Adenylyl Cyclase Type VI Increases Akt Activity and Phospholamban Phosphorylation in Cardiac Myocytes

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Increased expression of adenylyl cyclase VI has beneficial effects on the heart, but strategies that increase cAMP production in cardiac myocytes usually are harmful. Might adenylyl cyclase VI have beneficial effects unrelated to increased β-adrenergic receptor-mediated signaling? We previously reported that adenylyl cyclase VI reduces cardiac phospholamban expression. Our focus in the current studies is how adenylyl cyclase VI influences phospholamban phosphorylation. In cultured cardiac myocytes, increased expression of adenylyl cyclase VI activates Akt by phosphorylation at serine 473 and threonine 308 and is associated with increased nuclear phospho-Akt. Activated Akt phosphorylates phospholamban, a process that does not require β-adrenergic receptor stimulation or protein kinase A activation. These previously unrecognized signaling events would be predicted to promote calcium handling and increase contractile function of the intact heart independently of β-adrenergic receptor activation. We speculate that phospholamban phosphorylation, through activation of Akt, may be an important mechanism by which adenylyl cyclase VI increases the function of the failing heart.

Adenylyl cyclase (AC) catalyzes ATP to generate cAMP, a second messenger that is required for many intracellular events. AC is the effector molecule in the β-adrenergic receptor (βAR)-Gs-AC signaling pathway and for many other G-protein-coupled receptors in cardiac myocytes and other cells (1–2). Cardiac myocytes express predominantly AC type V and AC type VI (ACVI) (3). Cardiac-directed expression of ACVI in murine cardiomyopathy increases cardiac function, attenuates myocardial hypertrophy, and increases survival (4, 5). However, when cardiac-directed βAR expression is used in this same model, life is shortened (6, 7). Clearly there are marked differences in effects that are evoked by these two elements in the βAR-Gs-AC signaling pathway, even though both strategies increase cAMP.

The objective of the current study was to determine whether increased ACVI expression has effects not directly linked with βAR stimulation and protein kinase A (PKA) activation, thereby providing a mechanistic explanation for the unanticipated favorable effects of increased cardiac ACVI expression in heart failure. We previously reported that ACVI reduces cardiac phospholamban (PLB) expression through increased expression of activating transcription factor-3, which suppresses PLB promoter activity (8). We now focus on how ACVI influences PLB phosphorylation. We test the hypothesis that ACVI increases PLB phosphorylation independently of βAR stimulation and PKA activation. This would result in increased cardiac function but would circumvent deleterious effects of sustained PKA activation (9).

EXPERIMENTAL PROCEDURES

Materials—The reagents used (and supplier) included: phospholamban monoclonal antibody (clone A1), recombinant Akt1/PKBα, active (activated by 3’-phosphoinositide-dependent protein kinase-1 phosphorylation, Upstate, Temecula, CA); anti-phospholamban phospho-Ser-16 and anti-phospholamban phospho-Thr-17 antibodies (Badrilla Ltd., West Yorkshire, UK); anti-Akt, anti-phospho-Akt, anti-GSK3, anti-phospho-GSK3, anti-phospho-PKA catalytic subunit antibodies, and Akt kinase assay kit (Cell Signaling, Beverly, MA); anti-PKA catalytic subunit antibody (Dr. Susan Taylor at the University of California San Diego); QuikChange II kit (Strategene, San Diego, CA); EZ-detect Rap1 activation kit (Pierce); cAMP Biotrak Enzymeimmunoassay System (GE Healthcare); PKA activator (+)-Brefeldin A and inhibitors to Akt and PKA (PKI, 14–22 amide, cell-permeable, myristoylated; H-89 and Rp-8-CPT-cAMP, from Calbiochem); Epac activator (8-(4-chlorophenylthio)-2′-O-methyl-cAMP, from Biolog, Bremen, Germany); SigmaTECT cAMP-dependent Protein Kinase (PKA) Assay System and SigmaTECT Calcium/Calmodulin-dependent Protein Kinase Assay System (Promega, Madison, WI); protease inhibitor mixture (Roche Applied Science); Colloidal Blue Staining Kit (Invitrogen); adenylyl cyclase P-site inhibitors, 9-cyclopentyladenine monomethanesulfonate, and sodium phosphonoformate trisic acid (foscarnet).

