Increased intracellular Ca\(^{2+}\) concentrations prevent membrane localization of PH domains through the formation of Ca\(^{2+}\)-phosphoinositides

Jin Ku Kang\(^a\), Ok-Hee Kim\(^b\), June Hur\(^a\), So Hee Yu\(^c\), Santosh Lamicchane\(^d\), Jin Wook Lee\(^d\), Uttam Ojha\(^a\), Jeong Hee Hong\(^a\), Cheol Soon Lee\(^a\), Ji-Young Cha\(^b\), Young Jae Lee\(^b\), Seung-Soon Im\(^b\), Young Joo Park\(^d\), Cheol Soo Choi\(^a\), Dae Ho Lee\(^a\), In-Kyu Lee\(^e\), and Byung-Chul Oh\(^f\)

\(^a\)Department of Physiology, Lee Gil Ya Cancer and Diabetes Institute, Gachon University College of Medicine, Incheon 21999, Republic of Korea; \(^b\)Department of Biochemistry, Lee Gil Ya Cancer and Diabetes Institute, Gachon University College of Medicine, Incheon 21999, Republic of Korea; \(^c\)Department of Physiology, Keimyung University School of Medicine, Daegu 704-701, Republic of Korea; \(^d\)Department of Internal Medicine, Seoul National University College of Medicine, Seoul 03080, Republic of Korea; and \(^e\)Department of Internal Medicine, Graduate School of Medicine, Kyungpook National University, Daegu 702-701, Republic of Korea

Edited by C. Ronald Kahn, Section of Integrative Physiology, Joslin Diabetes Center, Harvard Medical School, Boston, MA, and approved September 29, 2017 (received for review April 19, 2017)

Insulin resistance, a key etiological factor in metabolic syndrome, is closely linked to ectopic lipid accumulation and increased intracellular Ca\(^{2+}\) concentrations in muscle and liver. However, the mechanism by which dysregulated intracellular Ca\(^{2+}\) homeostasis causes insulin resistance remains elusive. Here, we show that increased intracellular Ca\(^{2+}\) acts as a negative regulator of insulin signaling. Chronic intracellular Ca\(^{2+}\) overload in hepatocytes reduces obesity and hyperlipidemia attenuates the phosphorylation of protein kinase B (Akt) and its downstream signaling. These findings provide a mechanistic link between intracellular Ca\(^{2+}\) homeostasis and insulin resistance during obesity and hyperlipidemia attenuates the phosphorylation of Akt and its key downstream signaling in obese subjects and diabetic patients (12, 13), but also restored autophagy (4, 9) and insulin sensitivity in obese mouse models (14). However, the molecular mechanisms that link intracellular Ca\(^{2+}\) overload to insulin resistance have not been completely elucidated.

Insulin-stimulated phosphoinositide 3-kinase (PI3K) catalyzes the phosphorylation of phosphoinositides (PIPs) at the 3-position to produce PI(3,4)P\(_2\) or PI(3,4,5)P\(_3\), which recruit a variety of signaling proteins with pleckstrin homology (PH) domains, including phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (Akt) (1, 15). In turn, Akt acts as a key merge point of the PI(3,4,5)P\(_3\)-mediated insulin signaling pathway by phosphorylating the enzyme glycogen synthase kinase 3 beta (GSK3\(\beta\)), the forkhead transcription factors, the 160-kDa substrate of Akt (AS160), and cAMP response element-binding protein (CREB) (1). The activity of the insulin signaling pathway is transiently attenuated by dephosphorylation of PI(3,4,5)P\(_3\).

**Significance**

Insulin resistance is a metabolic disorder in which target cells fail to respond to physiological levels of circulating insulin, leading to hyperinsulinemia and glucose intolerance. The molecular mechanism underlying insulin resistance is still largely unknown. Here, we found that intracellular Ca\(^{2+}\) overloading in obesity attenuates insulin-stimulated phosphorylation of protein kinase B and its downstream signaling by preventing membrane localization of various pleckstrin homology (PH) domains. When at high intracellular levels, Ca\(^{2+}\) binds tightly with phosphoinositides to yield Ca\(^{2+}\)-phosphoinositides (PIPs), abrogating the membrane targeting of PH domains and disrupting insulin signaling. Thus, we identified a previously unknown physiological function of intracellular Ca\(^{2+}\) as a critical negative regulator of insulin signaling, especially through the formation of Ca\(^{2+}\)-PIPs.

