Akt regulates L-type Ca\(^{2+}\) channel activity by modulating Ca\(\alpha_{1}\) protein stability

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The insulin IGF-1–PI3K–Akt signaling pathway has been suggested to improve cardiac inotropism and increase Ca\(^{2+}\) handling through the effects of the protein kinase Akt. However, the underlying molecular mechanisms remain largely unknown. In this study, we provide evidence for an unanticipated regulatory function of Akt controlling L-type Ca\(^{2+}\) channel (LTCC) protein density. The pore-forming channel subunit Ca\(\alpha_{1}\) contains highly conserved PEST sequences (signals for rapid protein degradation), and in-frame deletion of these PEST sequences results in increased Ca\(\alpha_{1}\) protein levels. Our findings show that Akt-dependent phosphorylation of Ca\(\beta_{2}\), the LTCC chaperone for Ca\(\alpha_{1}\), antagonizes Ca\(\alpha_{1}\) protein degradation by preventing Ca\(\alpha_{1}\) PEST sequence recognition, leading to increased LTCC density and the consequent modulation of Ca\(^{2+}\) channel function. This novel mechanism by which Akt modulates LTCC stability could profoundly influence cardiac myocyte Ca\(^{2+}\) entry, Ca\(^{2+}\) handling, and contractility.

Introduction

The IGF-1 (insulin growth factor 1)–PI3K (phosphatidylinositol 3-kinase)–Akt pathway plays a crucial role in a broad range of biological processes involved in the modulation of local responses as well as processes implicated in metabolism, cell proliferation, transcription, translation, apoptosis, and growth. In the heart, the IGF-1–PI3K–Akt pathway is involved in the regulation of contractile function, and impairment of this signaling pathway is considered an important determinant of cardiac function (Condorelli et al., 2002; McMullen et al., 2003; Ceci et al., 2004; McMullen et al., 2004; Catalucci and Condorelli, 2006; Sun et al., 2006).

The Akt (also called PKB) family of Ser/Thr kinases consists of three isoforms (Akt-1, -2, and -3) that are activated by IGF-1 and insulin through PI3K, which is a member of the lipid kinase family involved in the phosphorylation of membrane phosphoinositides (Ceci et al., 2004). The product of PI3K binds to the pleckstrin domain of Akt and induces its translocation from the cytosol to the plasma membrane, where Akt becomes accessible for phosphorylation by PDK1 (phosphoinositide-dependent kinase 1), resulting in its activation (Ceci et al., 2004; Bayascas et al., 2008). The Ca\(^{2+}\) current (I\(_{\text{Ca,L}}\)) in both cardiomyocytes and neuronal cells has been shown to be increased by Akt activation (Blair et al., 1999; Viard et al., 2004; Catalucci and Condorelli, 2006; Sun et al., 2006) and decreased by Akt inhibition (Viard et al., 2004; Catalucci and Condorelli, 2006; Sun et al., 2006), suggesting a pivotal role of Akt in regulating L-type Ca\(^{2+}\) channel (LTCC) complex function.
In cardiomyocytes, the LTCC is composed of different subunits: the pore-forming subunit Ca\textsubscript{v}1 \text{L} and the accessory \( \alpha \) and \( \beta \) subunits (Catterall, 2000; Bourinet et al., 2004). The opening of the LTCC is primarily regulated by the membrane potential and by other factors, including a variety of hormones, protein kinases, phosphatases, and accessory proteins (Bodi et al., 2005). In healthy cardiomyocytes, electrical excitation starting during the upstroke of the action potential leads to cytosolic Ca\textsuperscript{2+} influx through opening of the LTCC (Bers and Perez-Reyes, 1999; Richard et al., 2006). This triggers the CICR (Ca-induced Ca release) of intracellular Ca\textsuperscript{2+} from the sarcoplasmic reticulum (SR) through activation of the ryanodine receptor (Ryr), eventually leading to cardiomyocyte contraction (Bers, 2002).

The importance and ubiquity of Ca\textsuperscript{2+} as an intracellular signaling molecule suggests that altered channel function could give rise to widespread cellular and organ defects. Indeed, a variety of cardiovascular diseases, including atrial fibrillation, heart failure, ischemic heart disease, Timothy syndrome, and diabetic cardiomyopathy, have been related to alterations in the density or function of the LTCC (Mukherjee and Spindloe, 1998; Quignard et al., 2001; Bodi et al., 2005; Pereira et al., 2006). However, the molecular basis for dysregulation of LTCC function and the possible involvement of Akt in I\textsubscript{Ca,L} regulation remain unresolved.

Recently, a seminal study in neuronal cells revealed the importance of Akt-dependent phosphorylation of the Ca\textsubscript{v}2 subunit in promoting the chaperoning of the Ca\textsubscript{v}2.2 pore-forming unit to the plasma membrane (Viard et al., 2004). In this study, we identify a novel posttranslational mechanism by which Akt modulates LTCC function under physiological conditions, highlighting the pivotal role of this kinase in cardiac function. Interestingly, our results show that the pore-forming channel subunit Ca\textsubscript{v,\alpha}1 contains highly conserved PEST sequences that direct rapid protein degradation and demonstrate that Akt-mediated phosphorylation of the Ca\textsubscript{v}\beta LTCC chaperone subunit prevents PEST site recognition, thereby slowing or preventing Ca\textsubscript{v,\alpha}1 degradation. This mechanism of action might be an essential process for Ca\textsuperscript{2+} channel functional regulation, thus contributing to normal or better cardiomyocyte contractile function.

