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A Critical Role for β-Catenin in Modulating Levels of Insulin Secretion from β-Cells by Regulating Actin Cytoskeleton and Insulin Vesicle Localization*

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Brie Sorrenson[‡§], Emmanuelle Cognard[‡], Kathryn L. Lee[‡], Waruni C. Dissanayake[‡], Yanyun Fu[¶], Weiping Han[¶], William E. Hughes[‖], and Peter R. Shepherd[‡§1]**

*From the [‡]Department of Molecular Medicine and Pathology, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand, the [§]Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand, the [¶]Singapore Bioimaging Consortium, Agency for Science, Technology and Research (A*STAR), Singapore 138667, the [‖]Department of Medicine, St. Vincent's Hospital, Victoria Street, Sydney, New South Wales 2010, Australia, and the **Garvan Institute of Medical Research, 384 Victoria Street, Sydney, New South Wales 2010, Australia*

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The processes regulating glucose-stimulated insulin secretion (GSIS) and its modulation by incretins in pancreatic β-cells are only partly understood. Here we investigate the involvement of β-catenin in these processes. Reducing β-catenin levels using siRNA knockdown attenuated GSIS in a range of β-cell models and blocked the ability of GLP-1 agonists and the depolarizing agent KCl to potentiate this. This could be mimicked in both β-cell models and isolated islets by short-term exposure to the β-catenin inhibitory drug pyrvinium. In addition, short-term treatment with a drug that increases β-catenin levels results in an increase in insulin secretion. The timing of these effects suggests that β-catenin is required for the processes regulating trafficking and/or release of pre-existing insulin granules rather than for those regulated by gene expression. This was supported by the finding that the overexpression of the transcriptional co-activator of β-catenin, transcription factor 7-like 2 (TCF7L2), attenuated insulin secretion, consistent with the extra TCF7L2 translocating β-catenin from the plasma membrane pool to the nucleus. We show that β-catenin depletion disrupts the intracellular actin cytoskeleton, and by using total internal reflectance fluorescence (TIRF) microscopy, we found that β-catenin is required for the glucose- and incretin-induced depletion of insulin vesicles from near the plasma membrane. In conclusion, we find that β-catenin levels modulate Ca^{2+}-dependent insulin exocytosis under conditions of glucose, GLP-1, or KCl stimulation through a role in modulating insulin secretory vesicle localization and/or fusion via actin remodeling. These findings also provide insights as to how the overexpression of TCF7L2 may attenuate insulin secretion.

Significant advances have been made in recent years in understanding the processes controlling biphasic insulin release from the β-cells of the pancreas (reviewed in Refs. 1–3). The overall amount of insulin secreted can be increased by incretin hormones, such as glucagon-like peptide-1 (GLP-1)[2] (4), but this still requires glucose to actually trigger the fusion of insulin-containing vesicles with the plasma membrane. Thus incretins increase insulin secretion by increasing the number of insulin vesicles capable of fusing with the membrane. In a normally functioning β-cell, there is a significant pool of insulin-containing vesicles that can be quickly mobilized for release in this way, and this is thought to allow for the rapid surge in insulin release known as first phase insulin secretion (reviewed in Ref. 2).

The final steps in the movement of insulin secretory vesicles to be primed for secretion requires remodeling of actin that involves small GTPases (5). This process can be modulated by incretins, but the release of insulin vesicles is triggered by the metabolism of glucose, which increases the ADP/ATP ratio within the cell, resulting in the closure of ATP-sensitive potassium channels and membrane depolarization. Subsequent influx of Ca^{2+} into the cells occurs due to the opening of voltage-dependent Ca^{2+} channels and triggers insulin vesicle exocytosis. The fusion mechanism of insulin vesicles involves classical v-SNARE (soluble NSF attachment protein receptor) interactions with t-SNARE on the plasma membrane, and subsequent fusion being triggered by Ca^{2+} (6, 7). The PDZ domain-containing protein Synip (also known as STXBP4) regulates the interactions of these vesicles with t-SNARE proteins such as syntaxin-4 and so regulates fusion events (8).

Evidence for new potential mechanisms regulating β-cell function has come from genetic studies. For example, the recent discovery that single nucleotide polymorphisms (SNPs) in the genomic region around the transcription factor 7-like 2 (*TCF7L2*), particularly those linked to the overexpression of

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[1] To whom correspondence should be addressed. Tel.: 64-9-923-9891; E-mail: peter.shepherd@auckland.ac.nz.

[2] The abbreviations used are: GLP-1, glucagon-like peptide-1; GSIS, glucose-stimulated insulin secretion; KRBH, Krebs-Ringer bicarbonate HEPES; SNARE, soluble NSF attachment protein receptor; NSF, *N*-ethylmaleimide-sensitive factor; t-SNARE, target SNARE; v-SNARE, vesicle SNARE; TIRF, total internal reflectance fluorescence; BIO, 6-bromoindirubin-3′-oxime; ANOVA, analysis of variance.