Author contributions: J.K.K., O.-H.K., I.-K.L., and B.-C.O. designed research; J.K.K., O.-H.K., J.H., S.H.Y., S.L., J.W.L., U.O., J.H.H., C.S.L., J.Y.C., Y.J.L., S.-S.I., Y.J.P., C.S.C., and D.H.L. performed research; O.-H.K., Y.I.P., D.H.L., I.-K.L., and B.-C.O. analyzed data; and I.-K.L. and B.-C.O. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

To whom correspondence should be addressed. Email: bcoh@gachon.ac.kr.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1706489114/-/DCSupplemental.
via phosphoinositide phosphatases such as PTEN and SHIP2, altering its binding specificity and affinity to PH domains (15). Thus, the binding of PH domains to PI(3,4,5)P3 has a critical role in regulating Akt function (16).Aside from enzymatic dephosphorylation of PI(3,4,5)P3 by phosphoinositide phosphatases, however, other regulatory mechanisms of the binding of PH domains to PI(3,4,5)P3 have not been reported.

PH domains are small protein modules that occur in a large variety of ~250 proteins, including Akt/Rac family serine/threonine kinases, Btk/Ik/Tec subfamily tyrosine kinases, phosphoinositide-specific phospholipase C (PLC), the Rho family of GTPases, insulin receptor substrates (IRSs), and cytoskeletal proteins (17), suggesting their broad and important roles in cell signaling and regulation. PH domains play essential roles in recruiting proteins to the plasma membrane by binding to their phosphoinositides with a broad range of specificity and affinity. PH domains of Akt, Bruton’s tyrosine kinase (BTK), and general receptor for phosphoinositides-1 (GRP1) are known to recognize highly specific PI(3)K products of PI(3,4)P2 and PI(3,4,5)P3 (17). Mutations disrupting PH domain function that abolish PI(3,4,5)P3 binding cause severe signaling defects such as X-linked agammaglobulinemia in humans and X-linked immunodeficiency in mice (18, 19). In contrast, mutations that promote constitutive membrane localization of Akt PH domains at the plasma membrane can cause cancer (20). These findings imply that membrane targeting of PH domains through PI(3,4,5)P3 recognition is essential for Akt activity.

In this study, we provide evidence that phosphoinositides tightly bind with Ca++, forming Ca++,PIPs under obesity-associated intracellular Ca++ overload. These Ca++,PIPs prevent membrane recruitment of PH domains by inhibiting their binding to PI(3,4,5)P3, leading to abnormal subcellular localization of PH domains. Our results demonstrate a molecular mechanism of Ca++,mediated inhibition of the recruitment of various PH domain-containing molecules to the plasma membrane, providing insights into diseases associated with abnormal subcellular localization of signaling proteins.

Results

High-Fat Diets and Palmitate Treatment Increase Intracellular Ca++ Levels and Attenuate Insulin Signaling. To investigate the molecular mechanisms of insulin resistance, we fed mice a high-fat diet (HFD) for normal chow for 8 wk, then fasted the mice overnight and subsequently refed them with normal chow or a HFD for 4 h. We then analyzed the effects of a HFD on the phosphorylation of key insulin signaling molecules, Akt, and its downstream signaling molecules GSK3β and FOXO3. Interestingly, postprandial phosphorylation of Akt at T308 and S473 and the phosphorylation of GSK3β and FOXO3 were dramatically decreased in mice livers after refeeding with a HFD (Fig. 1A), suggesting that insulin signaling was impaired in the livers of mice fed a HFD for 8 wk. Based on recent findings that dysregulation of intracellular Ca++ plays an important role in insulin resistance (5, 9, 10, 21), we next analyzed in vivo levels of intracellular Ca++ in the liver of mice fed a HFD or normal chow for diet for 10 wk using adenosine vectors to express calmodulin-based genetically encoded fluorescent calcium indicators (GCaMP6m) (22). This method results in robust expression of adenoviral GCaMP6m in the hepatocytes of mice fed a HFD (Fig. 1B), where we observed that the hepatocytes expressing GCaMP6m were significantly elevated in the livers of HFD-fed mice compared with controls (Fig. 1C). Quantification of fluorescent signals showed that the intracellular Ca++ level was almost threefold higher in the hepatocytes of HFD-fed mice than in control mice regardless of feeding status (Fig. 1C), demonstrating that intracellular Ca++ was highly elevated in the hepatocytes of HFD-fed mice.