### Results

To gain insight into the mechanism of action by which Akt regulates I\textsubscript{Ca,L} and Ca\textsuperscript{2+} handling in the heart, we studied a mouse line with tamoxifen-inducible (Sohal et al., 2001) and cardiac-specific deletion of PDK1, the upstream activator of all three Akt isoforms. Mice in which exons 3 and 4 of the pdk1 gene were flanked by loxP excision sequences (Lawlor et al., 2002) were crossed with transgenic (Tg) mice expressing an inducible and cardiac-specific MerCreMer \alpha-MHC promoter driving the cre recombinase gene (Sohal et al., 2001), resulting in MerCreMer \alpha-MHC PDK1 mice (knockout [KO]). As opposed to the previously described muscle creatine kinase–Cre PDK1 mouse model (Mora et al., 2003) in which PDK1 is embryonically deleted in all striated muscles, this model allows for specific deletion of PDK1 in the adult heart. A further advantage of this model is the inducible cardiac-specific deletion that was necessary to circumvent the embryonic lethality we observed in a mouse model with constitutive \alpha-MHC–Cre cardiac deletion of PDK1 (unpublished data). Similar to the muscle creatine kinase–Cre PDK1 mouse model (Lawlor et al., 2002), PDK1 gene deletion in the adult mouse heart (KO; Fig. S1, A and B) resulted in a lethal phenotype with a mortality that reached 100% at 10 d after tamoxifen injection (Fig. S1 C). Age-matched littermate control mice without cre (wild type [WT]) were unaffected by tamoxifen treatment. Consistent with findings from the previously reported analysis of the PDK1 KO mouse model (Lawlor et al., 2002), cardiac function evaluated by echocardiography at 7 d after tamoxifen injection revealed dramatically impaired systolic function with severe dilated cardiomyopathy and an abrupt drop in fractional shortening in KO but not in WT mice (Fig. S1 D, Table S1, and not depicted). Histological examination substantiated the echocardiographic findings, revealing dilatation of both ventricles and atria (Fig. S1 E) with apparently no evidence of significant apoptosis or interstitial fibrosis (Fig. S1, F and G). These observations indicate that PDK1/Akt activity plays a major role in maintaining adult heart function.

### Deficiency in Akt activity leads to a reduction in the Ca\textsubscript{v,\alpha}1 protein level

Using the cardiac-specific PDK1 KO mouse model, we investigated whether deficiency in Akt activity affects the expression or activation of signaling molecules that are implicated in Ca\textsuperscript{2+} handling and cardiac function. A time course analysis of extracts from WT and KO mouse ventricles revealed striking changes in protein expression upon induction of the PDK1 KO (Fig. 1, A and B). Notably, KO mice had decreased protein levels of the pore-forming Ca\textsuperscript{2+} channel subunit, Ca\textsubscript{v,\alpha}1, which progressed as PDK1 protein expression gradually declined. No change in the protein level of the regulatory Ca\textsubscript{v}\beta subunit was observed. As PDK1 expression decayed, levels of Akt activation also dramatically decreased (assessed by phosphorylation of Akt at the PDK1 phosphorylation site Thr308) despite unaltered expression of total Akt protein (Fig. 1, A and B). Furthermore, Akt activity (assessed using GSK-3\beta as a substrate) was virtually absent in KO hearts (Fig. 1 C). Based on this evidence, we decided to perform further experiments at day 6 after the beginning of treatment.

Although the main physiological action of PDK1 is on Akt activation, PDK1 can potentially influence other members of the cAMP-dependent, cGMP-dependent, and PKC (AGC) kinase protein family such as PKC and PKA, which could also affect cellular Ca\textsuperscript{2+} handling (Williams et al., 2000; Mora et al., 2004). However, PKC activity was unchanged in KO mice (1.15 ± 0.05–fold greater than WT; not statistically significant; assessed by an assay using a PKC-specific peptide as substrate). There was no apparent effect of PDK1 deletion on SERCA2a (Fig. S2 A) as well as PKA activity because the phosphorylation of specific PKA regulatory sites in two SR Ca\textsuperscript{2+} regulatory proteins, Ryr (Ryr2-P2809) and phospholamban (PLN; PLN-P16), were unchanged in KO mice (Fig. S2 B), although it cannot be
excluded that typical changes associated with heart failure and secondary to adrenergic receptor hyperactivation may take place at subsequent time points. Collectively, these data suggest that an acute reduction in Akt activation affects expression of proteins involved in the Ca\textsuperscript{2+} influx into the cell.

**Deficiency in Akt activity affects \textit{I}_{Ca,L}**

Ca\textsuperscript{2+} handling and inotropism were examined in adult cardiomyocytes freshly isolated from WT and KO mice. Using the whole cell voltage-clamp technique, we recorded and analyzed LTCC \textit{I}_{Ca,L} properties. No difference in cell size was observed between WT and KO cells as deduced from membrane capacitance measurements. Membrane capacitance was 116 ± 6 pF in WT cells (\(n = 18\)) and 115 ± 6 pF in KO cells (\(n = 18\)). However, the density of \textit{I}_{Ca,L} (picoampere/picofarad) was decreased in KO versus WT (Fig. 2 B). At 0 mV, the density of \textit{I}_{Ca,L} was -9.08 ± 0.96 pA/pF in KO cells (\(n = 12\)) versus -16.26 ± 0.96 pA/pF in WT cells (\(n = 12\); \(P < 0.001\)). In addition, there was no significant difference in either steady-state activation or inactivation curves (unpublished data). Indeed, mean half-activation occurred at -12.97 ± 0.53 mV in WT cells versus -15.07 ± 0.66 mV in KO cells, and mean half-inactivation occurred at -31.11 ± 0.48 mV in WT cells versus -30.77 ± 0.42 mV in KO cells.

The absence of a shift in the voltage dependence of these properties (Fig. 2 B) was consistent with the absence of modification in gating properties of the LTCC, suggesting that a reduction in the number of functional LTCCs can account for the observed decrease in \textit{I}_{Ca,L} in KO mice. Of note, the decay kinetics of \textit{I}_{Ca,L} were slower in KO cells compared with WT cells with a decrease in the early fast inactivating component (Fig. 2 A). Consistent with previous observations by us and others regarding the role of Akt in cardiac function (Blair et al., 1999; Condorelli et al., 2002; Kim et al., 2003; Sun et al., 2006), both contraction (Fig. 2 C) and systolic Ca\textsuperscript{2+} amplitudes (Ca\textsuperscript{2+} transients; Fig. 2 D and Fig. 3 A) were significantly depressed (by \(\sim 35\%\) and \(30\%\), respectively; \(P < 0.05\)) in KO cardiomyocytes compared with WT littermates.