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5The abbreviations used are: AC, adenylyl cyclase; ACVI, adenylyl cyclase VI; βAR, β-adrenergic receptor; PKA, protein kinase A; PLB, phospholamban; GST, glutathione S-transferase; EGFP, enhanced green fluorescent protein; Iso, isoproterenol; GSK3, glycogen synthase kinase 3; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropanesulfonic acid; CaMKII, calmodulin kinase II; 8-CPT, 8-pCPT-2′-O-Me-cAMP; Epac, 8-(4-chlorophenylthio)-2′-O-methyl-cAMP.
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(DH5\textsubscript{3}, Sigma); SQ22536 ( Biomol, Plymouth Meeting, PA). The Akt consensus peptide, PRPAATF, was purchased from Calbiochem. Phospholamban peptide (RSAIRRAST) was synthesized by Abgent (San Diego, CA).

Cardiac Myocyte Culture and Gene Transfer—Neonatal rat cardiac myocytes were isolated as previously described (10). One day after plating, an E1-deleted recombinant adenovirus encoding murine ACV_{VI} (with an AU1 tag, a 6-amino acid epitope: DTYRYI) was added to the culture media (600 viral particle/cell). An adenovirus encoding enhanced green fluorescence protein (EGFP) was used as a control vector. Twenty-four hours after adenovirus was added, cultured cells were stimulated with 10\mu M isoproterenol or 10\mu M NKH477, a water-soluble forskolin analog that directly stimulates AC. Kinase inhibitors were added 2 h after adenovirus infection in specific experiments (see below).

Cloning of GST-PLB and Site-directed Mutagenesis—A full-length rat PLB cDNA was cloned from neonatal rat cardiac myocytes using reverse transcription and polymerase chain reaction. The primer for reverse transcription was a random hexamer obtained from Qiagen (Valencia, CA). The primers for PCR included: PLBs, 5'-GGAATTC-AAAAAGTCTCAATCCCTTAC and PLBAs, 5'-GGAATTC-CAGAAGCATCACACTAATTG. The PLB cDNA was subcloned into pGEX1-T vector (GE Healthcare Life Sciences) at an EcoRI site. After the sequence was confirmed by PCR, the GST-PLB plasmid was transformed into Escherichia coli (DH5\alpha strain).

Site-directed mutagenesis was performed. The primers used for generating variants of GST-PLB included 5'-TGG CTC ATC AGG AGA GCC TCG ACT ATT for mutating amino acid Arg to Ala at -2 position (Mut-2), 5'-CGC TGG CTC ATC AGG AGA GCC TCG ACT for mutating Arg to Ala at -3 position (Mut-3), 5'-CTT ACT CGC TCG CTG ATC AGG AGA GCC for mutating Ala to Arg at -5 position (Mut-5, Akt consensus), and 5'-CAA TAC CTT ACT GCC TCG TGT ATC AGG for mutating Arg to Ala at -7 position (Mut-7).

Generation of GST-PLB Fusion Protein—The recombinant GST-PLB fusion proteins were expressed in bacteria after induction with 0.1\mu M isopropyl \beta-D-thiogalactoside for 2 h. To purify the GST-PLB protein, bacteria were collected in PBS, sonicated, and lysed with Triton X-100 (final concentration, 1%) for 30 min at room temperature. After centrifugation (15,000 \times g, 30 min), the GST-PLB proteins were purified by incubating the supernatant with glutathione-Sepharose 4B, which was followed by washing six times with PBS. GST-PLB proteins were confirmed (SDS-PAGE), and the amount of fusion proteins was determined using bovine serum albumin standard on the same SDS-PAGE.

Phosphorylation of GST-PLB by Akt—Different amounts of GST-PLB beads (50% in PBS) were incubated with recombinant Akt1 protein (100 ng) in kinase buffer (8 mM MOPS (pH 7.2); 0.2 mM EDTA; 1.0 mM EGTA; 15 mM MgCl\textsubscript{2}; 100 \mu M ATP; 5 mM \beta-glycerol phosphate; 0.2 mM sodium orthovanadate; 0.2 mM dithiothreitol) for 10 min at room temperature. The reaction was stopped by adding 4-fold SDS-Sample Buffer, and the sample was separated (SDS-PAGE). The phospho-Ser-16 on GST-PLB protein was detected using anti-phospho-PLB (S16) antibody (1:3000).