To assess whether impaired insulin signaling in mice fed a HFD is associated with increased intracellular Ca++ levels, we treated human HepG2 hepatoma cells for 24 h with palmitic acid, a long-chain saturated fatty acid that causes insulin resistance in animals (23). Similar to our in vivo findings, palmitic acid treatment markedly attenuated the insulin-stimulated phosphorylation of Akt at T308 and S473 and the phosphorylation of GSK3β and FOXO3 in a dose-dependent manner (Fig. 1D and SI Appendix, Fig. S1), indicating that palmitic acid impairs insulin signaling in vitro. Next, we examined the effects of palmitic acid on intracellular Ca++ levels in HepG2 cells using the fluorescent dye Fluo-3 acetoxymethyl (AM). We found that elevated intracellular Ca++ levels were almost threefold higher in HepG2 cells treated with palmitic acid (Fig. 1F). These results indicated that exposure to a HFD elevated palmitic acid levels, leading to increased intracellular Ca++ levels, suggesting a mechanism responsible for impaired insulin signaling. To further assess the importance of intracellular Ca++ overload, we measured intracellular Ca++ concentrations in HepG2 cells with Fura-2 AM after palmitic acid treatment for 24 h. Treatment with high concentrations of palmitic acid significantly elevated the baseline intracellular Ca++ concentrations (approximately threefold, SI Appendix, Fig. S2A). Strikingly, high concentrations of palmitic acid led to irregular patterns of sustained intracellular Ca++ overload in HepG2 cells (SI Appendix, Fig. S2B).

Thus, we hypothesized that the attenuation of insulin-stimulated Akt phosphorylation by a HFD is potentially driven by elevated intracellular Ca++ levels.
Concentration and M (24) and high intracellular Ca concentration prevents membrane localization of Akt, FOXO3A, and AS160, and the total amounts of the indicated proteins in HepG2 cells. Cells were incubated for 30 min with the indicated concentrations of PMA (A) or ionomycin (D), followed by treatment with 100 nM insulin for 15 min. (B, C, E, and F) Representative Fluo-3 AM images (B) and quantification (C) of intracellular Ca\(^{2+}\) in HepG2 cells treated with PMA. Data represent means ± SEM (n = 5, *P < 0.05).

**Akt Phosphorylation Is Modulated by Intracellular Ca\(^{2+}\) Concentration.**

To investigate the direct effects of elevated intracellular Ca\(^{2+}\) on Akt phosphorylation, we evaluated the effects of phorbol myristate acetate (PMA) and ionomycin on Akt phosphorylation, both of which are used to trigger intracellular calcium influx. After pretreating the HepG2 cells with PMA or ionomycin for 30 min to induce sustained intracellular Ca\(^{2+}\) overload, we examined insulin-stimulated phosphorylation of Akt after stimulating with insulin (100 nM) for 15 min. Immunoblotting clearly showed that PMA dramatically inhibited insulin-stimulated phosphorylation of Akt at T308 and S473 and its substrates AS160 and FOXO3, and the total amounts of the indicated proteins in HepG2 cells. Cells were incubated for 30 min with the indicated concentrations of PMA (A) or ionomycin (D), followed by treatment with 100 nM insulin for 15 min. (B, C, E, and F) Representative Fluo-3 AM images (B) and quantification (C) of intracellular Ca\(^{2+}\) in HepG2 cells treated with PMA. Data represent means ± SEM (n = 5, *P < 0.05).

**Fig. 2.** The catalytic activity of Akt is modulated by intracellular Ca\(^{2+}\) concentration. (A and D) Immunoblot analysis of the phosphorylation states of Akt, FOXO3A, and AS160, and the total amounts of the indicated proteins in HepG2 cells. Cells were incubated for 30 min with the indicated concentrations of PMA (A) or ionomycin (D), followed by treatment with 100 nM insulin for 15 min. (B, C, E, and F) Representative Fluo-3 AM images (B) and quantification (C) of intracellular Ca\(^{2+}\) in HepG2 cells treated with PMA. Data represent means ± SEM (n = 5, *P < 0.05).