The observed reduction in Ca\textsuperscript{2+} transient amplitude and cardiac contractility could be explained by reduced Ca\textsuperscript{2+} entry into cells via the LTCC, but decreased intracellular Ca\textsuperscript{2+} release from the SR may also contribute. However, although the Ca\textsuperscript{2+} transient amplitude between the systolic and diastolic phase (twich) was smaller in KO cardiomyocytes (Fig. 3 B, left bars), no difference in total SR Ca\textsuperscript{2+} content was found (Fig. 3 B, right bars), suggesting that the decrease in Ca\textsuperscript{2+} transient amplitude is only caused by reduced Ca\textsuperscript{2+} entry. This is consistent with the observed slowing of the early fast inactivation of \textit{I}_{Ca,L} (Fig. 2 A), which is highly dependent on CICR-triggered SR Ca\textsuperscript{2+} release during the action potential (Richard et al., 2006). Therefore, we conclude that the reduced \textit{I}_{Ca,L} may contribute to the reduced contractility in KO hearts.

**Akt regulates the Ca\textsubscript{\alpha1} protein level at the plasma membrane**

The properties of the Ca\textsubscript{\alpha1} subunit are known to be markedly affected by LTCC accessory subunits (Catterall, 2000; Bourinet et al., 2004). Among the LTCC accessory subunits expressed in the heart, Ca\textsubscript{\beta}2 is known to act as a chaperone for the Ca\textsubscript{\alpha1} subunit, both as a positive modulator of channel opening probability and for its trafficking from the ER to the plasma membrane (Yamaguchi et al., 1998; Viard et al., 2004). Therefore, supported by previous results (Viard et al., 2004) as well as corroborated by unchanged Ca\textsubscript{\alpha1} mRNA levels in KO compared with WT hearts (Fig. 3 A), we hypothesized that in the heart, an Akt-mediated phosphorylation of the LTCC accessory subunit would mainly affect trafficking of Ca\textsubscript{\alpha1} protein to the plasma membrane. However, because the amount of Ca\textsubscript{\alpha1} was reduced in both mitochondrial and membrane fractions from KO extracts compared with WT (Fig. 3 B), we hypothesized that the reduced Ca\textsubscript{\alpha1} level observed in KO mice was caused by enhanced protein degradation in addition to impaired protein translocation to the plasma membrane. To assess the pathway involved in the Akt-dependent Ca\textsubscript{\alpha1} protein degradation, three sets of specific cell degradation system inhibitors were examined for their ability to
The detection effect was abolished in the absence of Ca v1/H9252 cotransfection, a condition under which Ca v1/H9251 is retained in the ER (Fig. 3C, bottom). All together, these results confirm that Akt activity is regulating Ca v1/H9251 protein density and reveal that in the absence of Akt function, Ca v1/H9251 is susceptible to lysosome-mediated membrane protein degradation.

Because Ca v1/H9252 is the only LTCC accessory subunit containing an Akt phosphorylation consensus site (Viard et al., 2004), we hypothesized that Ca v1/H9251 protein degradation at the plasma membrane might result from loss of Ca v1/H9252 chaperone activity in the absence of Akt-induced phosphorylation. In support of this hypothesis, treatment of Ca v1/H9251- and Ca v1/H9252-cotransfected cells with bafilomycin-A1, an inhibitor of the lysosomal degradation system responsible for the degradation of many membrane proteins (Dice, 1987), prevented the decrease in Ca v1/H9251 protein induced by Akt inhibition (Fig. 3C, top). Conversely, a ubiquitin/proteasome inhibitor, MG132, failed to protect Ca v1/H9251 from protein degradation. Similar results were obtained by inhibiting calpain, the intracellular Ca2+-dependent Cys protease known to be involved in membrane protein degradation (Belles et al., 1988; Romanin et al., 1991). Intriguingly, the bafilomycin-A1–dependent protection effect was abolished in the absence of Ca v1/H9252 cotransfection, a condition under which Ca v1/H9251 is retained in the ER (Fig. 3C, bottom). All together, these results confirm that Akt activity is regulating Ca v1/H9251 protein density and reveal that in the absence of Akt function, Ca v1/H9251 is susceptible to lysosome-mediated membrane protein degradation.

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Figure 2. Impaired intracellular Ca2+ handling and contractility in PDK1 KO cardiomyocytes. (A and B) Smaller Ca2+ current in KO cardiomyocytes. (A) Whole cell representative I Ca,L currents normalized for difference in cell size. (B) I Ca,L IV current/voltage relationships (n = 12; *, P < 0.05; **, P < 0.01). (C and D) Cardiomyocyte contraction and Ca transients at different stimulation frequencies. (C) Cardiomyocyte shortening is decreased in KO compared with WT cardiomyocytes (*, P < 0.05; ANOVA). (D) Ca2+ frequency relationship indicates smaller peak systolic but not diastolic Ca2+ in KO compared with WT cells (*, P < 0.05; ANOVA). Error bars show SEM.

