Akt Increases Sarcoplasmic Reticulum Ca\(^{2+}\) Cycling by Direct Phosphorylation of Phospholamban at Thr\(^{17}\)*

Received for publication, June 23, 2009, and in revised form, August 5, 2009. Published, JBC Papers in Press, August 19, 2009.

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Cardiomyocytes adapt to physical stress by increasing their size while maintaining cell function. The serine/threonine kinase Akt plays a critical role in this process of adaptation. We previously reported that transgenic overexpression of an active form of Akt (Akt-E40K) in mice results in increased cardiac contractility and cell size, as well as improved sarcoplasmic reticulum (SR) Ca\(^{2+}\) handling. Because it is not fully elucidated, we decided to study the molecular mechanism by which Akt-E40K overexpression improves SR Ca\(^{2+}\) handling. To this end, SR Ca\(^{2+}\) uptake and the phosphorylation status of phospholamban (PLN) were evaluated in heart extracts from wild-type and Akt-E40K mice and mice harboring inducible and cardiac specific knock-out of phosphatidylinositol-dependent kinase-1, the upstream activator of Akt. Moreover, the effect of Akt was assessed in vitro by overexpressing a mutant Akt targeted preferentially to the SR, and by biochemical assays to evaluate potential interaction with PLN. We found that when activated, Akt interacts with and phosphorylates PLN at Thr\(^{17}\), the Ca\(^{2+}\)-calmodulin-dependent kinase II\(\beta\) site, whereas silencing Akt signaling, through the knock-out of phosphatidylinositol-dependent kinase-1, resulted in reduced phosphorylation of PLN at Thr\(^{17}\). Furthermore, overexpression of SR-targeted Akt in cardiomyocytes improved Ca\(^{2+}\) handling without affecting cell size. Thus, we describe here a new mechanism whereby the preferential translocation of Akt to the SR is responsible for enhancement of contractility without stimulation of hypertrophy.

Akt, also referred to as protein kinase B, is a serine/threonine kinase found as part of the insulin, insulin-like growth factor-1 (IGF-1)3/phosphatidylinositol 3-kinase (PI3K)/phosphatidylinositol-dependent kinase-1 (PDK1) pathway (1). Upon activation, Akt phosphorylates a broad range of substrates involved in metabolism, transcription, translation, cell growth, differentiation, proliferation, and survival (2, 3). In the heart the IGF-1/Akt axis is implicated in the control of physiological cardiac hypertrophy, contractile function, and Ca\(^{2+}\) handling (4–11).

The cardiac effects of Akt have been described in many transgenic (Tg) mouse models, and cardiac specific overexpression of different mutated forms of Akt results in varying phenotypes (5, 12–16). A common effect observed in most of these Tg mouse lines is increased cardiomyocyte (CM) size and either maintained or improved cardiac function (5, 12–14), with modest or no activation of either mitogen-activated protein kinases (MAPks) or fetal genes, two hallmarks of maladaptation to stress (5, 12, 13).

Our group has previously described a Tg mouse model (Akt-E40K Tg) expressing an Akt with a mutation (E40K) located in its pleckstrin homology domain. This mutation renders Akt constitutively active in a manner similar to stimulation with growth factor (5). The occurrence of increased CM size with preservation of cardiac contractility constitutes an intriguing aspect of this mouse model. Single cell studies revealed not only increased inotropic but also enhanced lusitropism and an increase in systolic calcium (Ca\(^{2+}\)) transients in adult CMs isolated from this Tg mouse (5, 15). Enhanced contractility secondary to improved Ca\(^{2+}\) handling is also a feature of a Tg mouse line with nuclear overexpression of Akt (16). However, in contrast to the Akt-E40K Tg mouse, an increase in protein kinase A (PKA) activity inducing phosphorylation of phospholamban (PLN) at residue Ser\(^{16}\) was reported to occur in that model. These features raise relevant questions regarding the specific mechanisms by which Akt controls Ca\(^{2+}\) handling.

