Akt regulates L-type Ca\textsuperscript{2+} channel activity by modulating Ca\textsubscript{v\alpha1} protein stability

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The insulin IGF-1–PI3K–Akt signaling pathway has been suggested to improve cardiac inotropism and increase Ca\textsuperscript{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\textsuperscript{2+} channel (LTCC) protein density. The pore-forming channel subunit Ca\textsubscript{v\alpha1} contains highly conserved PEST sequences (signals for rapid protein degradation), and in-frame deletion of these PEST sequences results in increased Ca\textsubscript{v\alpha1} protein levels. Our findings show that Akt-dependent phosphorylation of Ca\textsubscript{v\beta2}, the LTCC chaperone for Ca\textsubscript{v\alpha1}, antagonizes Ca\textsubscript{v\alpha1} protein degradation by preventing Ca\textsubscript{v\alpha1} PEST sequence recognition, leading to increased LTCC density and the consequent modulation of Ca\textsuperscript{2+} channel function. This novel mechanism by which Akt modulates LTCC stability could profoundly influence cardiac myocyte Ca\textsuperscript{2+} entry, Ca\textsuperscript{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\textsuperscript{2+} current (I\textsubscript{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\textsuperscript{2+} channel (LTCC) complex function.
In cardiomyocytes, the LTCC is composed of different subunits: the pore-forming subunit Ca_{α1} and the accessory β and α2β 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^{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^{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^{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 Spinaile, 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_{Ca,L} regulation remain unresolved.

Recently, a seminal study in neuronal cells revealed the importance of Akt-dependent phosphorylation of the Ca_{β2} subunit in promoting the chaperoning of the Ca_{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_{α1} contains highly conserved PEST sequences that direct rapid protein degradation and demonstrate that Akt-mediated phosphorylation of the Ca_{β2} LTCC chaperone subunit prevents PEST site recognition, thereby slowing or preventing Ca_{α1} degradation. This mechanism of action might be an essential process for Ca^{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_{Ca,L} and Ca^{2+} handling in the heart, we studied a mouse line with tamoxifen-inducible (Sohal et al., 2001) and cardiac-specific deletion of PKD1, 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 α-MHC promoter driving the cre recombinase gene (Sohal et al., 2001), resulting in MerCreMer α-MHC PKD1 mice (knockout [KO]). As opposed to the previously described muscle creatine kinase–Cre PKD1 mouse model (Mora et al., 2003) in which PKD1 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 α-MHC–Cre cardiac deletion of PKD1 (unpublished data). Similar to the muscle creatine kinase–Cre PKD1 mouse model (Lawlor et al., 2002), PKD1 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 PKD1 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_{α1} 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^{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^{2+} channel subunit, Ca_{α1}, which progressed as PKD1 protein expression gradually declined. No change in the protein level of the regulatory Ca_{β2} subunit was observed. As PKD1 expression decayed, levels of Akt activation also dramatically decreased (assessed by phosphorylation of Akt at the PKD1 phosphorylation site Thr308) despite unaltered expression of total Akt protein (Fig. 1, A and B). Furthermore, Akt activity (assessed using GSK-3β 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 PKD1 is on Akt activation, PKD1 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^{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 PKD1 deletion on SERCA2a (Fig. S2 A) as well as PKA activity because the phosphorylation of specific PKA regulatory sites in two SR Ca^{2+} regulatory proteins, Ryr (Ryr2-P2809) and phospholamban (PLN, PLN-P16), were unchanged in KO mice (Fig. S2 B), although it cannot be
Deficiency in Akt activity affects $I_{\text{Ca,L}}$