Figure 3. Akt mediates regulation of Ca v1/H9251 protein density at the plasma membrane. (A) RT-PCR analysis of Ca v1/H9251 mRNA expression from WT and KO ventricular extracts. GAPDH served as a loading control. (B) Western blot analysis of whole lysate, membrane, and microsomal fractions from WT and KO ventricular extracts. CSQ, calsequestrin. (C) YFP-Ca v1/H9251–transfected COS-7 cells alone or in combination with Ca v1/H9252 expression vector were serum starved and treated with Akt inhibitor (Akt inh.) and 1 μM bafilomycin-A1, 25 μM MG132, or 25 μM calpeptin. 6 h after drug administration, cell lysates were prepared and subjected to Western blot analysis for YFP. GAPDH served as a loading control. (D and E) Ca v1/H9251 protein levels in KO cardiomyocytes infected with empty (mock) or active E40K-Akt (AdAkt)–expressing adenoviral vector (D) and in whole lysates of WT and E40K-Akt (Tg Akt) hearts (E). Representative experiments are shown (n = 4).
of this hypothesis, forced expression of the active E40K-Akt mutant (AdAkt) restored Ca\(_{\beta2}\) protein levels in isolated cardiomyocytes from KO mice (Fig. 3 D). Similarly, cardiomyocytes from Tg mice expressing constitutively active HA-E40K-Akt (Tg Akt; Condorelli et al., 2002) showed increased Ca\(_{\alpha1}\) levels compared with WT controls (Fig. 3 E).

**Akt is a determinant for Ca\(_{\alpha1}\) protein level regulation by direct phosphorylation of the Ca\(_{\beta2}\) chaperone subunit**

To assess whether Akt is directly involved in modulation of Ca\(_{\beta2}\) chaperone activity in the heart, we first confirmed the interaction between Akt and Ca\(_{\beta2}\). Ventricular homogenates derived from either WT or Tg Akt mice were immunoprecipitated with anti-HA antibody and assayed for Ca\(_{\beta2}\), which revealed association of the Ca\(_{\beta2}\) subunit with active Akt (Fig. 4 A). Similarly, Ca\(_{\beta2}\) was found to coimmunoprecipitate with insulin-stimulated endogenous Akt (Fig. S4 A).

To determine whether Ca\(_{\beta2}\) can be phosphorylated by Akt, Ca\(_{\beta2}\) immunoprecipitates from cardiac homogenates were incubated with recombinant active Akt and \(\gamma\)-(\(^{32}\)P)ATP. A band corresponding to phosphorylated Ca\(_{\beta2}\) was detected only in the presence of the kinase (Fig. 4 B, left). To determine whether the Ca\(_{\beta2}\) subunit was phosphorylated by Akt in vivo, we treated overnight-starved mice with 1 mU/g insulin to induce activation of Akt (Bayascas et al., 2008). 20 min after treatment, Ca\(_{\beta2}\) was immunoprecipitated from ventricular homogenates, subjected to Western blot analysis, and probed for phosphorylated Akt consensus sites using phospho-Akt substrate antibody. This revealed insulin-stimulated phosphorylation of Ca\(_{\beta2}\) in WT but not in KO hearts (Fig. 4 B, right). Furthermore, a back phosphorylation assay, which is used to assess the basal state of Ca\(_{\beta2}\) phosphorylation, revealed a reduction of the basal phosphorylation level of Ca\(_{\beta2}\) by 36\% (P < 0.05) in KO mouse ventricle compared with WT (Fig. 4 C).

Collectively, these data demonstrate that active Akt binds to and phosphorylates Ca\(_{\beta2}\), the chaperone for Ca\(_{\alpha1}\).

To directly assess whether Akt phosphorylation of Ca\(_{\beta2}\) protects Ca\(_{\alpha1}\) from protein degradation, we constructed a mutant of Ca\(_{\beta2}\) in which Ser625, which is contained in the putative Akt consensus site (R-X-X-R-S/T), was replaced by glutamate (Ca\(_{\beta2}-\text{SE}\)) to mimic phosphorylation. Cotransfection of 293T cells with Ca\(_{\alpha1}\) and Ca\(_{\beta2}\)-SE resulted in Ca\(_{\alpha1}\) protein levels that were increased compared with those found when cotransfected with Ca\(_{\beta2}\)-WT (Fig. 5 A). Similarly, Ca\(_{\alpha1}\) expression was increased in insulin-treated Ca\(_{\beta2}\)-WT–cotransfected cells (Fig. 5 A). Notably, the active phosphomimic Ca\(_{\beta2}\)-SE also counteracted the down-regulation of Ca\(_{\alpha1}\) induced by an Akt inhibitor (Fig. 5 B). Opposite results were obtained with a dominant-negative (DN) Ca\(_{\beta2}\) mutant in which Ser was replaced by Ala (Ca\(_{\beta2}\)-SA) to prevent Akt phosphorylation. Indeed, Ca\(_{\alpha1}\) protein levels were reduced when coexpressed with Ca\(_{\beta2}\)-SA (Fig. 5 C). In addition, insulin stimulation failed to increase Ca\(_{\alpha1}\) in the presence of the DN Ca\(_{\beta2}\)-SA mutant (Fig. 5 C).

Consistent with the hypothesis that Ca\(_{\alpha1}\) protein down-regulation relies on Akt kinase activity, overexpression of a DN form of Akt (AdAktDN) resulted in a significant reduction in Ca\(_{\alpha1}\) protein levels, whereas forced expression of AdAkt was sufficient to counteract Ca\(_{\alpha1}\) reduction in a serum-free condition, in which Akt is not phosphorylated (Fig. S4 B). Furthermore, suppression of Akt expression in 293T cells by siRNA (small interfering Akt [siAkt]) resulted in reduction of the Ca\(_{\alpha1}\) protein level (Fig. 5 D).