At the sarcoplasmic reticulum (SR) the activity of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA)2a, a pump that

* This work was supported, in whole or in part, by National Institutes of Health Grant HL078797-01A1. This work was also supported in part by a Marie Curie International Fellowship within the 6th European Framework Programme (to D. C.) and the Italian Ministry of University and Research, the Italian Ministry of Health.

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2 Supported by the Canadian Institutes of Health Research, the Alberta Heritage Foundation for Medical Research, the Canada Foundation for Innovation, and the Alberta Science and Research Investments Program.

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4 The abbreviations used are: IGF-1, insulin-like growth factor-1; CAMKII\(\beta\), Ca\(^{2+}\)-calmodulin-dependent protein kinase II\(\beta\); CM, cardiomyocyte; KO, knockout; PDK1, phosphatidylinositol-dependent kinase-1; PKA, protein kinase A; PLN, phospholamban; SERCA2a, sarcoplasmic reticulum Ca\(^{2+}\)-ATPase 2a; SR, sarcoplasmic reticulum; Tg, transgenic; WT, wild type; P3K, phosphatidylinositol 3-kinase; PBS, phosphate-buffered saline; ANOVA, analysis of variance; AIP, autacamide 2-related inhibitory peptide.
transports Ca\(^{2+}\) from the cytosol to the SR lumenal space, is dependent on PLN. In its dephosphorylated state, PLN binds to SERCA2a and inhibits Ca\(^{2+}\) pump activity, whereas phosphorylation of PLN disrupts the interaction with SERCA2a, relieving Ca\(^{2+}\) pump inhibition and enhancing relaxation rate and contractility (17). Relief from the inhibitory effects of PLN is the principal contributor to the positive inotropic and lusitropic effects of \(\beta\)-adrenergic agonists (17). The activity of PLN is inhibited by protein kinase A (PKA) and Ca\(^{2+}\)-calmodulin-dependent protein kinase (CAMKII\(\alpha\), which phosphorylate PLN at Ser\(^{16}\) and Thr\(^{17}\), respectively (17). Phosphorylation of PLN by PKA was demonstrated to be a prerequisite for subsequent phosphorylation by CAMKII\(\beta\) following \(\beta\)-adrenergic agonist stimulation (18). Phosphorylation of PLN at Ser\(^{16}\) was also reported to be sufficient to mediate the inotropic responses of the heart to \(\beta\)-adrenergic agonists (19). However, during other types of stimuli, such as ischemia or frequency-dependent stimuli, Thr\(^{17}\), but not Ser\(^{16}\), becomes phosphorylated, suggesting that phosphorylation at Thr\(^{17}\) is more relevant than that of Ser\(^{16}\) under certain conditions (17, 20). Moreover, it was recently reported that PLN phosphorylation at Ser\(^{16}\) is determined by increased protein levels of adenyl cyclase VI (21). This adenyl cyclase overexpression was shown to be associated not with PKA activation but rather with phosphorylation of Akt and its substantial relocalization to the nucleus that, similar to results obtained in nuclear Akt Tg mice (16), resulted in Ser\(^{16}\) PLN phosphorylation.

In this study, we demonstrate that upon physiological activation Akt translocates to the SR where it interacts with and phosphorylates PLN at Thr\(^{17}\). This leads to improved contractility without stimulating hypertrophic growth normally associated with Akt.

**EXPERIMENTAL PROCEDURES**

**Mice**—All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. The generation of Tg mice with cardiac specific overexpression of constitutively active Akt (Akt-E40K) has been described previously (5). Cardiac specific PDK1-inducible knock-out (KO) mice (MerCreMer \(\alpha\)-MHC PDK1) were generated as described elsewhere (11). Adult and neonatal mouse CMs were isolated according to published procedures (15, 22). Adult CMs were from age-matched mice between 8 and 13 weeks of age.