Ca$^{2+}$ 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 $I_{\text{Ca,L}}$ properties. No difference in cell size was observed between WT and KO cells as deduced from membrane capacitance measurements. Membrane capacitance was $116 \pm 6 \text{ pF}$ in WT cells ($n = 18$) and $115 \pm 6 \text{ pF}$ in KO cells ($n = 18$). However, the density of $I_{\text{Ca,L}}$ (picoampere/picofarad) was decreased in KO versus WT (Fig. 2 B). At 0 mV, the density of $I_{\text{Ca,L}}$ was $-9.08 \pm 0.96 \text{ pA/pF}$ in KO cells ($n = 12$) versus $-16.26 \pm 0.96 \text{ pA/pF}$ 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 $-12.97 \pm 0.53 \text{ mV}$ in WT cells versus $-15.07 \pm 0.66 \text{ mV}$ in KO cells, and mean half-inactivation occurred at $-31.11 \pm 0.48 \text{ mV}$ in WT cells versus $-30.77 \pm 0.42 \text{ mV}$ 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 $I_{\text{Ca,L}}$ in KO mice. Of note, the decay kinetics of $I_{\text{Ca,L}}$ 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$^{2+}$ amplitudes (Ca$^{2+}$ transients; Fig. 2 D and Fig. S3 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$^{2+}$ transient amplitude and cardiac contractility could be explained by reduced Ca$^{2+}$ entry into cells via the LTCC, but decreased intracellular Ca$^{2+}$ release from the SR may also contribute. However, although the Ca$^{2+}$ transient amplitude between the systolic and diastolic phase (twitch) was smaller in KO cardiomyocytes (Fig. S3 B, left bars), no difference in total SR Ca$^{2+}$ content was found (Fig. S3 B, right bars), suggesting that the decrease in Ca$^{2+}$ transient amplitude is only caused by reduced Ca$^{2+}$ entry. This is consistent with the observed slowing of the early fast inactivation of $I_{\text{Ca,L}}$ (Fig. 2 A), which is highly dependent on CICR-triggered SR Ca$^{2+}$ release during the action potential (Richard et al., 2006). Therefore, we conclude that the reduced $I_{\text{Ca,L}}$ may contribute to the reduced contractility in KO hearts.

Akt regulates the Ca$\alpha_1$ protein level at the plasma membrane

The properties of the Ca$\alpha_1$ 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$\beta_2$ is known to act as a chaperone for the Ca$\alpha_1$ 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$\alpha_1$ 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$\alpha_1$ protein to the plasma membrane. However, because the amount of Ca$\alpha_1$ was reduced in both microsomal and membrane fractions from KO extracts compared with WT (Fig. 3 B), we hypothesized that the reduced Ca$\alpha_1$ 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$\alpha_1$ protein degradation, three sets of specific cell degradation system inhibitors were examined for their ability to...
A detection effect was abolished in the absence of Ca\textsubscript{v1}/H\textsubscript{925} cotransfection, a condition under which Ca\textsubscript{v1}/H\textsubscript{925} is retained in the ER (Fig. 3C, bottom). All together, these results confirm that Akt activity is regulating Ca\textsubscript{v1}/H\textsubscript{925} protein density and reveal that in the absence of Akt function, Ca\textsubscript{v1}/H\textsubscript{925} is susceptible to lysosome-mediated membrane protein degradation. Because Ca\textsubscript{v1}/H\textsubscript{925} is the only LTCC accessory subunit containing an Akt phosphorylation consensus site (Viard et al., 2004), we hypothesized that Ca\textsubscript{v1}/H\textsubscript{925} protein degradation at the plasma membrane might result from loss of Ca\textsubscript{v1}/H\textsubscript{925} chaperone activity in the absence of Akt-induced phosphorylation. In support of this hypothesis, treatment of Ca\textsubscript{v1}/H\textsubscript{925}- and Ca\textsubscript{v1}/H\textsubscript{925}2-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\textsubscript{v1}/H\textsubscript{925} protein induced by Akt inhibition (Fig. 3C, top). Conversely, a ubiquitin/proteasome inhibitor, MG132, failed to protect Ca\textsubscript{v1}/H\textsubscript{925} from protein degradation. Similar results were obtained by inhibiting calpain, the intracellular Ca\textsuperscript{2+}-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\textsubscript{v1}/H\textsubscript{925} cotransfection, a condition under which Ca\textsubscript{v1}/H\textsubscript{925} is retained in the ER (Fig. 3C, bottom). All together, these results confirm that Akt activity is regulating Ca\textsubscript{v1}/H\textsubscript{925} protein density and reveal that in the absence of Akt function, Ca\textsubscript{v1}/H\textsubscript{925} is susceptible to lysosome-mediated membrane protein degradation.