To support the evidence that Akt-dependent phosphorylation of Ca\(_{\beta2}\) is a determinant for Ca\(_{\alpha1}\) stability and functionality, we measured the effect of the Ca\(_{\beta2}\) mutants on Ca\(^{2+}\) current. Although cotransfection of cells with Ca\(_{\alpha1}\) and Ca\(_{\beta2}\)-WT resulted in significant depressed I\(_{\text{Ca,L}}\) in serum-free medium compared with serum-containing medium in which Akt is phosphorylated (not depicted), cotransfection of Ca\(_{\alpha1}\)
sequences obtained are highly conserved among species (Table I), and 1,839–1,865). Intriguingly, the highest scored potential PEST motifs (amino acids 435–460, 807–820, 847–858, 1,732–1,745, and 1,839–1,865) have been suggested to serve as signals for rapid protein degradation. (A–C) YFP-Caα1-Δcotransfected 293T cells with the indicated mutant variant of Caβ2. Cells were serum starved overnight and treated with 100 μM insulin (A and C) or 5 μM Akt inhibitor (Akt inh; B) as indicated. The expression of YFP-Caα1 in lysates was monitored by Western blot analysis with anti-YFP antibody and normalized based on transfection efficiency [Caβ2] and protein amount (tubulin; n = 3). (D) Caα1- and Caβ2-cotransfected 293T cells were treated with siAkt-expressing vector as indicated. 3 d after transfection, cell lysate was tested by Western blot analysis. Protein loading was normalized to GAPDH levels. Representative experiments are shown (n = 3).

Akt regulates Caα1 protein stability

PEST sequences have been suggested to serve as signals for rapid proteolytic degradation through the cell quality control system (Rechsteiner, 1990; Smith et al., 1993; Krappmann et al., 1996; Sandoval et al., 2006). Notably, PEST-mediated protein degradation has recently been suggested to play an essential role in modulating neuronal Ca2+ channel function through regulation of the Caβ3 accessory subunit (Sandoval et al., 2006). Our findings raise the possibility that processing of the Caα1 protein may be affected in a similar way. To test this hypothesis, we used the web-based algorithm PESTfind (Rogers et al., 1986) in a search for potential Caα1 PEST sequences and found several putative motifs (amino acids 435–460, 807–820, 847–858, 1,732–1,745, and 1,839–1,865). Intriguingly, the highest scored potential PEST sequences obtained are highly conserved among species (Table I), with one located in the I–II linker of the Caα1 subunit and overlapping with the α1-interacting domain (AID), which is the primary binding region for Caβ2 (Fig. 7 A; Bodi et al., 2005). To determine whether these PEST sequences are involved in Caα1 degradation control, we generated two in-frame deletion mutants encompassing either the I–II (Caα1-ΔP) or II–III (Caα1-ΔH) cytosolic linker region (Fig. 7 A). Western blot and immunofluorescence analyses of serum-starved 293T cells transfected with these mutants revealed higher protein expression levels for both Caα1-ΔP and Caα1-ΔH mutants compared with Caα1-WT, which is consistent with the hypothesis that these motifs determine Caα1 protein stability (Fig. 7 B). Furthermore, a pulse-chase analysis, with a chase starting 36 h after cell starvation, revealed markedly increased protein stability of Caα1-ΔP and Caα1-ΔH compared with Caα1-WT (Fig. 7 C). In particular, Caα1-WT showed a short half-life typical of proteins containing PEST sequences (Dice, 1987), with a rapid and progressive degradation starting 4 h after the chase and reaching 50% of degradation 25 h after the chase. In contrast, Caα1-ΔP and Caα1-ΔH mutants were less sensitive to degradation and were degraded by only 23% and 15% after 25 h, respectively (P < 0.001). Notably, cotransfection of Caβ2-SE with Caα1-ΔP resulted in a considerable increase in the half-life of Caα1-WT (Fig. 7 C). In addition, transfection of 293T cells with Caα1 PEST sequences fused in frame with GFP resulted in marked instability of GFP, as shown by both Western blot and immunofluorescence analyses (Fig. 7 D), providing further evidence that these motifs are determinants for Caα1 protein stability. Consistent with the hypothesis that Akt-mediated protection of Caα1 degradation acts through PEST sequences, overexpression of AdAktDN or siAkt had no significant effect on protein levels of either Caα1-ΔP or Caα1-ΔH mutants (Fig. S4, B and C). To assess whether the observed PEST mechanism is caused by a direct Akt-dependent interaction between Caβ2 and Caα1, we performed in vitro binding assays using in vitro–translated [35S]Met-labeled Caα1 cytosolic domains and a GST-fused Caβ2 C-terminal coiled-coil region. Notably, direct interaction took place between the Akt-phosphorylated Caβ2 C-terminal coiled-coil region and the Caα1 C-terminal domain (Fig. 7 E). No interactions were found with other Caα1 cytosolic domains (unpublished data), although it cannot be excluded that other binding sites may exist.

To assess whether PEST-deleted Caα1 channels are still functional, traffic appropriately to the membrane, and associate with the Caβ2 subunit, we measured Ca2+ current in Caα1-ΔH mutant–transfected cells. No significant differences in I_{Ca,L} were found in cells transfected with Caα1-ΔP compared with Caα1-ΔH (Fig. 7 F). Conversely, although serum deprivation resulted in I_{Ca,L} reduction in Caα1-WT–transfected cells, no significant changes were observed in Caα1-ΔH mutant–transfected cells (Fig. 7 F). This confirms that PEST-deleted Caα1-ΔH is resistant to rapid protein degradation and maintains its integrity and physiological function. Furthermore, current-voltage analysis (I–V curves) revealed that neither serum deprivation nor PEST-H deletion modifies steady-state activation parameters (Fig. S5). Also, all electrophysiological experiments were performed at a holding potential of −80 mV, which is a value far away from the potential for half steady-state inactivation (V0.5) of I_{Ca,L}, indicating that a change in the macroscopic current properties of Caα1.2 is unlikely.
Collectively, our results suggest that Akt-mediated phosphorylation of Ca_\(\beta_2\) regulates Ca_\(\alpha_1\) density through protection of Ca_\(\alpha_1\) PEST motifs from the cell protein degradation machinery. Impairment of this mechanism is expected to result in dysregulation of cardiomyocyte contractile function.