Phosphorylation of PLB Peptide by Akt—The Akt kinase assay reaction mixtures (25 \mu l) contained 10 mM MOPS/NaOH, pH 7.0, 0.2 mM EDTA, 15 MgCl\textsubscript{2}, 100 \mu M cold ATP, and 0.5 \mu Ci of [\gamma-\textsuperscript{32}P]ATP (3000 Ci/mmol) and various concentrations of substrate. The reaction was carried out for 10 min at 30 °C after adding 100 ng of recombinant Akt and stopped by adding 12.5 \mu l of termination buffer (7.5 mM guanidine hydrochloride). This amount of recombinant Akt and the reaction time was titrated to be in the lineage range. Ten microliters of reaction mix was spotted onto P81 phosphocellulose filter and washed extensively with 0.75% phosphoric acid. Radioactive \textsuperscript{32}P incorporation was counted using a scintillation counter. The \textit{K}_m and \textit{V}_max were determined by performing a nonlinear least-square best fit to the Michaelis-Menten equation using GraphPad Prism 5 (La Jolla, CA).

PKA and CaMKII Activities—Neonatal rat cardiac myocytes infected with Ad.ACV_{VI} or Ad.EGFP or uninfected were stimulated with isoproterenol (10\mu M) and NKH477 (10 \mu M) for 10 min. Cardiac myocytes then were homogenized in buffer (25 mM Tris-HCl (pH 7.4); 0.5 mM EDTA; 0.5 mM EGTA; 10 mM \beta-mercaptoethanol; 50 mM \beta-glycerophosphate; 10 mM NaF; 1 mM Na\textsubscript{3}VO\textsubscript{4}) in the presence of protease inhibitor mixture. The homogenates were centrifuged at 14,000 \times g for 5 min at 4 °C. The supernatant was incubated with PKA biotinylated peptide substrate provided in SigmaTECT\textsuperscript{®} cAMP-dependent Protein Kinase (PKA) Assay System or with CaMKII-biotinylated peptide substrate provided in SigmaTECT Calcium/Calmodulin-dependent Protein Kinase Assay System in the presence of [\gamma-\textsuperscript{32}P]ATP. The \textsuperscript{32}P-labeled and biotinylated substrate was recovered with a streptavidin matrix, and the specific activity of PKA or CaMKII in each sample was determined by measuring the amount of radioactive substrate.

Akt Activity—The Akt kinase assay kit was used for detection of Akt activity in cardiac myocytes. Cardiac myocyte extracts were prepared by lysing in Cell Lysis Buffer (provided in kit) supplemented with protease inhibitors. Lysates were centrifuged at 10,000 \times g (10 min, 4 °C). Akt protein then was specifically precipitated using an anti-Akt antibody. The precipitate then was incubated with recombinant glycogen synthase kinase 3 (GSK-3) \alpha/\beta fusion protein in the presence of ATP and kinase buffer to allow Akt to phosphorylate GSK-3-\alpha/\beta at Ser-21/9 sites. Phosphorylated GSK-3 was detected by immunoblotting using an anti-phospho-GSK-3-\alpha/\beta antibody (1:1000).

Rap1 activity was used to determine the effects of ACV_{VI} on Epac signaling pathways. Rap1 activity was detected using the EZ-Detect Rap1 activation kit and protocol from the manufacturer (Pierce). Cell lysates from ACV_{VI} virus-infected or uninfected cardiac myocytes were incubated with GST-RalGDS-RBD fusion protein. The active GTP-Rap1 pulled down from GST-RBD fusion protein. The active GTP-Rap1 pulled down from GST-RBD fusion protein in the presence of ATP and kinase buffer to allow Akt to phosphorylate GSK-3-\alpha/\beta at Ser-21/9 sites. Phosphorylated GSK-3 was detected by immunoblotting using an anti-phospho-GSK-3-\alpha/\beta antibody (1:1000).

Immunoblotting Analysis—Immunoblotting analysis for detection of Akt and its phosphorylated forms was performed
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Thr-17 phosphorylation (bottom row, lane 6 versus lane 4). Cardiac myocytes infected with Ad.EGFP acted similar to uninfected cells (data not shown). PLB with a single site (Ser-16) phosphorylation has been shown to be sufficient in mediating its maximal cardiac responses to β-agonists (13). The following studies were focused on determining the mechanisms for ACv1-associated Ser-16 phosphorylation of phospholamban.