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

Figure 3. Akt mediates regulation of Ca\textsubscript{v1}/H\textsubscript{925} protein density at the plasma membrane. (A) RT-PCR analysis of Ca\textsubscript{v1}, 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\textsubscript{v1}/H\textsubscript{925}– transfected COS-7 cells alone or in combination with Ca\textsubscript{v1}/H\textsubscript{925}2 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\textsubscript{v1}/H\textsubscript{925} 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,α1 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,α1 levels compared with WT controls (Fig. 3 E).

Akt is a determinant for Ca,α1 protein level regulation by direct phosphorylation of the Ca,β2 chaperone subunit

To assess whether Akt is directly involved in modulation of Ca,β2 chaperone activity in the heart, we first confirmed the interaction between Akt and Ca,β2. Ventricular homogenates derived from either WT or Tg Akt mice were immunoprecipitated with anti-HA antibody and assayed for Ca,β2, which revealed association of the Ca,β2 subunit with active Akt (Fig. 4 A). Similarly, Ca,β2 was found to coimmunoprecipitate with insulin-stimulated endogenous Akt (Fig. S4 A).

To determine whether Ca,β2 can be phosphorylated by Akt, Ca,β2 immunoprecipitates from cardiac homogenates were incubated with recombinant active Akt and γ-[32P]ATP. A band corresponding to phosphorylated Ca,β2 was detected only in the presence of the kinase (Fig. 4 B, left). To determine whether the Ca,β2 subunit was phosphorylated by Akt in vivo, we treated overnight-starved mice with 1 mU/g insulin to induce activation of Akt (Bayas et al., 2008). 20 min after treatment, Ca,β2 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,β2 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,β2 phosphorylation, revealed a reduction of the basal phosphorylation level of Ca,β2 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,β2, the chaperone for Ca,α1.

To directly assess whether Akt phosphorylation of Ca,β2 protects Ca,α1 from protein degradation, we constructed a mutant of Ca,β2 in which Ser625, which is contained in the putative Akt consensus site (R-X-X-R-S/T), was replaced by glutamate (Ca,β2-SE) to mimic phosphorylation. Cotransfection of 293T cells with Ca,α1 and Ca,β2-SE resulted in Ca,α1 protein levels that were increased compared with those found when cotransfected with Ca,β2-WT (Fig. 5 A). Similarly, Ca,α1 expression was increased in insulin-treated Ca,β2-WT–cotransfected cells (Fig. 5 A). Notably, the active phosphomimetic Ca,β2-SE also counteracted the down-regulation of Ca,α1 induced by an Akt inhibitor (Fig. 5 B). Opposite results were obtained with a dominant-negative (DN) Ca,β2 mutant in which Ser was replaced by Ala (Ca,β2-SA) to prevent Akt phosphorylation. Indeed, Ca,α1 protein levels were reduced when coexpressed with Ca,β2-SA (Fig. 5 C). In addition, insulin stimulation failed to increase Ca,α1 in the presence of the DN Ca,β2-SA mutant (Fig. 5 C). Consistent with the hypothesis that Ca,α1 protein down-regulation relies on Akt kinase activity, overexpression of a DN form of Akt (AdAktDN) resulted in a significant reduction in Ca,α1 protein levels, whereas forced expression of AdAkt was sufficient to counteract Ca,α1 reduction in a serum-free condition, in which Akt is not phosphorylated (Fig. 5 D). Furthermore, suppression of Akt expression in 293T cells by siRNA (small interfering Akt [siAkt]) resulted in reduction of the Ca,α1 protein level (Fig. 5 D).

To support the evidence that Akt-dependent phosphorylation of Ca,β2 is a determinant for Ca,α1 stability and functionality, we measured the effect of the Ca,β2 mutants on Ca2+ current. Although cotransfection of cells with Ca,α1 and Ca,β2-WT resulted in significant depressed Icalc in serum-free medium compared with serum-containing medium in which Akt is phosphorylated (not depicted), cotransfection of Ca,α1
with one located in the I–II linker of the Ca\textsubscript{v} and 1,839–1,865). Intriguingly, the highest scored potential PEST motifs (amino acids 435–460, 807–820, 847–858, 1,732–1,745, (A–C) YFP-Ca\textsubscript{v} degradation. [A–C] YFP-Ca\textsubscript{a,1}–cotransfected 293T cells with the indicated mutant variant of Ca\textsubscript{b,2}. 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-Ca\textsubscript{a,1} in lysates was monitored by Western blot analysis with anti-YFP antibody and normalized based on transfection efficiency [Ca\textsubscript{b,2}] and protein amount (tubulin; n = 3). (D) Ca\textsubscript{a,1}- and Ca\textsubscript{b,2}–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).

and Ca\textsubscript{b,2}-SE mutant but not Ca\textsubscript{b,2}-SA mutant completely counteracted this reduction (Fig. 6).

