PKA had no effect on RII proteolysis in either bacterial lysate (Figure 1, lanes 2 and 3) or purified protein (lanes 4 and 5), as determined by scanning densitometry of the RII band. Furthermore, the level of RII detected immunologically by anti–RII antibody did not change with RII phosphorylation (not shown).

Identification of AKAP100 by RII Overlay and Immunoblot Analysis

By using radioactively labeled RII in an overlay assay, we confirmed the presence of AKAP100 in the human heart, as previously reported in rat cardiac myocytes and myocyte H9C2 cell line. Consistent with previous findings from our laboratory, $32P$-labeled RII identified an $\approx 82$-kDa band in fresh human heart homogenates and a doublet of $\approx 85$ to $82$ kDa in rat cardiac myocytes (Figure 2A). The binding was inhibited by incubation with 1 $\mu$mol/L Ht31 peptide (not shown). The anti-AKAP100 antibody recognized the same band at $\approx 82$ kDa, with a proteolytic fragment at $\approx 56$ kDa (Figure 2B), although recent cloning studies suggest that the full-length protein may be larger. Thus AKAP100 is likely to be a major PKA II anchor in the human heart, consistent with our previous immunofluorescence studies in rat cardiac myocytes.

Phosphorylation State of RII In Vivo

Because little is known about the phosphorylation state of RII in the intact heart, we performed PKA-dependent backphosphorylation in fresh human hearts and rat cardiomyocytes. Freshly isolated rat left ventricular cardiomyocytes were treated with isoproterenol (1 $\mu$mol/L) or were untreated (vehicle control). The phosphorylation of known PKA substrates such as TnI, phospholamban (PLB), and C-protein (vehicle control). The phosphorylation of known PKA subunits by cAMP-dependent protein phosphorylation was determined by backphosphorylation, as described in Methods. Fold increase in protein phosphorylation after $\beta$-adrenergic stimulation by isoproterenol was for rat left ventricular cardiac myocytes.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fold Stimulation by Isoproterenol</th>
<th>$P$ (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-protein</td>
<td>4.17±0.70</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>RII</td>
<td>0.97±0.08</td>
<td>NS</td>
</tr>
<tr>
<td>Tnl</td>
<td>3.62±0.37</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PLB</td>
<td>1.79±0.17</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

PKA-dependent protein phosphorylation was determined by backphosphorylation, as described in Methods. Fold increase in protein phosphorylation after $\beta$-adrenergic stimulation by isoproterenol was for rat left ventricular cardiac myocytes.
the heart. This is consistent with data from a freshly obtained, unfrozen, explanted human heart (cold ischemic time in cardioplegia 0.5 hours), which indicates a high level of in vivo phosphorylation (70 ± 2%; mean ± SD, n = 3 determinations).

Ht31 Overlay
To investigate the importance of RII autophosphorylation in regulating AKAP-PKA interactions in the heart, we first used an Ht31 peptide overlay assay to measure binding affinities between Ht31 and the regulatory subunit. RII or RII-P was transferred to nitrocellulose, and the amount of bound Ht31B was determined by densitometric scanning. Ht31B recognized RII-P and RII at ≈57 and ≈55 kDa, respectively (Figure 4A). At 70 ng recombinant protein, Ht31B binding was significantly increased 145 ± 7% (P < 0.01) for RII-P relative to control RII (Figure 4B). At saturating amounts of RII protein (280 ng), no difference in Ht31B binding was detected. With high protein loading, however, a proteolytic product of RII (≈37 kDa) was visible by Ht31 overlay. Binding of Ht31B to this fragment was significantly greater for RII-P, relative to RII (not shown). No binding of Ht31B was observed in left ventricular homogenates of mouse (45 mg total protein/lane), rat (45 mg total protein), or human (30 mg total protein) by overlay analysis, suggesting that the protein was not sufficiently concentrated in the samples to be detected by this technique.

In a separate series of experiments, Ht31 overlays were performed under nondenaturing conditions on samples of bacterial lysate containing RII. This alternative approach was used to avoid possible alterations resulting from RII purification and denaturation in SDS gels. Aliquots of lysate (0.25 to 1.0 mg/mL) were spotted directly to nitrocellulose in triplicate, then probed with Ht31B. Under these conditions, binding of Ht31B was also significantly increased for RII-P relative to control RII (Figure 4C).