**Ca\(^{2+}\) Transient Measurements**—1-Day-old neonatal mouse CMs were loaded with the Ca\(^{2+}\) indicator fura 4-AM (Molecular Probes, Eugene, OR) by incubating the cells in Tyrode’s solution (in mM, 121 NaCl, 4.6 KCl, 1.2 CaCl\(_2\), 1.2 MgSO\(_4\), 1.2 NaH\(_2\)PO\(_4\), 15 glucose, and 10 HEPES, pH 7.4) containing 2 \(\mu\)mol/liter fura 4-AM for 20 min. The cells were then washed with Tyrode’s solution and incubated for 30 min. One more wash was performed before mounting the chamber on an epifluorescence inverted microscope (Nikon TS100) with an attached CCD camera (Ionoptix MyoCam, Ionoptix, Milton, MA). The coverslip was superfused with Tyrode’s solution at room temperature at a rate of 20 ml/h. Cells were stimulated using an external stimulator, and Ca\(^{2+}\) concentration was measured using a dual excitation spectrofluorometer that detects the fluorescence excited by UV light at 360 and 380 nm with an emission at 505 nm.

**SR-enriched Microsomes and Cytosolic Fractions**—Homogenization of pulverized ventricular tissue from WT and Tg mouse hearts, as well as isolation of SR-enriched microsomes and cytosolic fractions, was done according to published procedures (23, 24).

**Measurements of SR Ca\(^{2+}\) Uptake**—SR Ca\(^{2+}\) uptake assays were performed on ventricular homogenates at room temperature based on a protocol by Pagani and Solaro (25) and modified in the Dillmann laboratory (26). When used, 40 \(\mu\)M Akt-IV (Calbiochem), 5 \(\mu\)M AIP, or 5 \(\mu\)M KN93 was preincubated with homogenized aliquots at room temperature before adding ATP.

**Cell Culture and Insulin Induction Assays**—CMs were cultured in serum-free medium for 3 h prior to stimulation with 0.1 mM insulin for 40 min. When necessary, cells were pretreated with inhibitors (0.1 mM KN93 or 10 \(\mu\)M Akt inhibitor IV) for 10 min. These reagents were obtained from Calbiochem. At the end of the stimulation period, cells were rinsed in ice-cold PBS and scraped on ice in lysis buffer (in mM, 150 NaCl, 50 Tris, pH 7.5, 30 NaF, 0.1 Na\(_3\)VO\(_4\), and 1 phenylmethylsulfonyl fluoride) containing 1% Triton X-100 and a protease inhibitor mixture (Complete, Roche Applied Science). Lysates were cleared (14,000 rpm for 10 min at 4 °C) and analyzed by Western blotting.

**Immunoprecipitation of Hemagglutinin (HA)-tagged Akt-E40K and Kinase Assays**—Hearts from Tg and WT mice were homogenized at 4 °C in 1 ml of homogenization buffer (in mM, 25 imidazole, pH 7.0, 150 KCl, containing 0.1% Triton, 0.1% Nonidet P-40, and protease inhibitor mixture) with a Teflon glass Thomas tissue grinder. After a 20-min centrifugation at 14,000 rpm at 4 °C, 50 ml of mono-HA11 beads (Covance Babco, affinity matrix mono-HA11) was added to the supernatant and incubated for 60 min at 4 °C with gentle shaking. For coimmunoprecipitation experiments, beads were washed three times with homogenization buffer and then resuspended in 5× sample buffer without reducent. Samples were boiled and loaded onto SDS-PAGE. For in vitro kinase assay and SR uptake, beads were washed with homogenization buffer and added to the homogenized heart obtained from WT mice in kinase buffer (in mM, 25 imidazole, 10 MgCl\(_2\), 0.1 sodium vanadate, 20 NaF, and 0.05 ATP). Reaction mixtures were incubated for 25 min at 30 °C. Subsequently, beads were resuspended in 5× sample buffer without reducent for Western blot analysis or used to measure SR Ca\(^{2+}\) uptake, as described above. An in vitro Akt kinase assay was performed as follows: recombinant PLN was obtained as described previously (27), whereas purified active Akt was purchased from Cell Signaling (Waltham, MA). The kinase assay was performed according to the manufacturer’s instructions, with or without active Akt; detection of total PLN and PLN phosphorylated at Ser\(^{16}\) or Thr\(^{17}\) was performed by Western blot analysis. PKA activity was measured using a cAMP assay kit (Upstate, Charlottesville, VA) according to the manufacturer’s instructions.