**Discussion**

This study reveals a mechanism through which the insulin IGF-1–PI3K–PDK1–Akt pathway can sustain or modulate Ca\(^{2+}\) entry in cardiac cells via the voltage-gated LTCC and eventually affect cardiac contractility. Using a mouse model with an inducible and cardiomyocyte-specific deletion of the upstream activator PDK1, we showed that Akt is of key importance for the structural organization and functionality of the LTCC complex at the plasma membrane. This regulation of LTCC activity is directly related to the Akt-mediated phosphorylation of the accessory subunit Ca_\(\beta_2\), which in turn results in increased protein density of the pore-forming Ca_\(\alpha_1\) subunit through protection of PEST sequences from the proteolytic degradation system. In the absence of phosphorylated Akt, the Ca\(^{2+}\) current is reduced, resulting in a decreased Ca\(^{2+}\) transient and contractility. Therefore, it is tempting to speculate that the Akt-mediated phosphorylation of Ca_\(\beta_2\) and the consequent direct association of the Ca_\(\alpha_1\) C-terminal region (Fig. 7E) may induce conformational changes that prevent PEST sequences from being recognized by the cell degradation system (Fig. 8). In addition, one cannot exclude the possibility that phosphorylated Ca_\(\beta_2\) might also act indirectly through other, as of yet unknown, LTCC protein partners.

The identified mechanism alone is unlikely to be responsible for the detrimental cardiac defects observed in the PDK1 KO mouse model. To assess whether a reduction in the Akt antiapoptotic activity could lead to increased cell death, we measured caspase 3 activation (Fig. S1). However, consistent with previous evidence reported by Mora et al. (2003), our results failed to prove any significant involvement of this mechanism in the PDK1 KO phenotype. Our PDK1 KO mouse model does not appear to progress through slow transitional states, which are typical of heart failure, but rather progresses directly to a dilated cardiac phenotype, which eventually leads to premature failure.

**Table I. PEST sequences are highly conserved in Ca_\(\alpha_1\)**

| Species | Fragment | Sequences | PESTfind score |
|---------|----------|-----------|---------------|
| Mouse   | PEST I   | 435-KGYLDWITQAEIDPENEDGMDEDK 460 | 8.45 |
| Rat     | PEST I   | 476-KGYLDWITQAEIDPENEDGMDEDK 501 | 8.45 |
| Human   | PEST I   | 446-KGYLDWITQAEIDPENEDGMDEDK 471 | 8.66 |
| Mouse   | PEST II  | 807-KSITADGESPPTTK 820 | 9.45 |
| Rat     | PEST II  | 848-KSITADGESPPTTK 861 | 9.45 |
| Mouse   | PEST III | 837-HSNPDTAGEDEEEPEMPVGR 858 | 19.51 |
| Rat     | PEST III | 878-HSNPDTAGEDEEEPEMPVGR 899 | 19.51 |
| Human   | PEST II  | 845-KSYPNPETGDEEEPEMPVGR 869 | 20.26 |
| Mouse   | PEST IV  | 1,732-KTGNNGADTSEPSh1 1,745 | 5.5 |
| Rat     | PEST IV  | 1,772-KTGNNGADTSEPSh1 1,785 | 5.5 |
| Mouse   | PEST V   | 1,839-RMSEEAEYSEPLSLTDMFSYQEDEH 1,865 | 5.86 |
| Human   | PEST IV  | 1,937-HDTEACSEPSLSTEMLSQGDDENR 1,961 | 7.54 |
| Human   | PEST V   | 2,214-RGAPSEEIIGD5R 2,226 | 7.71 |

Occurrence of PEST sites within the amino acid sequence of Ca_\(\alpha_1\) from the mouse, rat, and human. Amino acid identity is underlined.
and change the energy metabolism. Further studies are required to unravel the complex mechanisms that contribute to the establishment of the observed PDK1 KO mouse heart phenotype.

death (Fig. S1). Therefore, we hypothesize that the lethal phenotype is caused by activation of more complex systems that rapidly remodel the extracellular matrix and cell to cell contacts and change the energy metabolism. Further studies are required to unravel the complex mechanisms that contribute to the establishment of the observed PDK1 KO mouse heart phenotype.
Several findings have shown the importance of the insulin IGF-1–PI3K–Akt pathway in heart function. Our group has previously demonstrated that overexpression of an active form of Akt-1 results in improved cardiac inotropism both in vivo (Condorelli et al., 2002) and in vitro (Kim et al., 2003), augmenting \( I_{\text{Ca,L}} \). Similar results were recently obtained in a mouse model with cardiac-specific Akt-1 nuclear overexpression (Rota et al., 2005) and in mice deficient for PTEN (phosphatase and tensin homologue deleted on chromosome 10), an antagonist of PI3K 2 activity (Sun et al., 2006). In addition, short-term administration of IGF-1 in animal experiments has also been reported to increase cardiac contractility (Duerr et al., 1995). However, the mechanism through which the insulin IGF-1–PI3K–Akt pathway affects \( Ca^{2+} \) current has remained elusive. In an elegant in vitro study, Viard et al. (2004) demonstrated that a region of the \( \alpha1 \) subunit is involved in the PI3K-induced chaperoning of \( \alpha2 \) subunits in neurons. This PI3K-induced regulation was shown to be mediated by Akt phosphorylation of the \( \alpha2a \) subunit, which in turn regulates \( \alpha2 \) trafficking from the ER to the plasma membrane. Notably, the C-terminal region containing the putative Akt phosphorylation consensus site is conserved in all variants of the \( \alpha1 \) C-terminal domain. In turn, a conformation shift prevents PEST sequence recognition, stabilizing \( \alpha1 \) protein levels. The blue and red ribbons in \( \alpha1 \) represent AID and PEST sequences, respectively.

![Proposed mechanism.](image)

**Figure 8. Proposed mechanism.** Akt, followed by PDK1 activation, phosphorylates \( \alpha2 \) at the C-terminal coiled-coil domain. The phosphorylation allows association of the C-terminal portion of \( \alpha2 \) with the \( \alpha1 \) C-terminal domain. In turn, a conformation shift prevents PEST sequence recognition, stabilizing \( \alpha1 \) protein levels. The blue and red ribbons in \( \alpha1 \) represent AID and PEST sequences, respectively.