**Fig. 3.** Higher intracellular Ca\(^{2+}\) concentrations prevent membrane localization of PH domains. (A) Fluorescence images of Akt-PH mCherry. CHO-IR cells were transfected with Akt PH domain-mCherry fusion vector, serum starved for 3 h, and treated with or without ionomycin (10 μM)/PMA (100 nM) for 30 min before a 15-min stimulation with 100 nM insulin. (B) Fluorescence images of PLCδ-PH GFP. CHO-IR cells were transfected with PLCδ-PH GFP fusion vector, followed by incubation with 10 μM ionomycin or 100 nM PMA for 15 min. After 100 nM PMA for 15 min treatment, the cells were incubated with 1 mM EDTA for 5 min to chelate Ca\(^{2+}\). (C) Representative fluorescence images of adenoviral Akt-PH mCherry from mice fed normal chow or a HFD for 10 wk following 7 d of adenoviral infection. Ex-vivo hepatocytes expressing adenoviral Akt-PH mCherry were visualized using confocal microscopy from formalin-fixed liver sections of mice following overnight fasting and subsequent refeeding with normal chow or a HFD for 4 h. (Scale bars: 5 μm.)

**High Intracellular Ca\(^{2+}\) Concentration Prevents Membrane Localization of PH Domains.**

Given that intracellular Ca\(^{2+}\) concentration in cells transiently increases up to 10^{-4} M (24) and high intracellular Ca\(^{2+}\) concentration inhibits the phosphorylation of Akt and its downstream signaling proteins, we asked whether intracellular Ca\(^{2+}\) leaks can modulate the subcellular localization of PH domains required for kinase activity. For the experiment, we selected two different PH domains, Akt-PH and PLCδ-PH. To examine the effects of intracellular Ca\(^{2+}\) on the membrane localization of these two PH domains, we transiently expressed Akt-PH domain mCherry (Akt-PH mCherry) or PLCδ-PH domain GFP (PLCδ-PH GFP) fusion proteins in CHO cells that stably express the IR (CHO-IR cells) (25).

The Akt-PH domain recognizes the highly specific PI3K products of PI(3,4)P\(_2\) and PI(3,4,5)P\(_3\), which are generated transiently upon activation of almost all surface receptors such as insulin and growth factors (1). We treated CHO-IR cells with or without insulin (100 nM). The localization of Akt-PH mCherry was primarily cytoplasmic in unstimulated cells (Fig. 3A). After stimulation with insulin, Akt-PH mCherry was preferentially localized to the plasma membrane (Fig. 3A). In contrast, pretreatment with PMA/ionomycin inhibited insulin-stimulated membrane recruitment of Akt-PH mCherry (Fig. 3A). Similarly, pretreatment with ionomycin led to the inhibition of insulin-stimulated membrane localization of endogenous Akt in CHO-IR cells (SI Appendix, Fig. S8A), suggesting that intracellular Ca\(^{2+}\) overload prevents membrane translocation of Akt, potentially by inhibiting PH domain interactions with PI(3,4)P\(_2\) or PI(3,4,5)P\(_3\).

Because the PLCδ-PH domain recognizes PI(4,5)P\(_2\), which is present at 10- to 20-fold higher levels than those of PI3K-dependent
products PI(3,4)P₂ and PI(3,4,5)P₃ (17), we monitored the subcellular localization of PLCδ-PH GFP in CHO-IR cells. As shown in Fig. 3C, PLCδ-PH GFP was localized to the plasma membrane when transiently expressed in CHO-IR cells. However, PLCδ-PH GFP was rapidly moved from the plasma membrane to the cytosol after stimulation with insulin or PMA (Fig. 3C), which was consistent with a previous study (26). Interestingly, the inhibitory effects of PMA on membrane localization of PLCδ-PH GFP was completely reversed by subsequent chelation of intracellular Ca²⁺ by EDTA (Fig. 3C), implying that higher intracellular Ca²⁺ is a negative regulator for membrane targeting of PH domains. Next, we also monitored the subcellular localization of endogenous IRS1 protein containing a PH domain with broad substrate specificity in CHO-IR cells pretreated with insulin stimulation. Again, pretreatment with PMA/ionomycin completely blocked insulin-stimulated membrane localization of endogenous IRS1 protein (SI Appendix, Fig. S8B), indicating that intracellular Ca²⁺ overload prevents membrane translocation of various PH domains by a common mechanism, potentially inhibiting interactions with PIPs.