**Electrophoresis and Western Blot Analysis**—SDS-PAGE and Western blot analysis were performed on total cytosolic lysate and the SR fraction from WT, Tg, or KO mouse ventricles. The
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following antibodies were used: anti-total PLN and anti-phospho Thr\(^{17}\) PLN antibodies (Badrilla, Leeds, UK); anti-phosphoser\(^{16}\) PLN antibody (Upstate, Charlottesville, VA); anti-phospho-Ser\(^{173}\) Akt and anti-total Akt antibodies (Cell Signaling); anti-phospho-Thr\(^{387}\) CAMKII\(\delta\) antibody (Affinity Bioreagents, Golden, CO); anti-total CAMKII\(\delta\) antibody (kindly provided by Dr. Harold Singer) (28); and anti-PDK1 antibody (Calbiochem).

Adenovirus Generation—Three adenoviruses were produced. One (mock) was an infective virus with no transgene expression. The second (Ad Akt-E40K) contained the HA-tagged E40K Akt transgene used for production of our Tg mouse line. The third (Ad Akt-PLN) contained an active form of Akt linked to an SR localization sequence at the C terminus. Constitutive activity of the T308D/S473D Akt mutant, in which the aspartic acid residues mimic phosphorylation, has been described previously (1, 29). This mutant is biologically active and able to mediate some of the effects of Akt, including transformation of chicken embryo fibroblasts (30). To serve as an SR localization signal, amino acids 23–52 of the transmembrane region of PLN were used. In addition, this PLN sequence contained mutations L31A and N34A to eliminate its ability to inhibit SERCA activity. This sequence was obtained from the consensus sequence of PLN for CAMKII activity. This sequence was obtained from the

\[5\text{tcgcccagacgcaatcagagcgtcgtagccgacgccgatcaggagtttgag}-5\]

was used for amplification. Finally, viral vectors were amplified and purified—Isolated Ca\(^{2+}\) tolerant CMs were plated with Reduced Phosphorylation of PLN at Thr\(^{17}\) in this, cytosolic and SR extracts were probed with antibodies for constitutive activity is reduced in Tg mice hearts and, consequently, plays a minor, if any, role in phosphorylation of PLN at Thr\(^{17}\) in this model.

\[5\text{tcgcccagacgcaatcagagcgtcgtagccgacgccgatcaggagtttgag}-5\]

activity might be higher in the hearts of Tg than WT. To test this, cytosolic and SR extracts were probed with antibodies for total and active, Thr\(^{446}\)-phosphorylated CAMKII\(\delta\). Surprisingly, phosphorylation of CAMKII\(\delta\) at Thr\(^{446}\) was decreased in Tg compared with WT (Fig. 1B). This indicated that CAMKII\(\delta\) activity is reduced in Tg mice hearts and, consequently, plays a minor, if any, role in phosphorylation of PLN at Thr\(^{17}\) in this model.

\[5\text{tcgcccagacgcaatcagagcgtcgtagccgacgccgatcaggagtttgag}-5\]

were repeated at least twice to confirm the fluorescence patterns.

\[5\text{tcgcccagacgcaatcagagcgtcgtagccgacgccgatcaggagtttgag}-5\]

Analysis was performed using GraphPad Prism version 4.0 for Mac (GraphPad Software, San Diego).