**Akt regulates Ca\textsubscript{a,1} 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 Ca\textsuperscript{2+} channel function through regulation of the Ca\textsubscript{b,3} accessory subunit (Sandoval et al., 2006). Our findings raise the possibility that processing of the Ca\textsubscript{a,1} 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 Ca\textsubscript{a,1} 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 Ca\textsubscript{a,1} subunit and overlapping with the α1-interacting domain (AID), which is the primary binding region for Ca\textsubscript{b,2} (Fig. 7 A; Bodi et al., 2005). To determine whether these PEST sequences are involved in Ca\textsubscript{a,1} degradation control, we generated two in-frame deletion mutants encompassing either the I–II (Ca\textsubscript{a,1}-ΔP) or II–III (Ca\textsubscript{a,1}-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 Ca\textsubscript{a,1}-ΔP and Ca\textsubscript{a,1}-ΔH mutants compared with Ca\textsubscript{a,1}-WT, which is consistent with the hypothesis that these motifs deter- mine Ca\textsubscript{a,1} 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 Ca\textsubscript{a,1}-ΔP and Ca\textsubscript{a,1}-ΔH compared with Ca\textsubscript{a,1}-WT (Fig. 7 C). In particular, Ca\textsubscript{a,1}-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, Ca\textsubscript{a,1}-ΔP and Ca\textsubscript{a,1}-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 Ca\textsubscript{b,2}-SE with Ca\textsubscript{a,1}-WT resulted in a considerable increase in the half-life of Ca\textsubscript{a,1}-WT (Fig. 7 C). In addition, transfection of 293T cells with Ca\textsubscript{a,1} 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 Ca\textsubscript{a,1} protein stability. Consistent with the hypothesis that Akt-mediated protection of Ca\textsubscript{a,1} degradation acts through PEST sequences, overexpression of AdAktDN or siAkt had no significant effect on protein levels of either Ca\textsubscript{a,1}-ΔP or Ca\textsubscript{a,1}-ΔH mutants (Fig. S4, B and C). To assess whether the observed PEST mechanism is caused by a direct Akt-dependent interaction between Ca\textsubscript{b,2} and Ca\textsubscript{a,1}, we performed in vitro binding assays using in vitro–translated [35]S-labeled Ca\textsubscript{a,1} cytosolic domains and a GST-fused Ca\textsubscript{b,2} C-terminal coiled-coil region. Notably, direct interaction took place between the Akt-phosphorylated Ca\textsubscript{b,2} C-terminal coiled-coil region and the Ca\textsubscript{a,1} C-terminal domain (Fig. 7 E). No interactions were found with other Ca\textsubscript{a,1} cytosolic domains (unpublished data), although it cannot be excluded that other binding sites may exist.

To assess whether PEST-deleted Ca\textsubscript{a,1} channels are still functional, traffic appropriately to the membrane, and associate with the Ca\textsubscript{b,2} subunit, we measured Ca\textsuperscript{2+} current in Ca\textsubscript{a,1}-ΔH mutant–transfected cells. No significant differences in I\textsubscript{Ca,L} were found in cells transfected with Ca\textsubscript{a,1}-WT compared with Ca\textsubscript{a,1}-ΔH (Fig. 7 F). Conversely, although serum deprivation resulted in I\textsubscript{Ca,L} reduction in Ca\textsubscript{a,1}-WT–transfected cells, no significant changes were observed in Ca\textsubscript{a,1}-ΔH mutant–transfected cells (Fig. 7 F). This confirms that PEST-deleted Ca\textsubscript{a,1}-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\textsubscript{Ca,L}, indicating that a change in the macroscopic current properties of Ca\textsubscript{a,1,2} is unlikely.
Collectively, our results suggest that Akt-mediated phosphorylation of Ca\(_{\beta2}\) regulates Ca\(_{\alpha1}\) density through protection of Ca\(_{\alpha1}\) 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\(_{\text{2+}}\) 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\(_{\beta2}\), which in turn results in increased protein density of the pore-forming Ca\(_{\alpha1}\) subunit through protection of PEST sequences from the proteolytic degradation system. In the absence of phosphorylated Akt, the Ca\(_{\text{2+}}\) current is reduced, resulting in a decreased Ca\(_{\text{2+}}\) transient and contractility. Therefore, it is tempting to speculate that the Akt-mediated phosphorylation of Ca\(_{\beta2}\) and the consequent direct association of the Ca\(_{\alpha1}\) C-terminal coiled-coil region (Fig. 7 E) 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\(_{\beta2}\) 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 anti-apoptotic 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