Finally, to investigate whether physiological elevation of intracellular Ca²⁺ in mice fed a HFD inhibits membrane localization of the Akt-PH domain, we examined the subcellular localization of Akt-PH domains in mice fed normal chow or a HFD using adenosine-mediated overexpression of Akt-PH mCherry. Adenoviral Akt-PH mCherry was mostly localized to the plasma membrane in the hepatocytes of normal chow-fed mice in response to refeeding (insulin stimulation). Concurrent with the increased intracellular Ca²⁺ levels in mice fed a HFD (Fig. 1 B and C), however, adenoviral Akt-PH mCherry did not translocate to the plasma membrane in the hepatocytes of HFD-fed mice (Fig. 3C). This provides direct evidence for the inhibition of PH domain localization to the plasma membrane via physiological elevation of intracellular Ca²⁺ in HFD-fed mice. Taken together, these results demonstrate that sustained intracellular Ca²⁺ overload in mice fed a HFD prevents membrane localization of Akt in vivo by inhibiting membrane localization of the PH domain.

Ca²⁺ Inhibits the Binding of PH Domains to PIPs with Two Adjacent Phosphate Groups. The PH domains of Akt, BTK, and GRP1 recognize highly specific PI3K products PI(3,4)P₂ and PI(3,4,5)P₃, which are generated transiently upon stimulation of almost all cell surface receptors (17). Because the activation and phosphorylation of Akt are regulated by direct interactions of PI(3,4)P₂ or PI(3,4,5)P₃ with PH domains (16), we reasoned that PH domains play an important role in Ca²⁺-mediated inhibition of Akt phosphorylation. To address this question, we expressed and purified the PH domain of Akt and examined its binding properties toward various PIPs (Fig. 4A). Protein-lipid overlay experiments showed selective binding of Akt PH domain to PI(3,4)P₂ and PI(3,4,5)P₃ in the absence of Ca²⁺ (Fig. 4B). However, increasing the Ca²⁺ concentration inhibited the binding of Akt PH domain to PI(3,4)P₂ and PI(3,4,5)P₃, suggesting that high concentrations of intracellular Ca²⁺ inhibit electrostatic interactions between PH domains and PIPs.

This result also raises the possibility that high intracellular Ca²⁺ concentrations may inhibit the binding of other PH domains to various PIPs. We purified PH domains from phospholipase C-δ1 (PLC-δ1), which binds most tightly to PI(4,5)P₂ (27), and other PH domains from adapter proteins for several members of the tyrosine kinase receptor family, such as IRS1 (25). Consistent with previous findings (27), protein-lipid overlay experiments showed that the PLC-δ1 PH domain (PLCδ-PH) bound tightly to PI(4,5)P₂ only in the absence of Ca²⁺ (Fig. 4C). However, increasing the Ca²⁺ concentration completely abolished the binding of PLCδ-PH to PI(4,5)P₂. Interestingly, the IRS1-PH domain bound to all of the PIPs, including PI(3,4)P₂, PI(3,5)P₂, PI(4,5)P₂, PI(3,4,5)P₃, and PI(3,5)P₃, in the absence of Ca²⁺, suggesting that IRS1 has a broad binding specificity for various PIPs (28). Consistently, higher Ca²⁺ concentrations abolished the binding affinity of the IRS1 PH domain to PIPs, including PI(3,4)P₂, PI(4,5)P₂, and PI(3,4,5)P₃ (Fig. 4D), suggesting that Ca²⁺ inhibits the binding of PH domains to PIPs with two adjacent phosphate groups. Thus, these results demonstrate that higher intracellular Ca²⁺ prevents the binding of PIPs to the PH domains of Akt, PLCδ-1, and IRS1.

Elevated Ca²⁺ Causes the Formation of Ca²⁺-PIP₃s, Which Abolish Electrostatic Interactions Between PH Domains and PIPs. The crystal structure of Akt PH domain bound to inositol-1,3,4,5-tetraphosphate (Ins(1,3,4,5)P₄), a head group of PI(3,4,5)P₃, provides mechanistic clues to the Ca²⁺-mediated inhibition of PH domain binding to PIPs (29). The PH domain of Akt anchors the phosphates at the 3, 4, and 5 positions of PI(3,4,5)P₃ through electrostatic interactions with positively charged side chains of K14, K23, R25, and R86 (Fig. 4E),
signifying that Ca\(^{2+}\) may inhibit the electrostatic interactions by binding to either the PH domain of Akt or PI(3,4,5)P\(_3\).