ACv1-induced PLB Phosphorylation Does Not Require βAR Stimulation—To determine whether catecholamines in the media were contributing to PLB phosphorylation after ACv1 gene transfer, two approaches were used. First, fetal bovine serum was dialyzed to eliminate catecholamines. No difference in ACv1-induced PLB phosphorylation was found between myocytes cultured in dialyzed and undialyzed serum (Fig. 2, lanes 3 and 4 versus lanes 1 and 2), indicating that increased PLB phosphorylation associated with ACv1 gene transfer did not stem from low levels of catecholamines in media. Second, βAR blockade with a β1AR antagonist (CGP20712A, 1 μM and 10 μM) and a β2AR antagonist (ICI118551, 1 μM and 10 μM) did not reduce phosphorylation of PLB (Fig. 2, lanes 5–8 versus lane 2), confirming that ACv1-induced PLB phosphorylation was independent of βAR stimulation.

ACv1 Gene Transfer Increases PLB Phosphorylation Independently of PKA and Epac—Using several approaches, we examined the potential role of PKA activation in PLB phosphorylation associated with ACv1 gene transfer. First, we measured PKA catalytic α-isozyme (PKA-α) expression (immunoblotting) using an antibody against total PKA-α and phospho-PKA-α (Thr-197). ACv1 gene transfer did not affect PKA-α subunit expression or its phosphorylation (Fig. 3A). Second, although PKA activity was increased 5-fold in response to βAR (isoproterenol) and AC (NKH477) stimulations in ACv1-infected cells, basal PKA activity was unaltered by ACv1 (Fig. 3B). Finally, specific inhibition of PKA did not block Ad.ACv1-associated PLB phosphorylation (shown below in Fig. 8, E and F). These data correlate with the effects of ACv1 on intracellular cAMP levels: ACv1 gene transfer does not change basal cAMP levels, but cAMP production in response to βAR or AC stimulation are increased (10, 14, 15). Taken together, these data indicate that PLB phosphorylation evoked by ACv1 occurs by a mechanism that does not involve PKA. ACv1 gene transfer was not associated with activation of calmodulin kinase.
**AC_v1 Increases PLB Phosphorylation via Akt**

A. 

|          | Control | Ad.AC_v1 | Ad.EGFP |
|----------|---------|----------|---------|
| PKA-Cα   | B       | Iso      | NKH     |
| phos-PKA-Cα | B       | Iso      | NKH     |

**FIGURE 3. PKA and Epac. A, detection of PKA-Cα-subunit and phospho-PKA-C (Thr-197) by Western blot analysis. AC_v1 gene transfer did not affect expression or phosphorylation of protein kinase C-Cα. B, analysis of PKA activity. Cultured cardiac myocytes were infected with Ad.AC_v1 or Ad.EGFP or uninfected (Con). Isoproterenol (Iso, 10 μM) and N84077 (NKH, 10 μM) were added for 10 min. Cell homogenates were centrifuged (14,000 × g, 5 min, 4°C), and supernatant was incubated with PKA-biotinylated peptide substrate in the presence of [γ-32P]ATP. The 32P-labeled, biotinylated substrate was recovered with a streptavidin matrix, and the specific activity of CaMKII in each sample was determined after measuring the amount of radioactive substrate. AC_v1 gene transfer did not alter basal (B) PKA activity, but greatly increased PKA activity in response to βAR or AC stimulation. C, analysis of CaMKII activity. Cell homogenates of Ad.AC_v1 virus-infected (AC_v1) or uninfected (Con) cardiac myocytes were incubated with CaMKII biotinylated peptide substrate in the presence of [γ-32P]ATP, and with (Activation) or without (Basal) calmodulin, a CaMKII activator. The 32P-labeled, biotinylated substrate was recovered with a streptavidin matrix, and the specific activity of CaMKII in each sample was determined after measuring the amount of radioactive substrate. AC_v1 gene transfer did not alter basal (basal) CaMKII activity, neither the activity in response to calmodulin (activation). D, Rap1 activity. Cardiac myocytes were incubated with 100 μM [8-4-chlorophenylthio]2′-O-methyl-cAMP (8-CPT) or Brefeldin A (BFA) for 24 h. GTP-bound active Rap1 was pulled down using GST-RalGDS-RBD fusion protein. The Rap1 was detected by immunoblotting using anti-Rap1 antibody. Total Rap1 protein was detected from cell lysate without pulldown.

II (CaMKII) (Fig. 3C), which was in line with the lack of PLB phosphorylation on Thr-17 (Fig. 1).