RESULTS

Overexpression of Akt-E40K Induces Phosphorylation of PLN at Thr\(^{17}\) Independently of CAMKII\(\delta\)—We decided first to determine SERCA2a activity in Tg mice by analyzing the phosphorylation status of PLN at Ser\(^{16}\) and Thr\(^{17}\) by Western blotting. We found that in Tg heart extracts phosphorylation of PLN at Thr\(^{17}\) was increased when compared with WT, whereas phosphorylation at Ser\(^{16}\) was unchanged or even reduced (Fig. 1A). The partial reduction of PLN phosphorylation at Ser\(^{16}\) in Tg could be explained by a decrease in PKA activity that we found in these mice compared with WT, as evaluated by measurement of the level of cAMP (0.270 \(\pm\) 0.015 pmol/mg protein, \(p < 0.05\), respectively).

Because phosphorylation at Thr\(^{17}\) is an indirect measurement of CAMKII\(\delta\) activity, we hypothesized that CAMKII\(\delta\) activity might be higher in the hearts of Tg than WT. To test this, cytosolic and SR extracts were probed with antibodies for total and active, Thr\(^{446}\)-phosphorylated CAMKII\(\delta\). Surprisingly, phosphorylation of CAMKII\(\delta\) at Thr\(^{446}\) was decreased in Tg compared with WT (Fig. 1B). This indicated that CAMKII\(\delta\) activity is reduced in Tg mice hearts and, consequently, plays a minor, if any, role in phosphorylation of PLN at Thr\(^{17}\) in this model.
Akt phosphorylates PLN at Thr\(^{17}\)

**FIGURE 1.** Phosphorylation of PLN at Thr\(^{17}\) is not CAMKII-dependent in Akt-E40K Tg mice. A, left, total extracts from WT and Tg mouse hearts were separated by SDS-PAGE under reducing conditions and incubated with anti-phospho-Thr17-PLN, anti-phospho-Ser16-PLN, anti-total PLN, and anti-actin antibodies. Increased phosphorylation of PLN at Thr\(^{17}\) is evident in the Tg lane. Right, densitometric analysis presented relative to WT \((n = 3)\), \(* p < 0.05\) Tg versus WT. B, left, blots of cytosolic and SR extracts from WT and Tg mouse hearts were incubated with anti-phospho-CAMKII\(^{\alpha}\), anti-total CAMKII\(^{\alpha}\), and anti-actin antibodies. Surprisingly, CAMKII\(^{\alpha}\) phosphorylation is decreased in Tg hearts compared with WT. Right, densitometric analysis presented relative to WT \((n = 3)\), \(* p < 0.05\) Tg versus WT.

**FIGURE 2.** Reduced phosphorylation of PLN at Thr\(^{17}\) in PDK1 knock-out (KO) mouse hearts were processed for Western blotting. The results obtained suggest that phosphorylation of PLN at Thr\(^{17}\) is Akt-dependent and not CAMKII\(^{\alpha}\)-dependent. Right, densitometric analysis \((n = 3)\) for each of the presented data. \(* p < 0.05\); \(** p < 0.01\) KO versus WT.

and WT (Fig. 2). These results corroborated the hypothesis that Akt might be involved in PLN phosphorylation at Thr\(^{17}\).

**Active Akt Localizes to the SR and Directly Interacts with and Phosphorylates PLN—**Direct phosphorylation of PLN by Akt implies that these two proteins colocalize and physically interact with each other at the SR. In fact, Western blotting with both anti-HA-tagged and anti-phospho-Akt antibodies revealed that Akt-E40K was indeed found at the SR in Tg (Fig. 3A). Importantly, Akt translocates to the SR also in a more physiological setting, i.e. treatment of WT CMs with insulin. In fact, confocal microscopy images of in vitro WT CMs stained for total Akt revealed a rather homogeneous and diffuse staining in untreated CMs but a striated pattern, which was superimposed to that for PLN, after insulin stimulation (Fig. 3B).