To distinguish between these two possibilities, we used isothermal titration calorimetry (ITC), the gold standard for measuring binding affinity, to analyze whether Ca\(^{2+}\) binds to either the PH domain of Akt or PIPs. We examined the thermodynamics of Ca\(^{2+}\) binding to the PH domain of Akt at 25 °C. ITC analysis showed that Ca\(^{2+}\) does not bind to the PH domain of Akt (Fig. 4F), suggesting that Ca\(^{2+}\) may directly interact with PIPs, including PI(3,4)P\(_2\) and PI(3,4,5)P\(_3\). For the ITC analysis of Ca\(^{2+}\) binding to PIPs, we made liposomes composed of di-palmitoyl-sn-glycero-3-phosphocholine (POPC)/PI(3,4)P\(_2\) or PI(3,4,5)P\(_3\) (molar ratio of 80:20) (30). ITC analysis showed that PI(3,4)P\(_2\) bound two molecules of Ca\(^{2+}\) with strong affinity (K\(_d\) = 4.6 ± 0.7 μM, K\(_d2\) = 6.5 ± 0.4 μM) (Fig. 4G). Ca\(^{2+}\) also bound PI(4,5)P\(_2\) liposomes with a very high affinity (K\(_d\) = 6.7 ± 0.12 μM) (Fig. 4H). Interestingly, PI(3,4,5)P\(_3\) tightly bound two molecules of Ca\(^{2+}\), one with high affinity (K\(_d\) = 15.1 ± 1.5 nM) and the second with low affinity (K\(_d\) = 4.6 ± 0.14 μM) (Fig. 4I). These results indicate that Ca\(^{2+}\) has a high affinity for PIPs with two adjacent phosphate groups and forms Ca\(^{2+}\)-PIP complexes, which are highly compatible with physiological concentrations of elevated intracellular Ca\(^{2+}\) (24). Importantly, these results are consistent with a well-known property of inositol phosphate, which mediates the formation of a bidentate (P-Ca\(^{2+}\)2-3) between Ca\(^{2+}\) and the two acidic phosphate groups of inositol phosphates (31, 32). Consistent with this observation, previous computational modeling studies (33) have suggested that Ca\(^{2+}\) can form Ca\(^{2+}\)-induced PI(4,5)P\(_2\) clusters through electrostatic interactions. Furthermore, Bilkova et al. (34) showed that Ca\(^{2+}\) directly interacts with the head group phosphates of PI(4,5)P\(_2\), which further blocks the interactions of the PLC-6-PH domain to PI(4,5)P\(_2\). Taken together, we demonstrated that intracellular Ca\(^{2+}\) overload causes the formation of Ca\(^{2+}\)-PIP complexes, which prevent the recognition of PIPs by PH domains, likely due to electrostatic repulsion between positively charged side chains of PH domains and Ca\(^{2+}\)-PIPs (Fig. 4J).

Discussion

Dysregulation of intracellular Ca\(^{2+}\) homeostasis is one of the primary causes of insulin resistance in obesity and type 2 diabetes (5, 9, 10), although the molecular mechanisms that underlie these associations are not completely elucidated. Here, we provide evidence that an increased intracellular Ca\(^{2+}\) concentration prevents insulin-stimulated membrane translocation of Akt or IRS1 to their specific membrane PIPs. Finally, the crystal structure of Akt PH domain with Ins(1,3,4,5)P\(_4\) (29) and ITC studies verified that Ca\(^{2+}\)-mediated inhibition of targeting PH domains to the membrane resulted from the tight binding of Ca\(^{2+}\) rather than PH domains to PIPs, so that Ca\(^{2+}\)-PIPs eventually abrogated the binding of PH domains to the membrane due to electrostatic repulsion.