To determine whether the Epac signaling pathway was activated by AC_v1 gene transfer, intracellular active Rap1 was quantified. We found no difference in Rap1 activity between AC_v1 virus-infected and uninfected myocytes, with or without serum. The Epac activator (8-CPT) slightly increased active Rap1, but again, in both AC_v1 virus-infected and uninfected myocytes (Fig. 3D). Therefore, gene transfer of AC6 did not activate the Epac signaling pathway.

**AC Catalytic Activity Is Not Required for Phosphorylation of PLB and Akt**—To determine whether catalytic activation of AC is required for PLB or Akt phosphorylation, we used AC P-site inhibitors. Two P-site inhibitors (SQ22536 and 9-CPA) (16–18) greatly reduced forskolin stimulated AC activity in AC_v1 virus-infected myocytes (Fig. 4A). Another P-site inhibitor, foscanet (a pyrophosphate analog), was able to inhibit AC activity in uninfected cardiac myocytes (Fig. 4B), but did not inhibit AC activity in AC_v1 virus-infected myocytes (Fig. 4, A and C), which suggests that endogenous AC isoforms and increased AC_v1 have different sensitivities to foscarnet inhibition (19). None of these reagents inhibited AC_v1-associated phosphorylation of PLB or Akt (Fig. 4D), indicating that the catalytic activation of transgene AC_v1 is not required for its effects on PLB or Akt phosphorylation.

**AC_v1 Gene Transfer Increases Akt Phosphorylation and Activity**—AC_v1 gene transfer increased phosphorylation of Akt at both the Ser-473 and Thr-308 sites but did not change the levels of Akt protein (Fig. 5A). To determine whether Akt phosphorylation was associated with increased Akt activity, Akt was immunoprecipitated from cardiac myocyte lysates, and activity was assessed using recombinant GSK3αβ fusion protein as a substrate in vitro. AC_v1 gene transfer increased Akt activity 5-fold versus Ad.EGFP-infected cardiac myocytes (Fig. 5B, lane 2 versus lane 1). Sustained (22 h) βAR stimulation increased Akt activity in control cells (Fig. 5B, lane 3 versus lane 1) but not further enhance Akt activity in Ad.AC_v1-infected cells (Fig. 5B, lane 4 versus lane 2). We next determined whether phosphorylation of GSK-3, an endogenous Akt substrate, was increased after AC_v1 gene transfer. GSK-3 phosphorylation was detected using antibodies against its α- and β-isoforms. We found that AC_v1 gene transfer increased phosphorylation of Ser-21 on the α-isoform and Ser-9 on the β-isoform (Fig. 6).

**AC_v1 Gene Transfer Increases Nuclear Phospho-Akt**—To detect the intracellular location of Akt, we used anti-phospho-Akt (Ser-473) antibody and immunofluorescence staining followed by deconvolution analysis. In uninfected cardiac myocytes, phospho-Akt was detected in the cytoplasm, but was barely detectable in the nucleus. In contrast, after AC_v1 gene transfer, nuclear phospho-Akt was substantially increased (Fig. 7). As previously shown (Fig. 5), Akt activation did not require βAR stimulation.

**AC_v1 Gene Transfer Increases PLB Phosphorylation via Akt**—To determine if PLB was an Akt substrate in the setting of increased AC_v1 expression, we used three approaches. First, using GST-PLB fusion as substrate, we asked whether recombinant Akt protein could phosphorylate PLB Ser-16 in vitro. Phospho-PLB Ser-16 was detected using anti-phospho-PLB (Ser-16) antibody in immunoblotting after kinase
Akt1 in the presence of concentrations of PLB substrate were phosphorylated by 100 ng of Akt1, the Thr-17 mutant, was used to evaluate the specificity of the phosphorylation of the Ser-16 mutant as a negative control. An additional mutant on a consensus peptide, RPRAATF, as a positive control and PLB determined the kinetics of recombinant Akt1 phosphorylation critical for Akt to phosphorylate PLB.

These data confirmed that PLB is one of the Akt substrates and that the three arginines at position -3 was changed to arginine (called mut-2, mut-3, and mut-7, respectively). In addition, the alanine at position -5 was changed to arginine (called mut-5). To determine the amino acids around Thr-17 did not affect Akt phosphorylation of PLB on Ser-16, but mutation at Ser-16 completely abolished PLB phosphorylation, confirming the specificity of the phosphorylation (data not shown). These results indicated that PLB is one of the relative specific Akt substrates, which is consistent with the findings of others for these non-consensus Akt substrates (20, 21).