**Further evidence for direct interaction between Akt and PLN came from pulldown experiments in which Akt-E40K was immunoprecipitated from heart extracts with an anti-HA antibody and PLN probed for Western blotting with an anti-PLN antibody. We found that in Tg extracts, PLN coprecipitated with Akt-E40K (Fig. 3C). In addition, in an in vitro kinase assay in which recombinant full-length PLN was incubated with or without a commercially available purified active Akt in an in vitro kinase assay. Samples were subsequently separated by SDS-PAGE and probed with anti-phospho-Thr\(^{17}\)-PLN, anti-phospho-Ser\(^{16}\)-PLN, and total PLN antibodies. A band corresponding to PLN was detected with anti-phospho-Thr\(^{17}\)-PLN but not with anti-Ser\(^{16}\)-PLN. The blots shown are representative of three independent experiments.

**Akt Improves SR Ca\(^{2+}\) Uptake—**The effect of Akt on SR Ca\(^{2+}\) handling was assessed by an SR \(^{45}\)Ca\(^{2+}\) uptake assay on myocardial extracts from Tg and WT mice. SERCA2a pump activity can be monitored by \(^{45}\)Ca\(^{2+}\) uptake at a free \([\text{Ca}^{2+}]_o\) of 20 nm, a concentration at which the inhibitory effects of PLN are amplified (25). We found that \(^{45}\)Ca\(^{2+}\) uptake was significantly enhanced in homogenates of Tg heart compared with those of WT (4.9 and 0.8% of the WT maximal rate, respectively) (Fig. 5). Inhibition of Akt reduced the increased \(^{45}\)Ca\(^{2+}\) uptake of Tg homogenates to the level seen in WT. On the other hand, the presence of CAMKII inhibitors (either 5 \(\mu\)M AIP (data not shown) or 5 \(\mu\)M KN93) did not result in any significant effect. These data support the notion that phosphorylation of PLN by Akt at residue Thr\(^{17}\) contributes to the significantly increased SR Ca\(^{2+}\) uptake observed in Tg.

**Functional Characterization of an Active Akt Mutant with an SR Localization Signal—**We generated a viral vector (AdAktPLN) containing an active form of Akt fused to an HA
Akt Phosphorylates PLN at Thr^{17}

![Akt Phosphorylates PLN at Thr^{17}](image)

FIGURE 4. Akt phosphorylates PLN at Thr^{17} in WT CMs stimulated with insulin. Top, cultured WT CMs were treated with 0.1 \( \mu \)M insulin for 40 min and, where given, pretreated with inhibitors (0.1 \( \mu \)M KN93 or 10 \( \mu \)M AKT inhibitor IV) for 10 min. Representative blot of phosphorylated Akt, GSKα/β, and PLN at Thr^{17} and Ser^{16} is shown. Bottom, densitometric analysis of PLN phosphorylation in insulin-treated cells relative to untreated cells, expressed as mean ± S.D. (n = 3). Statistical significance was assayed by ANOVA. *, p < 0.05; **, p < 0.01 versus insulin-only treated CMs; \( \alpha \), p < 0.05 insulin-only treated versus untreated cells.

![Akt Phosphorylates PLN at Thr^{17}](image)

FIGURE 5. SR \( ^{45} \)Ca^{2+} uptake in WT and Tg heart homogenate. SR \( ^{45} \)Ca^{2+} uptake assays were performed at room temperature with homogenates of Tg and WT ventricles at 20 mM free Ca^{2+} (n = 3). \( ^{45} \)Ca^{2+} uptake (nmol/mg protein/min) was calculated from the slope of the linear regression analysis. The effects of Akt and CAMKII inhibitors (40 \( \mu \)M Akt-IV and 5 \( \mu \)M KN93, respectively) on SR \( ^{45} \)Ca^{2+} uptake are also given. Values are mean ± S.D. (n = 3). Statistical significance was assayed by ANOVA. *, p < 0.001 versus WT; \( \zeta \), p < 0.001 versus untreated Tg.

tag at the 5′-end (15) and a PLN-SR localization signal at the 3′-end. The PLN-SR localization signal was included to ensure targeting of activated Akt only to the SR, preventing it from localizing to other compartments such as the nucleus. In CMs transduced with AdAktPLN, increased SR Ca^{2+} uptake was expected. As a control, we used an HA-tagged Akt-E40K viral vector (AdAktE40K).