Determination of Kinetic Parameters by SPR
To examine the effect of RII autophosphorylation on AKAP binding, $k_+$, $k_-$, and $K_D$ were determined for the interaction of RII and RII-P with Ht31. Injection of soluble Ht31 but not
control Ht31P competitively removed RII bound to the active surface, indicating specificity of the measured RII-Ht31 interaction (Figure 5A). Similarly, preincubation of RII with a 10-fold molar excess of soluble Ht31 competitively blocked the binding of RII to the active Ht31B surface, whereas preincubation with Ht31P did not (not shown). The level of RII-P bound after 1-minute contact time was significantly increased by 192 ± 6 2% (mean ± SD, n = 5) relative to RII (Figure 5B). The $K_D$ for RII-P–Ht31 interaction was significantly decreased 2-fold (n = 3) compared with RII–Ht31 (Table 2) as a result of increased association rates. These data are in agreement with previously obtained $K_D$ values of 11.2 nmol/L by SPR.\textsuperscript{17}

**RII Autophosphorylation in DCM**

Because the results of experiments described above suggest that RII autophosphorylation may enhance the binding of PKA to AKAPs, we investigated the role of this mechanism in failing and NF human hearts. We measured baseline values of PKA-dependent RII phosphorylation in left ventricular homogenates of 4 human hearts with DCM and 4 NF control hearts (Figure 6A). The highly phosphorylated band at 40 kDa in both lanes is most likely autophosphorylated PKA cat , exogenously added as part of the assay procedure. PKA-dependent RII phosphorylation was significantly decreased 32% ($P<0.05$) in DCM compared with NF (Figure 6B). Because we previously found decreased RII protein in DCM,\textsuperscript{5} phosphorylation values were normalized to RII protein levels, as determined by immunoblot analysis of the same sample preparations used in the backphosphorylation assay.

### TABLE 2. Rate and Affinity Constants for Interaction of RII and RII-P With Ht31 With Use of SPR

<table>
<thead>
<tr>
<th>$k_a$, 1/Ms</th>
<th>$k_o$, 1/s</th>
<th>$K_a$, 1/M</th>
<th>$K_o$, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>RII</td>
<td>4.1 ± 0.2 × 10$^6$</td>
<td>1.4 ± 0.01 × 10$^{-2}$</td>
<td>2.9 ± 0.1 × 10$^5$</td>
</tr>
<tr>
<td>RII-P</td>
<td>9.5 ± 0.3 × 10$^6$</td>
<td>1.5 ± 0.02 × 10$^{-2}$</td>
<td>6.4 ± 0.2 × 10$^5$</td>
</tr>
</tbody>
</table>

RII or RII-P was injected over the Ht31B surface at 3.7, 7.5, 15, 30 and 60 nmol/L. Cycle of binding, dissociation, and regeneration was repeated 3 times for each concentration. Kinetic constants were calculated by global fitting of data to 1:1 Langmuir binding model, as described in Methods.

$^*$P<0.01 vs control.
Discussion

The main findings are that RII autophosphorylation by C enhances its binding to the Ht31 peptide and that baseline PKA-dependent RII phosphorylation is significantly decreased in DCM compared with NF. These results demonstrate that RII autophosphorylation may increase local concentrations of C by increasing holoenzyme affinity for its anchoring protein. Therefore these results suggest that PKA anchoring to specific subcellular substrates may be decreased in failing cardiac myocytes.

We found that autophosphorylation of RII by PKA in vitro significantly increases its binding to Ht31. The Ht31 peptide is widely used to mimic AKAP-RII interactions or to inhibit them in vivo. Ht31 forms an amphipathic helix, which represents the essential RII binding domain of the full-length protein. On the basis of this and the fact that the proline substituted mutant peptide (Ht31P) has no effect in either Ht31 overlay, RII overlay, or SPR experiments, our results imply that RII autophosphorylation enhances binding to full-length AKAPs.