Finally, we used Akt inhibitors to attempt to prevent phosphorylation of endogenous PLB. All three Akt inhibitors, in contrast to PKA inhibitors, blocked ACV1-associated PLB phosphorylation (Fig. 8, E and F). Data on Akt V not shown. These experiments indicate that increased ACV1 expression results in phosphorylation of PLB via activation of Akt.

**DISCUSSION**

The most important finding of this study is that increased ACV1 expression in cardiac myocytes leads to increased PLB phosphorylation on Ser-16, but not on Thr-17, via activation of Akt, and that this occurs independently of activation of cAMP, PKA, or Epac or catalytic activity of AC. This previously unrecognized interaction between Akt and PLB provides a mechanism by which cardiac myocyte function may be increased independent of βAR activation and cAMP generation.

**PLB Phosphorylation**—Typically, PKA phosphorylates PLB at Ser-16 (22, 23). However, in seeking a mechanism for the increased level of PLB phosphorylation associated with ACV1 expression, we found that the process was not the result of PKA activation (Figs. 3 and 6). Other kinases known to phosphorylate PLB include protein kinase C, cGMP-dependent protein kinase, and myotonic dystrophy protein kinase (24–27). However, cGMP-dependent protein kinase and myotonic dystrophy protein kinase expression were unchanged by ACV1 expression (data not shown), and we previously reported that cardiac protein kinase C activity and content were not altered by increased ACV1 expression (28).

To seek a PKA-independent means for PLB phosphorylation, we asked whether Akt, an important kinase in multiple signaling pathways, might be involved. We found that increased ACV1 was associated with increased phosphorylation and activity of Akt. Akt-specific inhibitors, but not a PKA inhibitor, diminished ACV1-induced phosphorylation, which strongly indicates that Akt is responsible for increased phosphorylation of PLB after ACV1 gene transfer. ACV1-associated Akt phosphorylation was also inhibited by phophatidylinositol 3-kinase inhibitors (data not shown). The precise mechanism by which ACV1 influences phosphatidylinositol 3-kinase and Akt activation is the focus of ongoing studies in our laboratory.
ACVI Increases PLB Phosphorylation via Akt

**A.**

|   | Con | ACVI | EGFP |
|---|-----|------|------|
| P-Ser473 | ![P-Ser473](image1) | ![P-Ser473](image2) | ![P-Ser473](image3) |
| P-Thr308 | ![P-Thr308](image4) | ![P-Thr308](image5) | ![P-Thr308](image6) |
| Total Akt | ![Total Akt](image7) | ![Total Akt](image8) | ![Total Akt](image9) |

**B.**

|   | Basal | Iso |
|---|-------|-----|
| EGFP | ![EGFP](image10) | ![EGFP](image11) |
| ACVI | ![ACVI](image12) | ![ACVI](image13) |
| P-GSK3 α/β fusion protein | ![P-GSK3 α/β fusion protein](image14) | ![P-GSK3 α/β fusion protein](image15) |

**FIGURE 5.** ACVI gene transfer increased phosphorylation and activity of Akt kinase. A, cardiac myocyte lysates were separated on a SDS-PAGE and phospho-Akt protein was detected by Western blot analysis using anti-phospho-Akt (Ser473) and anti-phospho-Akt (Thr308) antibodies. ACVI gene transfer increased Akt phosphorylation (lane 2). B, Akt activity was determined using recombinant GSK3 α/β fusion protein as substrate. Total Akt was immunoprecipitated from cell lysates from each condition using anti-Akt antibody. Immunoprecipitant then was incubated with recombinant GSK3 α/β fusion protein in the presence of ATP and kinase buffer. Phosphorylated GSK3 α/β was detected by Western blot analysis using anti-GSK3 α/β antibody. ACVI gene transfer increased basal Akt activity (lane 2 versus lane 1); isoproterenol (Iso) stimulation (22 h) did not increase activity (lane 2 versus lane 4), although Ad.EGFP cells did show a response (lane 1 versus lane 3).