C-terminal region in all \( \alpha2 \) splice isoforms corroborates the relevance of identifying new functional motifs that may give important insights into LTCC modulation. Consistent with an important functional role of the conserved \( \alpha2 \) C-terminal region, Lao et al. (2008) recently showed that, in the absence of the main \( \alpha2 \) protein domain, the selected C-terminal essential determinant is sufficient for \( I_{\text{Ca,L}} \) stimulation. All together, this evidence supports the notion that this region is a potential pharmacological target.

In conclusion, we show that the insulin IGF-1–PI3K–PDK1–Akt pathway regulates \( \alpha2 \) chaperone activity through phosphorylation by Akt and suggest that, in turn, this controls \( \alpha1 \) channel density by protection of \( \alpha1 \) from PEST-dependent protein degradation (Fig. 8). This paradigm highlights an unanticipated regulatory function for Akt in modulating LTCC function and provides evidence for an essential role of Akt in the control of cardiomyocyte \( Ca^{2+} \) handling and contractility. Interestingly, the high level of conservation of PEST sequences in the \( \alpha1 \) subunit throughout evolution (Table I) indicates that our proposed mechanism may play a universal role in regulating cell \( Ca^{2+} \) handling and survival. Because pathophysiological states are often accompanied by alterations in LTCC function (Mukherjee and Spinale, 1998), the elucidation of this novel regulatory pathway may open new therapeutic perspectives.

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**Materials and methods**

**Generation of genetically modified mice**

Cardiocyte-specific PDK1-inducible KO mice (MerCreMer \( \alpha\)-MHC PDK1) were generated by breeding PDK1 \( ^{\text{Flox/Flox}} \)/tg mice (provided by D.R. Alessi, Medical Research Council Protein Phosphorylation Unit, University of Dundee, Dundee, Scotland, UK; Williams et al., 2000) with mice expressing the cardiocyte-specific MerCreMer \( \alpha\)-MHC promoter-driven cre recombinase gene (provided by J.D. Molkentin, University of Cincinnati, Cincinnati, OH; Sahal et al., 2001). The resulting background strain of the MerCreMer mice was C57Bl/6-6SV129 and was unchanged throughout all experiments. Control animals used in this study were PDK1 \( ^{\text{Flox/Flox}} \)/tg littermates not expressing the cre recombinase gene and were treated with the same tamoxifen regimen. Tamoxifen dissolved in maize oil was injected intraperitoneally once a day at a dose of 75 mg/kg body weight. Male animals 7–8-wk old were used. 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.

**Culture and treatment of mouse cardiomyocyte cells**

Isolation of ventricular myocytes was performed as previously described (Care et al., 2007). Cells were infected with an adenovector expressing either no transgene (mock), HA-E40K-Akt (AdAkt), or AktK179M (AdAktDN) at MOI 100 and harvested 48 h after infection. The viral vector was amplified and purified in 3% sucrose/PBS by ViraQuest, Inc.

**Cell culture and cDNA mutagenesis**

Cell transfection was performed in serum-starved medium using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. 5 mM Akt XI inhibitor (EMD), insulin (Sigma–Aldrich), 1 mM bafilomycin-A1 (Sigma–Aldrich), 25 mM MG132 (EMD), and 25 mM calpeptin (EMD) were used as described in Results. Cacnb2 cDNA [complete coding sequence, cDNA clone MGC:129335, IMAGE:40047531; American Type Culture Collection) was cloned in the pcDNA3 vector. Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies). \( \alpha1 \) PEST deletion mutants and GFP fusion proteins were generated by PCR. \( \gamma\)-\( \alpha1 \) expression plasmids were provided by N. Soldatov (National Institute on Aging, National Institutes of Health, Baltimore, MD). A lentivirus vector was generated and used as an expression vector for siRNA-mediated silencing of the \( \alpha1 \) gene (siAkt). The sequence used (5’-TGCCCTCTACACCCAG-GAT-3’) was chosen in a conserved region between rat, mouse, and human and has been validated for targeting Akt-1 and -2 (Katome et al., 2003). All constructs were confirmed by DNA sequencing.
Ca\textsuperscript{2+} current measurement

Macroscopic \( I_{\text{Ca}} \) was recorded at room temperature (\(-22^\circ \text{C} \)) using the whole cell patch-clamp technique in native cells as previously described [Maier et al., 2003; Aaimond et al., 2005]. External recording solution contained 136 mM tetraethylammonium (TEA)-Cl, 2 mM CaCl\textsubscript{2}, 1.8 mM MgCl\textsubscript{2}, 10 mM Hepes, 5 mM 4-aminopyridine, and 10 mM glucose, pH 7.4, with TEA-OH. Pipette solution contained 125 mM CsCl, 20 mM TEA-Cl, 10 mM EGTA, 10 mM Hepes, 5 mM phosphocreatine, 5 mM Mg\textsubscript{2+}-ATP, and 0.3 GTP, pH 7.2, with CsOH. Myocytes were held at \(-80\text{ mV} \) and 10 mV depolarizing steps from \(-50 \text{ mV} \) to 30 mV for 300 ms were applied. Analysis was performed using a microscope (Diaphot 200; Nikon) equipped with 10X NA 20 objective lenses (CFVWN; Nikon). pCLAMP 9 (MDS Analytical Technologies) was used as acquisition software. For electrophysiological recordings of reconstituted, native Ca\textsubscript{2+}-negative Akt as well as siAkt on the Ca\textsubscript{2+}-negative Akt isoform, dominant-negative PKA regulatory proteins, Ryr (Ryr2-P2809) and PLN (PLN-P16). Fig. S3 shows representative Ca traces and twitch Ca\textsuperscript{2+} transient amplitude in KO compared with WT cardiomyocytes. Fig. S4 shows common immunoprecipitation of Ca\textsubscript{2+} with insulin-activated Akt isoforms and the effects of dominant-active and \(-25^\circ \text{C} \) for 30 min after injection, the hearts were rapidly extracted, freeze-clamped in liquid nitrogen, and homogenized to a powder in liquid nitrogen. In vitro phosphorylation assays on immunoprecipitated proteins were performed as described previously [Haase et al., 1999].