Ca\(^{2+}\) is one of the most versatile and universal signaling components, and it exerts allosteric regulatory effects on many enzymes and proteins (36). Intracellular Ca\(^{2+}\) signaling is initiated by a hormone or other agonist binding to a G protein-coupled receptor (GPCR) and subsequent signaling cascades, including the activation of inositol trisphosphate receptor (IP3R) (37). At physiological levels, glucagon and catecholamine transiently raises intracellular Ca\(^{2+}\) levels through the activation of IP3R, whereby the elevated Ca\(^{2+}\) antagonizes insulin signaling by complexing with Ca\(^{2+}\)-phosphoinositides and inhibiting the membrane recruitment of proteins containing PH domains to phosphoinositides. However, at the pathological conditions such as obesity or type 2 diabetes (38), activation of GPCRs may lead to sustained elevation of cytosolic Ca\(^{2+}\) levels in hepatocytes through IP3R (37). Thus, dysregulation of intracellular Ca\(^{2+}\) homeostasis in obesity may disrupt insulin action and mediate insulin resistance by inhibiting membrane localization and activation of proteins with PH domains through sustained formation of Ca\(^{2+}\)-phosphoinositides (Fig. 5). Alternatively, sustained high intracellular Ca\(^{2+}\) in obesity may also activate several Ca\(^{2+}\)-responsive proteins, such as c-Jun N-terminal kinases (21), NFAT transcription factors (39), and PKCs (40) that contribute to the development of insulin resistance.

Conversely, Akt is frequently hyperactivated in human cancer (41). Mutations that lead to either constitutive membrane localization of PH domains or disruption of the inhibitory interactions between PH domain and kinase domain promote oncogenesis in vivo (42), suggesting that the Akt PH domain acts as an inhibitor of kinase activation. Although many gaps remain in our understanding of the inhibitory functions of the PH domain
in Akt, our findings suggest that Ca\textsuperscript{2+}-PIPs in elevated intracellular Ca\textsuperscript{2+} conditions may act as negative regulators that eventually block the dissociation of the inhibitory interactions between the PH domain and the kinase domain in Akt. Interestingly, pretreatment of PMA or ionomycin inhibited both EGF-stimulated membrane localization of endogenous Akt and EGF-stimulated phosphorylation of Akt and its downstream target molecules in HaCaT cells (SI Appendix, Fig. S9). This suggested that intracellular Ca\textsuperscript{2+} overload prevents membrane localization of PH domains by other growth factors. These results may explain why increased intracellular Ca\textsuperscript{2+} levels induce apoptosis in multiple cell types, including thymocytes (43), neurons (44), and various cancer cells (45). Therefore, drugs that inhibit membrane localization of PH domain in Akt may be effective against many human cancers. Further elucidation of the role of Ca\textsuperscript{2+}-PIPs in cell biology and physiology may require additional studies to provide new potential targets for pharmacological interventions for major human diseases, including cancer and diabetes. In conclusion, dysregulation of intracellular Ca\textsuperscript{2+} homeostasis may contribute to the pathogenesis of insulin resistance, obesity, and type 2 diabetes by preventing the localization of PH domains to the plasma membrane by coupling Ca\textsuperscript{2+}-PIPs.

**Materials and Methods**

C57BL/6 male mice from Orient Bio, Inc. were studied under protocols approved by the animal ethics committee of Gachon University, Lee Gil Yar Cancer and Diabetes Institute (LCDI-2014-0088). For full details of all these processes, see SI Appendix.

**ACKNOWLEDGMENTS.** We thank Drs. Steven E. Shoelson (Harvard Medical School) and Jae Young Park (Korea University) for helpful discussions. This work was supported by grants from the Korea Health Technology R&D Project, Korea Ministry of Health & Welfare (HI14C1135, A111345), and the Basic Science Research Program (2017R 1D 1A 1803031094) of the National Research Foundation.