The intracellular localization of transgene Akt in transduced WT CMs was determined first. When transduced with AdAktE40K, HA tag staining of CMs demonstrated a diffuse pattern, with cytoplasmic and nuclear localization of Akt as well as its colocalization with PLN at the SR, indicating that Akt-E40K can localize freely to all cellular compartments (Fig. 6, D–F). In cells transduced with AdAktPLN, there was marked colocalization of Akt with PLN but complete absence of the kinase within the nucleus, suggestive of preferential localization of this mutant to the SR (Fig. 6, G–I).

To ensure that our observations were not due to any compensatory mechanisms, neonatal WT CMs were acutely transduced with either AdAktE40K or AdAktPLN and (i) phosphorylation of PLN at Ser^{16} and Thr^{17} and (ii) Ca^{2+} transients was then analyzed. As expected, PLN was phosphorylated at Thr^{17} after transduction with either AdAktE40K or AdAktPLN (Fig. 6F). Phosphorylation of Ser^{16} was unaffected or partially reduced with transduction, and thus in line with results obtained from Tg mice. In addition, Ca^{2+} transients were significantly higher in both AdAktE40K- and AdAktPLN-transduced CMs compared with mock controls (Fig. 6K). Ca^{2+} measurements, expressed as the percentage of increased peak to baseline, were 25.94 ± 5.98 for AdAktE40K-transduced CMs (p < 0.05 versus mock) and 26.00 ± 5.08 for AdAktPLN-transduced CMs (p < 0.05 versus mock), compared with 16.24 ± 4.42 for the mock control. These data confirm that targeting of active Akt to the SR results in phosphorylation of PLN at Thr^{17} and a consequent improvement in Ca^{2+} handling.

Targeting Akt to the SR Prevents Induction of CM Hypertrophy—A \(^{3} \)Hleucine incorporation assay was performed to evaluate the effect of AdAktE40K and AdAktPLN on hypertrophic growth. Although increased protein synthesis was detected as expected in CMs transduced with AdAktE40K, no increase was produced with AdAktPLN (Fig. 6L). This observation suggests that the preferential localization of Akt to the SR does not activate the mRNA translation machinery and thus hypothetic growth.

DISCUSSION

The critical role of Akt in the development of physiological hypertrophy, such as that seen in athletes, has been well documented (32, 33). Moreover, procedures that stimulate Akt activity, such as the administration of insulin (34) or IGF-1 (35) or the overexpression of the IGF-1 receptor (7), have been reported to produce beneficial effects through the improvement of cardiac contractility. Coherently, deletion of Akt-1 resulted in depressed cardiac function after physical stress in vivo (36). Thus, it is conceivable that Akt intervenes in ameliorating cardiac function during physiological stresses, such as training, also through the enhancement of Ca^{2+} handling.