**FIGURE 6.** Consequences of ACVI gene transfer and Akt activation. Phosphorylation of GSK3, an Akt substrate. Cardiac myocyte lysates from each condition were separated (SDS-PAGE), and phosphorylation of GSK3 α on Ser21 and Ser9 was detected by immunoblotting analysis using anti-phospho-GSK3 antibodies. ACVI gene transfer increased phosphorylation of GSK3 in cardiac myocytes (lane 2).

**FIGURE 7.** Localization of phospho-473-Akt. Immunofluorescence staining with anti-phospho-Akt (Ser473) (green). In control cardiac myocytes, a low level of endogenous phospho-Akt is distributed throughout the cell (top row). After ACVI gene transfer, increased phospho-Akt was detected not only in cytosol, but also in nucleus (bottom row). Nuclei were stained with Hoechst dye (blue). Experiments were repeated four times with similar results. The images were obtained with a 40× lens using a DeltaVision system and were subjected to deconvolution. Exposures were captured with a CoolSnap camera. To enable comparison, exposure times were identical for each fluorophore and kept within the linear range of the camera.

Does PLB possess structural features that are typical of other Akt substrates? Akt belongs to the AGC kinase family and has 47% sequence homology with the catalytic subunit of PKA (29). Akt can phosphorylate proteins containing a PKA site such as cAMP-response element-binding protein (30). Most of the Akt substrates contain an Akt consensus site RXRXXS, where X represents any amino acid and S represents a serine (21). Other substrates identified through peptide and protein library screening defined Akt substrate motifs as RXRXXXS where the R at position −7 (i.e. 7 amino acids upstream of S) is important for Akt phosphorylation (31), and R at −5 is dispensable (32). Akt also can phosphorylate actin on sequences XXXXXX(XS) (33), which is divergent from its consensus sequences. PLB contains sequences RXXXXXXS around serine 16 that are similar to the above identified sequences with R at −7 (34). The 3 arginines at −2, −3, and −7 are critical for Akt phosphorylation. Mutating any one of them abolished PLB phosphorylation by Akt (Fig. 8, C and D). Thus, PLB possesses structural features of other Akt substrates, making its phosphorylation by Akt structurally feasible. Although the K_m for PLB peptide phosphorylation in vitro is relatively high compared with the other Akt substrates (21), the maximal PLB phosphorylation induced by ACVI gene transfer (Fig. 1) indicate that ACVI might facilitate Akt to phosphorylate PLB in vivo with unknown mechanisms.

Gene transfer of ACVI did not increase PLB phosphorylation on Thr-17 (Fig. 1) and even inhibited Thr-17 phosphorylation in response to forskolin stimulation. Lack of Thr-17 phosphorylation after ACVI gene transfer indicates that the effects of ACVI differ from βAR agonist stimulation on cardiac myocytes (35). Absence of Thr-17 phosphorylation reflects the absence of CaMKII activation by ACVI expression (Fig. 3D). CaMKII inhibition is beneficial to the heart by protecting myocytes against necrosis, apoptosis, and hypertrophy in animals (36–39).
Therefore, lack of CaMKII activation and Thr-17 PLB phosphorylation could be another mechanism for the salutary effects of ACVI.

Akt and Cardiac Function—ACVI expression increased nuclear phospho-Akt. Whether this represents nuclear translocation or, alternatively, increased activation of Akt already present in the nucleus was not determined. Nuclear-targeted Akt expression was found to increase cardiac PLB phosphorylation in transgenic mice (40), so nuclear Akt activation, seen after ACVI gene transfer in the present study, is likely to be an important mechanism in PLB phosphorylation.

Increased phospho-Akt is likely to have additional potentially important consequences. For example, Akt signaling affects the balance between survival and programmed cell death (apoptosis) in cardiac myocytes and other cells (41, 42). This is achieved by phosphorylation and deactivation of pro-apoptotic factors such as Bad, caspase-9, and Forkhead transcription factors (AFX, Daf-16, and FKHR) (41, 43–48), and by increasing the expression of anti-apoptotic proteins.