In freshly isolated rat left ventricular myocytes, PKA-dependent RII phosphorylation did not increase above baseline after β-stimulation. In addition, baseline PKA-dependent RII phosphorylation was ~70% (sites phosphorylated in vivo) in the failing human heart. RII is thus likely to be constitutively phosphorylated in the heart. These results are in agreement with previous studies that suggest that bovine cardiac RII exists in vivo primarily in the autophosphorylated form. Although β-adrenergic stimulation of isolated myocytes had no significant effect on the phosphorylation state of RII, phosphorylation of other known PKA substrates such as PLB, TnI, and C-protein did significantly increase above baseline, indicating that the β-adrenergic pathway was intact in these myocytes and the isoproterenol treatment was effective.

Our results indicate that RII is constitutively phosphorylated by PKA in the heart and that this basal, steady-state phosphorylation may be decreased under pathological conditions. Although previous in vitro studies indicate that RII autophosphorylation occurs as an intramolecular event, recent evidence suggests that CAMP can induce catalytic activity of PKA without subunit dissociation. Furthermore, the presence of PKI (with substrate) in the cell may allow for catalytic subunit dissociation independent of cAMP binding, with subsequent phosphorylation of nearby RII. This decreased targeted holoenzyme in DCM (initially caused by decreased RII protein) may result in decreased RII phosphorylation and thus a further decrease in the subcellular localization of the kinase. We observed decreased PKA phosphorylation of other proteins in DCM such as C-protein (reduced ~30%) and TnI (reduced ~30%) but not PLB, which is consistent with previous results. Arguing against overall downregulation of the β-adrenergic pathway in DCM, these results favor the idea of specific localized defects in DCM, mediated perhaps by decreased local availability of C as a result of altered AKAP anchoring in those hearts.

The regulatory subunit of PKA is responsible for AKAP binding and targeting of the holoenzyme. AKAP-mediated anchoring of PKA is functionally important to the extent that disruption of AKAP-RII interaction by Ht31 can result in loss of PKA catalytic activity and loss of PKA modulation of L-type channel activity. Therefore, in addition to decreased RII protein in DCM, decreased RII autophosphorylation may limit PKA targeting to functionally important regions in the failing human heart. Thus the reduced PKA-dependent phosphorylation of substrates that we observed in DCM may be due, at least in part, to altered AKAP-RII interaction in those hearts.

The use of tissue from failing and NF human hearts presents several potential limitations. All the failing hearts were receiving inotropic support (digoxin or dobutamine), which could potentially increase β-adrenergic tone. However, long-term elevated circulating catecholamines are believed to trigger downregulation of β-receptors. Furthermore, although brain death may be associated with massive catecholamine release in the donor hearts, circulating catecholamines are washed out by flushing and transport of the explanted hearts in ice-cold cardioplegia. Because transport time to the laboratory for donor hearts is longer than for failing hearts, any residual phosphatase activity would be expected to be greater in the donor hearts, resulting in decreased substrate phosphorylation, yet this is the opposite of what we observed.

In summary, we have shown by Ht31 overlay analysis and by SPR that RII autophosphorylation enhances RII binding to Ht31 peptide, the RII-binding domain of AKAPs. In addition, we found decreased RII autophosphorylation in DCM compared with NF. Overall, decreased autophosphorylation of RII may lead to a reduced amount of targeted RII in the failing human heart, with subsequent decreased local concentrations of C and decreased ability for phosphorylation of PKA substrates.

Acknowledgments

This work was supported by National Heart, Lung, and Blood Institute grants HL-56256 (M. Bond) and HL-49929 (C. Moravec), National Institutes of Health cardiovascular training grant HL-07653 (D. Zakhary), and by an American Heart Association Established Investigator Award and grant-in-aid to C. Moravec. We thank Dr. Norman Ratliff, Department of Pathology, Drs Robert Stewart and Patrick McCarthy, Department of Cardiovascular Surgery, CCF, and Life Banc of Northeast Ohio for providing human tissue. We also thank Drs Tom Egelhoff, J.P. Jin, Mary Russell, and Jiacheng Yang for helpful discussions and Russell Desnoyer for expert technical assistance.

References

Regulation of PKA Binding to AKAPs in the Heart: Alterations in Human Heart Failure
Daniel R. Zakhary, Christine S. Moravec and Meredith Bond

Circulation. 2000;101:1459-1464
doi: 10.1161/01.CIR.101.12.1459

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/101/12/1459

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/