Increasing Ca^{2+} current (\( I_{\text{Ca,L}} \)) through the L-type Ca^{2+} channel complex and increasing Ca^{2+} release from the SR are two mechanisms through which inotropism may be enhanced. Regarding the first mechanism, we recently demonstrated that
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FIGURE 6. Features of SR-targeted active Akt. WT CMs were mock-transduced (A–C), AdAktE40K-transduced (D–F), or AdAktPLN-transduced (G–I). Dual immunostaining with anti-HA tag (A, D, and G) (green) and anti-PLN (B, E, and H) (red) antibodies was performed and analysis carried out by confocal microscopy. Images were merged (C, F, and I) to determine colocalization (yellow) of transgene HA-tagged Akt with PLN, presumably at the SR. Bar is 10 \(\mu\)m.J. Western blot analysis of mock-, AdAktE40K-, and AdAktPLN-transfected WT CMs. Increased phosphorylation at the CAMKII site, but not at the PKA site, of PLN is evident in CMs transduced with either AdAktE40K or AdAktPLN. Representative blots are shown. K, representative Ca\(^{2+}\) transient traces. L, targeting of active Akt to the SR does not cause CM hypertrophy. Hypertrophy was evaluated as the rate of incorporation of [\(^3\)H]leucine. Statistical significance was assessed by ANOVA. *, \(p < 0.05, n = 3\).
Akt directly controls L-type Ca$^{2+}$ channel complex protein density and $I_{\text{Ca,L}}$ by preventing degradation of Ca$_\alpha$1, the pore-forming subunit of the L-type Ca$^{2+}$ channel complex (11). We had previously described enhanced $I_{\text{Ca,L}}$ in our Akt-E40K Tg mouse model, a finding suggestive of the presence of an Akt-dependent mechanism capable of modulating Ca$^{2+}$ handling at the sarcoplasmic level (15). In line with this, enhanced $I_{\text{Ca,L}}$ was reported as a feature of CMs isolated from mice deficient in phosphatase and tensin homolog, an antagonist of PI3K, an upstream activator of Akt (6). However, cardiac function was shown to be depressed in vivo in that KO model (37). The negative inotropic effect was explained to be due to the up-regulation of the $\gamma$ isoform of PI3K (PI3K$\gamma$), which regulates cAMP phosphodiesterase and leads to decreased cAMP levels. Most probably, decreased cAMP levels masked the increase in $I_{\text{Ca,L}}$ mediated by the $\alpha$ isoform of PI3K (PI3K$\alpha$) responsible for the Akt-dependent enhanced inotropism described by Backx and co-workers (6).

This study gives evidence that Akt may positively affect inotropism also at the sarcoplasmic reticulum level through regulation of the diastolic Ca$^{2+}$ concentration. The results presented confirm the hypothesis that Akt is a direct effector in modulating SR Ca$^{2+}$ uptake and in increasing the availability of Ca$^{2+}$ for the next contraction. We propose a mechanism whereby upon its activation, Akt translocates to the SR where it selectively phosphorylates PLN at Thr$^{17}$, releasing SERCA2a from inhibition. This increases SR Ca$^{2+}$ cycling and results in the enhancement of cardiac contractility.

This result contrasts with that of the study by Gao et al. (21) where overexpression of adenyl cyclase was responsible for Akt activation and consequent PLN phosphorylation at Ser$^{16}$. This discrepancy might rely on differences in the spatiotemporal compartmentalization of active-Akt, which could thereby preferentially affect PLN phosphorylation at either Ser$^{16}$ or Thr$^{17}$. Indeed, Gao et al. (21) showed that adenyl cyclase overexpression resulted in a substantial nuclear relocalization of phospho-Akt. In line with this, increased Ser$^{16}$ phosphorylation, together with a reduced protein level of protein phosphatase-1, was found in transgenic mice overexpressing a nuclearly targeted Akt (16). On the other hand, our results derive from conditions where Akt has a strong localization at the SR level, i.e., Akt-E40K transgenic mice (Fig. 3A) and WT cardiomyocytes subjected to physiological Akt activation via insulin stimulation (Fig. 3B).

Support for this hypothesis might be derived from the peculiar spatial distribution features that have been attributed to the Ser$^{16}$ and Thr$^{17}$-phosphorylated forms of PLN. In fact, whereas phosphorylation of PLN at the Ser$^{16}$ site seems to be an exclusively peri-nuclear phenomenon, PLN localized at the SR membrane can be phosphorylated on Ser$^{16}$ and/or Thr$^{17}$ (38). Finally, of potential importance is the fact that preferential targeting of Akt to the SR does not seem to induce CM hypertrophy. This could be exploited for improving cardiac inotropism and lusitropism specifically.

Acknowledgment—We are grateful to Dr. Marie-Louise Bang for critical reading of the manuscript.

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