![Figure 6. Akt phosphorylation of Ca\(_{\beta2}\) preserves Ca\(_{\alpha1}\) currents.](image-url) Ca\(_{\text{2+}}\) currents recorded in cotransfected tsA-201 cells with YFP-Ca\(_{\alpha1}\) and Ca\(_{\beta2}\)-WT, Ca\(_{\beta2}\)-SE, or Ca\(_{\beta2}\)-SA mutant cultivated for 36 h in the presence or absence of 10% fetal bovine serum. Currents were recorded 1–2 min after the whole cell configuration was achieved (i.e., after stabilization of the current) and were elicited by a 0-mV depolarization of 200-ms duration applied from a holding potential of −80 mV. Currents are normalized to cell capacitance (current density, picoampere/pico-farad). Representative current traces are shown. n > 35 at each condition (*, P < 0.05 compared with YFP-Ca\(_{\alpha1}\); ANOVA). Error bars show SEM.

### Table I. PEST sequences are highly conserved in Ca\(_{\alpha1}\)

| Species  | Fragment | Sequences | PESTfind score |
|----------|----------|-----------|----------------|
| Mouse    | PEST I   | 435-KGYLDWITQAEIDPENEDGMDEK-460 | 8.45 |
| Rat      | PEST I   | 476-KGYLDWITQAEIDPENEDGMDEK-501 | 8.45 |
| Human    | PEST I   | 446-KGYLDWITQAEIDPENEDGMDEK-471 | 8.66 |
| Mouse    | PEST II  | 807-KSITADGESPPTTK-820 | 8.45 |
| Rat      | PEST II  | 848-KSITADGESPPTTK-861 | 9.45 |
| Mouse    | PEST III | 837-HSNPDTAGEEDEEEPEMPVGR-858 | 19.51 |
| Rat      | PEST III | 878-HSNPDTAGEEDEEEPEMPVGR-899 | 19.51 |
| Human    | PEST II  | 845-KSPYPNPTETEDEEEEEPEMPVGR-869 | 20.26 |
| Mouse    | PEST IV  | 1,732-KTGNMGATUESPH-1,745 | 5.5 |
| Rat      | PEST IV  | 1,772-KTGNMGATUESPH-1,785 | 5.5 |
| Mouse    | PEST V   | 1,839-RMSEAEYEPSLSTDMSYQDEH-1,865 | 5.86 |
| Human    | PEST IV  | 1,937-HDTEACSEPSLSTEMSQYQDDN-1,961 | 7.54 |
| Human    | PEST V   | 2,214-RGAPSEEEIGDERS-2,226 | 7.71 |

Occurrence of PEST sites within the amino acid sequence of Ca\(_{\alpha1}\) 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