1. Taniguchi CM, Emanuelli B, Kahn CR (2006) Critical nodes in signalling pathways: Insights into insulin action. Nat Rev Mol Cell Biol 7:85–96.
2. Kahn SE, Hull RL, Utzschneider KM (2006) Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 444:840–847.
3. Samuel VT, Shulman GI (2012) Mechanisms for insulin resistance: Common threads and missing links. Cell 148:852–871.
4. Park HW, Lee JH (2014) Calcium channel blockers as potential therapeutics for obesity-associated autophagy defects and fatty liver pathologies. Autophagy 10:2385–2396.
5. Arruda AP, et al. (2014) Chronic enrichment of hepatic endoplasmic reticulum-mitochondria contact leads to mitochondrial dysfunction in obesity. Nat Med 20:1427–1435.
6. Byyny RL, LoVerde M, Lloyd S, Mitchell W, Draznin B (1992) Cytosolic calcium and insulin resistance in elderly patients with essential hypertension. Am J Hypertens 5:459–464.
7. Gwiazda KS, Yang TL, Lin Y, Johnson JD (2009) Effects of palmitoleate on ER and cytosolic Ca\textsuperscript{2+} homeostasis in beta-cells. Am J Physiol Endocrinol Metab 296:E699–E701.
8. Standley PR, Ali S, Bapna C, Sowers JR (1993) Increased platelet cytosolic calcium responses to low density lipoprotein in type II diabetes with and without hypertension. Am J Hypertens 6:938–943.
9. Park HW, et al. (2014) Pharmacological correction of obesity-induced autophagy arrest using calcium channel blockers. Nat Commun 5:4834.
10. Fu S, et al. (2011) Aberrant lipid metabolism disrupts calcium homeostasis causing liver endoplasmic reticulum stress in obesity. Nature 473:528–531.
11. Gustavo Vazquez-Jimenez B, et al. (2016) Palmitic acid but not palmitoleic acid lowers serum cortisol, in insulin-resistant obese and hypertensive men. J Clin Endocrinol Metab 101:1437–1444.
12. Xiu G, Chen J, Jing G, Shaker A (2012) Preventing –p cell loss and diabetes with calcium channel blockers. Diabetes 61:848–866.
13. Kim JH, et al. (1996) The effects of calcium channel blockade on agouti-induced obesity. FASEB J 10:1646–1652.
14. Saltiel AR, Kahn CR (2001) Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414:799–806.
15. Franke TF, Kaplan DR, Cantley LC, Toker A (1997) Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4,5-trisphosphate. Science 275:665–668.
16. Lemmon MA (2008) Membrane recognition by phospholipid-binding domains. Nat Rev Mol Cell Biol 9:99–111.
17. Rawlings DJ, et al. (1993) Mutation of unique region of Bruton’s tyrosine kinase in immunodeficient XID mice. Science 261:358–361.
18. Lindvall JH, et al. (2005) Bruton’s tyrosine kinase: Cell biology, sequence conservation, mutation spectrum, siRNA modifications, and expression profiling. Immuno Rev 203:200–215.
19. Carpent JD, et al. (2007) A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature 448:439–444.
20. Ozcan L, et al. (2013) Activation of calcium/aldolmin-dependent protein kinase II in obesity mediates suppression of hepatic insulin signaling. Cell Metab 18:803–815.
21. Chen TW, et al. (2013) Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature 499:295–300.
22. Berotti SC, et al. (2009) Palmitic acid mediates hypothalamic insulin resistance by altering PKC-β subcellular localization in rodents. J Clin Invest 119:2577–2589.
23. Xu N, Francis M, Cioffi DL, Stevens T (2014) Studies on the resolution of subcellular free calcium concentrations: A technological advance. Focus on “detection of differentially regulated subcellular calcium signals activated by vasoactive agonists in rat pulmonary artery smooth muscle cells.” Am J Physiol Cell Physiol 306:C636–C638.
24. Werner ED, Lee J, Hansen L, Yuan M, Shoelson SE (2004) Insulin resistance due to phosphorylation of insulin receptor substrate-1 at serine 302. J Biol Chem 279:35328–35335.
25. Vârnai P, Balla T (1998) Visualization of phosphoinositides that bind pleckstrin homology domains: Calcium- and agonist-induced dynamic changes and relationship to myo-[3H]inositol-labeled phosphoinositide pools. J Cell Biol 143:501–510.
26. Karran JM, et al. (1998) Specificity and promiscuity in phosphoinositide binding by pleckstrin homology domains. J Biol Chem 273:30497–30508.
27. Dhe-Paganon S, Ottinger EA, Nolte RT, Eck MJ, Shoelson SE (1999) Crystal structure of the pleckstrin homology-phosphorytrosine binding (PH-PTB) targeting region of insulin receptor substrate 1. Proc Natl Acad Sci USA 96:8378–8383.
28. Milburn CC, et al. (2003) Binding of phosphatidylinositol 3,4,5-trisphosphate to the pleckstrin homology domain of protein kinase B induces a conformational change. Biochim Biophys Acta 1652:856–866.
29. Berridge MJ, Bootman MD, Lipp P (1998) Calcium: A life and death signal. J Physiol 506(Pt 3):331–361.