FIGURE 8. A, phosphorylation of PLB by recombinant Akt1. GST/PLB recombinant protein was produced and purified from bacteria. GST/PLB-bound agarose beads (2 μl) were incubated (10 min, 30°C) with increasing amounts of Akt1 in the presence of ATP and kinase buffer. Phospho-Ser-16-PLB was detected using anti-phospho-PLB (Ser-16) antibody. Recombinant Akt phosphorylated PLB at Ser-16. B, wild type and mutants of GST-PLB around serine 16. Serine 16 is defined as "0," and amino acids at 5' of serine 16 were defined as negatives. The mut-7, mut-3, and mut-2 have an "R" mutated to "A," and mut-5 has an "A" mutated to an "R" by site-directed mutagenesis. C, immunoblots of WT and mutants of GST-PLB phosphorylated by Akt1. WT or mutant GST-PLB protein was phosphorylated by Akt1 (100 ng) as described in A, and phospho-Ser-16 was detected using anti-phospho-PLB (Ser-16) antibody. Mutation at −2 and −3 completely abolished the ability of Akt1 to phosphorylate PLB Ser-16. D, phosphorylation of WT and mutants of GST-PLB by Akt1 at varies substrate concentrations. Phospho-Ser-16 was detected in immunoblotting using anti-phospho-PLB (Ser-16) antibody. The graph was plotted by intensity of phospho-PLB-Ser-16 on immunoblotting versus concentration of each GST-PLB protein. Because mutation at −2 and −3 completely abolished the ability of Akt1 to phosphorylate PLB at Ser-16, these mutants were not included in the graph. E, Akt phosphorylation of PLB in cardiac myocytes. Ad.ACVI-infected cardiac myocytes received no inhibitor (−), Akt inhibitor (Akt, 10 μM); Akt inhibitor II (Akt II, 10 μM); Akt inhibitor V (10 μM, data not shown); or PKA inhibitor (PKI, 10 μM) for 24 h. Lysates from each condition were separated (SDS-PAGE), and phospho-Ser-16-PLB was detected using phospho-specific antibody. ACVI gene transfer increased phosphorylation of PLB (lane 2 versus lane 1). Inhibition of Akt prevented PLB phosphorylation (lane 3), but PKA inhibition had no effect (lane 4). F, PKA inhibitors (PKI, 20 μM, Rp-8-CPT-cAMP (Rp8), 20 μM and H89, 10 μM) were incubated with cardiac myocytes for 24 h and phospho-PLB at Ser-16 or phospho-Akt at Ser473 were detected in immunoblotting as described above. PKA was not involved in ACVI-induced PLB and Akt phosphorylation in vivo.
such as Bcl-2 and Bcl-xL (49, 50). Cardiac myocyte number is reduced in heart failure, which is thought to be due, at least in part, to increased apoptosis (51). Mice with cardiac-directed expression of a constitutively active Akt show cardiac hypertrophy and increased systolic function (52), but sustained expression of Akt can lead to heart failure (53). In contrast, cardiac-directed and nuclear-targeted Akt expression increases contractile function (40, 54), inhibits apoptosis, and protects the heart during ischemia-reperfusion injury (55). In the present studies, we found nuclear translocation of activated Akt evoked by increases in ACV1 levels. We also found that increased cardiac levels of ACV1 are associated with reduced apoptosis in an animal model of congestive heart failure (56), suggesting that our findings in cultured cells may have important physiological implications.

**PLB and Cardiac Function—**PLB plays an important role as an inhibitor of the sarcoplasmic reticulum Ca2+ ATPase in cardiac myocytes. For example, βAR stimulation, through PKA activation, phosphorylates PLB, which disinhibits sarcoplasmic reticulum Ca2+ ATPase and facilitates Ca2+ uptake into the sarcoplasmic reticulum, increasing cardiac function (57). However, sustained βAR activation is associated with cardiac myocyte apoptosis (58, 59), which is not seen in animals expressing nuclear Akt (40) or ACV1 (56). Reduction of PLB through antisense suppression or genetic ablation increases sarcoplasmic reticulum calcium cycling and increases cardiac function (60, 61), and cardiac-directed expression of PLB decreases heart function (62). In contrast, increased expression of ACV1 in cardiac myocytes not only reduces the expression of PLB (8), but also, as we show here, increases PLB phosphorylation through increased Akt activity, effects which would be predicted to have beneficial effects on cardiac function.

**CONCLUSION**

Increased ACV1 protein activates Akt, which then phosphorylates PLB. ACV1 gene transfer is also associated with increased nuclear phospho-Akt. These previously unrecognized signaling events occur in the absence of βAR and PKA activation, which may be why this adrenergic intervention, unlike others, has beneficial effects. The discovery that ACV1 increases activation of Akt and phosphorylation of PLB, proteins that influence contractile function and cell survival, provides a plausible mechanism for the beneficial effects of ACV1 on the failing heart.

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