Figure 7. Rapid protein degradation PEST sequences determine Ca\(_{\alpha 1}\) protein instability. [A] Schematic representation of Ca\(_{\alpha 1}\) mapping the AID and PEST sequences in the I-II and II-III cytosolic loops. Deleted PEST sequences (P and H) are highlighted in red. [B] Western blot and immunofluorescence analyses showing relative levels of WT and PEST-deleted mutants of YFP-Ca\(_{\alpha 1}\) (n = 3). The asterisk indicates a YFP-Ca\(_{\alpha 1}\) degradation fragment. (C) Half-lives of WT Ca\(_{\alpha 1}\) subunit (alone or cotransfected with Ca\(_{\beta 2}\)-SE) and its in-frame ΔPEST mutants (Ca\(_{\alpha 1}\)-ΔP and Ca\(_{\alpha 1}\)-ΔH) were determined in COS-7 cells. After overnight starvation, transfected cells were pulse chased and analyzed along a time course (*, P < 0.001 compared with Ca\(_{\alpha 1};\) ANOVA; n = 3). Error bars show SD. (D) Western blot and immunofluorescence analyses showing relative levels of WT GFP and N-terminal fusion PEST mutants (n = 3). (E) The Ca\(_{\alpha 1}\) C terminus interacts with the Akt-phosphorylated GST-Ca\(_{\beta 2}\) coiled-coil region. Bacterially expressed GST or GST-C-Ca\(_{\beta 2}\) (Ca\(_{\beta 2}\), amino acids 480–655) fusion protein and glutathione–Sepharose beads were incubated with equal amounts of in vitro–translated \([^{35}S]\)Met-labeled C-Ca\(_{\alpha 1}\) (Ca\(_{\alpha 1}\), amino acids 1,477–2,169). Binding occurred only with Akt-phosphorylated GST-C-Ca\(_{\beta 2}\). Bound proteins were resolved by SDS-PAGE (4–12%). 10% of the input protein in each binding reaction is shown. Coomassie staining of SDS-PAGE is shown in the bottom panel. (F) Ca\(^{2+}\) currents recorded in tsA-201 cells cotransfected with Ca\(_{\beta 2}\)-WT and either Ca\(_{\alpha 1}\)-WT or Ca\(_{\alpha 1}\)-ΔH and cultivated for 36 h in the presence or absence of 10% fetal bovine serum. Current densities (pico-ampere/picofarad) are normalized to the control condition. n > 35 at each condition (*, P < 0.05 compared with Ca\(_{\alpha 1}\); ANOVA). Error bars show SEM. Bars: (B) 5 μm; (D) 20 μm.
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_{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 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 Ca_{\alpha}2.2a subunit is involved in the PI3K-induced chaperoning of Ca_{\alpha}2.2a in neurons. This PI3K-induced regulation was shown to be mediated by Akt phosphorylation of the Ca_{\alpha}2.2a subunit, which in turn regulates Ca_{\alpha}2.2a 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 Ca_{\alpha}2 subunit both in neurons and the heart (Viard et al., 2004), thus illustrating the importance of this site. Interestingly, two very short human cardiac splice isoforms, Ca_{\beta}2F and Ca_{\beta}2g, with preserved Akt sites have been shown to be essential for modulating Ca^{2+} channel function and Ca_{\alpha}1 channel density (De Waard et al., 1994; Kobrinsky et al., 2005). Strikingly, the same two Ca_{\beta}2 variants do not contain the PKA phosphorylation site (Kamp and Hell, 2000), which is consistent with our data suggesting no PKA involvement in the modulation of LTCC density (Fig. S2 B). As a corollary, the presence of this conserved C-terminal region in all Ca_{\beta}2 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 Ca_{\beta}2 C-terminal region, Lao et al. (2008) recently showed that, in the absence of the main Ca_{\beta}2 protein domain, the selected C-terminal essential determinant is sufficient for I_{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- and Akt-mediated regulation of Ca_{\alpha}1 channel density by protection of Ca_{\alpha}1 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 Ca_{\alpha}1 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.