Western blot analysis and antibodies

Protein expression was evaluated in total lysates or cell fractions by Western blot analysis according to standard procedures. Antibodies against the following proteins were used: Ca\textsubscript{2+}1 (Novus Biologicals), CA\textsubscript{2+}1 and CA\textsubscript{2+}2 (provided by H. Haase, Max Delbrück Center for Molecular Medicine, Berlin, Germany), Ryr and Ryr2-P2809 (provided by A. Marks, Columbia University, New York, NY); PKD1 (EMD); Akt, 1-2, and -3, Akt, AktP308, and anti-phospho-Ser/Thr/Akt substrate [Cell Signaling Technology]; PLN and PLP-N16 (Novus Biologicals); calsecqurin (BD); calsequestrin (BD); caspase 3 (Cell Signaling Technology); HA (Roche); GFP/YFP (GeneTex, Inc.); tubulin (Novus Biologicals); PLN-P16 (Novus Biologicals); calsequestrin (BD); caspase 3 (Cell Signaling Technology); carbonic anhydrase (CA) (Novus Biologicals); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology). ImageJ software (National Institutes of Health) was used to perform densitometry analyses.

Tissue preparation, immunoprecipitation, and in vitro phosphorylation

When described, overnight-fasted mice were injected intra peritoneally with 1 mg/kg insulin or saline solution 30 min after injection, the hearts were rapidly extracted, freeze-clamped in liquid nitrogen, and homogenized to a powder in liquid nitrogen. In vitro phosphorylation assays on immunoprecipitated proteins were performed as described previously [Haase et al., 1999].

Cell fractionation

Pulverized hearts were homogenized in ice-cold solution 1 [300 mM sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM Na\textsubscript{3}VO\textsubscript{4}, and protease inhibitors] at 1.5 mL/ventricle by three bursts of a mortar. Pulverized hearts were homogenized in ice-cold solution 1 (300 mM sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM Na\textsubscript{3}VO\textsubscript{4}, and protease inhibitors) and saved as ER fractions. All aliquots were stored at \(-80^\circ \text{C} \).

Histology and confocal microscopy

Fixation, staining, and confocal analysis were performed as previously described [Care et al., 2007]. Confocal microscopy was performed with a confocal microscope (Radiance 2000; Bio-Rad Laboratories) with a 60X Plan-Neofluar NA 1.4 objective [Carl Zeiss, Inc.]. Individual images (1,024 x 1,024 pixels) were converted to tiff format and merged as pseudo- color RGB images using Imaris (Bitplane AG).

Pulse-chase and immunoprecipitation experiments

36 h after transfection, 200 cells were starved for 30 min in Met and Ca\textsubscript{2+}-negative Akt isoform, dominant-negative PKA regulatory proteins, Ryr (Ryr2-P2809) and PLN (PLN-P16). Fig. S3 shows representative Ca traces and twitch Ca\textsuperscript{2+} transient amplitude in KO compared with WT cardiomyocytes. Fig. S4 shows common immunoprecipitation of Ca\textsubscript{2+} with insulin-activated Akt isoforms and the effects of dominant-active and \(-25^\circ \text{C} \) for 30 min after injection, the hearts were rapidly extracted, freeze-clamped in liquid nitrogen, and homogenized to a powder in liquid nitrogen. In vitro phosphorylation assays on immunoprecipitated proteins were performed as described previously [Haase et al., 1999].

Statistical analysis

Statistical comparison was performed within at least three independent experiments by paired or unpaired Student’s t test, whereas comparison between groups was analyzed by one-way repeated-measures analysis of variance (ANOVA) combined with a Newman-Keuls post-test to compare different values using Prism 4.0 software (GraphPad Software, Inc.). Differences with \( P < 0.05 \) were considered statistically significant.

Online supplemental material

Fig. S1 shows additional biochemical, histological, and echocardiographic analyses of mice lacking PKD1 expression. Fig. S2 shows SERCA2 level and phosphorylation of specific PKA regulatory sites in two SR Ca\textsuperscript{2+} regulatory proteins, Ryr (Ryr2-P2809) and PLN (PLN-P16). Fig. S3 shows representative Ca\textsuperscript{2+} traces and twitch Ca\textsuperscript{2+} transient amplitude in KO compared with WT cardiomyocytes. Fig. S4 shows common immunoprecipitation of Ca\textsubscript{2+} with insulin-activated Akt isoforms and the effects of dominant-active and \(-25^\circ \text{C} \) for 30 min after injection, the hearts were rapidly extracted, freeze-clamped in liquid nitrogen, and homogenized to a powder in liquid nitrogen. In vitro phosphorylation assays on immunoprecipitated proteins were performed as described previously [Haase et al., 1999].

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Akt regulates L-type Ca^{2+} channel activity by modulating Ca_{\alpha1} protein stability

Daniele Catalucci, Deng-Hong Zhang, Jaime DeSantiago, Franck Aimond, Guillaume Barbara, Jean Chemin, Désiré Bonci, Eckard Picht, Francesca Rusconi, Nancy D. Dalton, Kirk L. Peterson, Sylvain Richard, Donald M. Bers, Joan Heller Brown, and Gianluigi Condorelli

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The Tubulin panel in Fig. 3 B in the original version of this figure was a duplicate of the GAPDH panel in Fig. 3 C. The authors have indicated that this was due to a clerical error during figure preparation. A corrected version of Fig. 3 B is shown below.

The html and pdf versions of this article have been corrected. The error remains only in the print version.

**Figure 3.** (B) Western blot analysis of whole lysate, membrane, and microsomal fractions from WT and KO ventricular extracts. CSQ, calsequestrin.