Materials and methods

**Generation of genetically modified mice**
Cardiomyocyte-specific PDK1 inducible KO mice (MerCreMer α-MHC PDK1) were generated by breeding PDK1FLoxed/FLoxed 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 cardiomyocyte-specific MerCreMer α-MHC promoter-driven cre recombinase gene (provided by J.D. Molkentin, University of Cincinnati, Cincinnati, OH; Sohal et al., 2001). The resulting background strain of the MerCreMer mice was C57BL/6-SV129 and was unchanged throughout all experiments. Control animals used in this study were PDK1FLoxed/FLoxed 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 Akt-K179M (AdAktDN) at MOI 100 and harvested 48 h after infection. The viral vector was amplified and purified in 3% sucrose/PBS by ViralQuest, 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 μM Akt XI inhibitor (EMD), insulin (Sigma-Aldrich), 1 μM bafilomycin-A1 (Sigma-Aldrich), 25 μM MG132 (EMD), and 25 μM colcemid (EMD) were used as described in Results. Cacnb2 cDNA (complete coding sequence, cDNA clone MGC:129335, IMAGE:4047531; American Type Culture Collection) was cloned in the pcDNA3 vector. Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies). Ca_{\alpha}1 PEST deletion mutants and GFP fusion proteins were generated by PCR. YFP–Ca_{\alpha}1 expression plasmids were provided by N. Soldatov (National Institute on Aging, National Institutes of Health, Baltimore, MD). A lentiviral vector was generated and used as an expression vector for siRNA-mediated silencing of the akt gene (siAkt). The sequence used (5'-TGCCCTCTACAACCCAGGAT-3') was chosen in a conserved region between rat, mouse, and human and has been validated for targeting Akt1 and -2 (Katome et al., 2003). All constructs were confirmed by DNA sequencing.
Ca	extsuperscript{2+} current measurement

Macroscopic I_{Ca,L} was recorded at room temperature (~22°C) using the whole cell patch-clamp technique in native cells as previously described (Maier et al., 2003; Aimond 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-Cl. Pipette solution contained 125 mM CsCl, 20 mM TEA-Cl, 0.1 M 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 mV, and 10 mV depolarization steps from ~50 to 50 mV for 30 ms were applied. Analysis was performed using a microscope (Diaphot 200; Nikon) equipped with 10X NA 20 objective lenses (CFVN; Nikon). pCLAMP 9 (MDS Analytical Technologies) was used as acquisition software. For electrophysiological recordings of recombinant proteins, naive-Ca\textsubscript{v}2.1a cells were transfected in OptiMEM (Invitrogen) with a DNA mix containing plasmids encoding YFP-Ca\textsubscript{v}2.1a, Ca\textsubscript{2+} subunit (either Ca\textsubscript{v}2.2-WT, Ca\textsubscript{v}2.5E, or Ca\textsubscript{v}2.5A), Ca\textsubscript{2+} subunit, and CDB (in a ratio of 1:2.0:5.0:1). After 24 h, cells were cultured in DME with or without serum for 36 h, and electrophysiologival recordings were performed on cells expressing both YFP-Ca\textsubscript{v}2.1a and CDB, which is identified using anti-CDB-coated beads (Dynabeads; Invitrogen). The ~330 mosM extracellular solution contained 135 mM NaCl, 20 mM TEA-Cl, 5 mM CaCl\textsubscript{2}, 1 M MgCl\textsubscript{2}, and 10 mM Hepes (pH adjusted to 7.4 with KOH). Borosilicate glass pipettes have a typical resistance of 1.5-3 MW when filled with an ~310 mosM internal solution containing 140 mM CsCl, 10 mM EGTA, 10 mM Hepes, 3 mM Mg\textsubscript{2+}-ATP, 0.6 mM GTP-Na, and 2 mM CaCl\textsubscript{2} (pH adjusted to 7.2 with KOH). Analysis was performed using a microscope (x71; Olympus). Data acquisition was performed with pCLAMP 9 software.

Fluorescent measurement of [Ca\textsuperscript{2+}]

Isolated myocytes were loaded with 5 μM Fura-PE3 acetoxymethyl (TefLabs) and analyzed as previously described (Bassani et al., 1994; DeSantagious et al., 2002). Analysis was performed using a Diaphot 200 microscope. Data acquisition and analysis were performed using pCLAMP software (Clampex and Clampfit version 8.2; MDS Analytical Technologies).

Akt and PKC kinase assay

Myocardial tissue lysates were tested using the Akt Kinase Assay kit (Cell Signaling Technology) and PKC (Millipore) according to the manufacturer’s instructions.

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{v}1.1 (Novus Biologicals), Ca\textsubscript{v}1.3 and Ca\textsubscript{v}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, Akt-P308, and anti-phospho-Ser/Thr/Akt substrate (Cell Signaling Technology); PTK and PLN-P16 (Novus Biologicals); calsoquen (BD); calsoquestrin (Cell Signaling Technology); HA (Roche); GFP/YFP (GeneTex, Inc.); tubulin (Novus Biologicals); PLN-P16 (Novus Biologicals); calsequestrin (BD); caspase 3 (Cell Signaling Technology); PKA (Novus Biologicals); PKC ε and PKC δ (Cell Signaling Technology); GAPDH; Cardiac-specific Ca\textsupscript{2+} ATPase; and phosphorylation site-specific antibodies, Dr. Nikolai Soldatov for YFP-Ca\textsubscript{v}2.1a and circulating antibodies, 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 to WT cardiomyocytes. Fig. S4 shows coimmunoprecipitation of Ca\textsubscript{v}2.2 with insulin-activated Akt isoforms and the effects of dominant-active and -negative Akt as well as siAkt on the Ca\textsubscript{v}1.1 protein level. Fig. S5 shows current-voltage analysis (IV curves) of cells transfected with Ca\textsubscript{v}1.1-WT or Ca\textsubscript{v}1.1-L31H in normal or serum-free conditions. Table S1 shows electrophysiological analysis values of WT and KO mice. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200805063/DC1.

Histology and confocal microscopy

Fixation, staining, and confocal analysis were performed as previously described (Care et al., 2007). Confocal microscopy was performed using a confocal microscope (Radiance 2000; Bio-Rad Laboratories) with a 60x Plan-Apochromat 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, 293T cells were starved for 30 min in Met and Ca\textsubscript{v}2.1a (Sigma-Aldrich) were then labeled for 30 min by adding 500 μCi 35S-labeled Met and 2 mM L-Cys. Radioactive media was eventually washed out with PBS (time 0 pulse) and replaced with normal DME. Time points were at 4, 10, and 25 h after pulse. Anti-GFP polyclonal IgG (GTIX20290) was used for immunoprecipitation. Radioactivity was quantified with ImageQuant 5.2 software (GE Healthcare).

GST pull-down assay

Affinity-purified GST fusion proteins were generated using a pGEX system (GE Healthcare) and phosphorylated as described below. GST fusion protein bound to glutathione—Sepharose 4B beads (GE Healthcare) was incubated with 25 μl of 35S-labeled Met protein with moderate shaking at 25°C for 2 h in 200 μl of binding buffer containing 20 mM Hepes, pH 7.9, 1 mM EDTA, 10% glycerol, 0.15 M KCl, 0.05% Nonidet P-40, and 1 mM DTT. 35S-labeled probes were generated from the Cterminal region of Ca\textsubscript{v}1.1 cDNA fragments under control of the T7 promoter using the TnT Quick Coupled Reticulocyte Lysate System (11797; Promega), washed three times with washing buffer (20 mM Hepes, pH 7.9, 1 mM EDTA, 10% glycerol, 250 mM KCl, and 0.1% Nonidet P-40), and centrifuged. Bound proteins were eluted in SDS sample buffer, subjected to SDS-PAGE, and detected by autoradiography. Recombinant GST-Ca\textsubscript{v}2.2 beads or GST beads were phosphorylated by incubation with recombinant Akt (Millipore). In brief, 5 μg GST-Ca\textsubscript{v}2.2 or GST beads were incubated at 30°C for 45 min in a 50-μl solution containing 2 μg activated Akt kinase, 10 mM Hepes-KOH, pH 7.5, 50 mM γ-glycerophosphate, 50 mM NaCl, 1 mM dithiothreitol, 10 mM MnCl\textsubscript{2}, and 1 mM AIP.

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 PDK1 expression. Fig. S2 shows SERCA2a level and phosphorylation of specific PKA regulatory sites in two Ca\textsubscript{v}2.1 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 coimmunoprecipitation of Ca\textsubscript{v}2.2 with insulin-activated Akt isoforms and the effects of dominant-active and -negative Akt as well as siAkt on the Ca\textsubscript{v}1.1 protein level. Fig. S5 shows current-voltage analysis (IV curves) of cells transfected with Ca\textsubscript{v}1.1-WT or Ca\textsubscript{v}1.1-L31H in normal or serum-free conditions. Table S1 shows electrophysiological analysis values of WT and KO mice. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200805063/DC1.

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**Akt regulates L-type Ca$^{2+}$ channel activity by modulating Ca$_{v}$a1 protein stability**

Daniele Catalucci, Deng-Hong Zhang, Jaime DeSantiago, Franck Aimon, 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.

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