TCF7L2, are associated with a significant increased risk of β-cell dysfunction and type-2 diabetes, and these are associated with defects in insulin secretion (9–12). Although such variants have been determined to adversely affect β-cell mass and function (13), there appears to be a beneficial effect on insulin-responsive tissues, particularly with respect to hepatic glucose metabolism (discussed in Ref. 14). One of the most established functions of TCF7L2 is as a transcription factor that, when bound to β-catenin, regulates the expression of a wide range of genes (15). This has generated interest in the potential role of β-catenin in β-cell function. Previous studies had focused on the potential role of β-catenin in β-cell development. For example, gene knock-out studies have shown that Cre-mediated deletions of β-catenin, driven by either the rat insulin promoter or the PDX-1 promoter, result in defects in insulin secretion and mild glucose intolerance (16, 17). However, we recently provided evidence that suggests β-catenin is necessary for glucose-stimulated insulin secretion (GSIS) in the INS-1E β-cell model (18). This indicates that β-catenin could be playing a role in regulating insulin secretion in adult β-cells. The effect of β-catenin on insulin secretion in β-cells could involve effects mediated via β-catenin/TCF7L2 effects on gene expression (13, 19). However, other potential explanations for β-catenin's role in regulating insulin secretion in adult β-cells are possible. It is likely that the β-catenin/TCF7L2-mediated gene expression plays some role in these effects, but a second mechanism must also be considered. The second major role of β-catenin is at the plasma membrane as a component of the cadherin complexes that control adherens junctions regulating cell-cell adhesion (20–22). Adherens junctions between β-cells are required for the correct regulation of insulin release (23–26), and cell junction-associated β-catenin has been found to co-localize with insulin granules in MIN6 pseudoislets (27). Interestingly, β-catenin also participates in a range of interactions with proteins involved in vesicle trafficking and cytoskeleton organization (8, 28, 29), which suggests that β-catenin may play an important role in insulin vesicle exocytosis. In this case, it would be possible to measure the rapid effects of β-catenin on insulin granule dynamics in addition to the potential effects mediated in the longer term by gene expression. To date, the role of β-catenin in mediating the effects of incretin hormones on GSIS has not been studied, and neither has a role for β-catenin in regulating insulin vesicle localization. In this study, we investigate whether β-catenin is necessary for the effect of GLP-1 agonists on GSIS and explore a role for β-catenin in regulating insulin vesicle distribution near the plasma membrane using total internal reflectance fluorescence (TIRF) microscopy. We report that β-catenin is required for glucose- and incretin-stimulated insulin secretion in a range of β-cell models. These data indicate that the effects are due to a role for β-catenin in vesicle trafficking, and this is supported by the finding that inhibition or reduction of β-catenin perturbs insulin vesicle distribution near the cell surface and disrupts the actin cytoskeleton. Further, the overexpression of the β-catenin transcriptional co-activator TCF7L2 protein attenuates insulin secretion, which supports a function for β-catenin in insulin secretion that is independent of gene transcription. Together this demonstrates a role for β-catenin in the regulation of insulin secretory vesicle localization and exocytosis.

Results

Potentiation of Insulin Secretion by Incretins Requires β-Catenin—In the current study, we investigate the role of β-catenin in GSIS and incretin-induced potentiation of GSIS using INS832/3 and MIN6 β-cells because these are models that are responsive to GLP-1 agonists. In INS832/3 and MIN6 cells, exenatide potentiated GSIS at 15 min (Fig. 1, *A* and *C*) and at 2 h (Fig. 1, *B* and *D*). Treatment of INS832/3 and MIN6 β-cells with 100 nM pyrvinium, a potent inhibitor of the β-catenin signaling pathway (30) that reduces β-catenin protein level (Fig. 1*F*), prevented glucose- and incretin-stimulated insulin secretion, demonstrating that β-catenin is required for GSIS and the potentiating effect of incretins at 15 min and at 2 h (Fig. 1, *A–D*). Pyrvinium also prevented the effect of 30 mM depolarizing agent KCl on insulin secretion (Fig. 1*E*). Importantly, the effects of pyrvinium on insulin secretion were observed within 15 min, which is prior to any effect on gene transcription (data not shown). This strongly suggests that the effects were not due to a reduction in β-catenin-mediated gene expression but were an acute effect of changes in functional β-catenin. In isolated mouse islets, pyrvinium treatment attenuated the effect of high glucose (Fig. 2*A*), incretin (Fig. 2*B*), and 30 mM KCl (Fig. 2*C*), showing that β-catenin is required for appropriate insulin secretion from pancreatic islets and is not just restricted to β-cell models.

To confirm the results obtained using pyrvinium, we also suppressed the expression of β-catenin using siRNA. In INS832/3 cells transfected with β-catenin-specific siRNA, the level of β-catenin protein was reduced to ~25% of that seen in control siRNA transfected cells (Fig. 3*A*). The reduction in β-catenin protein level was associated with an 87% reduction in both glucose-stimulated and exenatide-stimulated insulin secretion as compared with the control transfected cells (Fig. 3*B*). These results were confirmed using a second β-catenin siRNA, which reduced β-catenin protein level by 50% and was associated with glucose- and exenatide-stimulated insulin secretion that was reduced by 58 and 62%, respectively (data not shown). Control siRNA had no effects as compared with untransfected cells (data not shown). β-Catenin siRNA transfected cells also had an attenuated insulin secretion in response to the depolarizing agent KCl (Fig. 3*C*).

Conversely, when we treated cells with the glycogen synthase kinase 3 (GSK3) inhibitor BIO, which prevents proteasomal degradation of β-catenin by inhibiting the β-catenin destruction complex (Fig. 1*F*), glucose- and exenatide-stimulated insulin secretion was increased (Fig. 3, *D* and *E*). Similarly, in islets, BIO treatment potentiated not only glucose-stimulated insulin secretion but the effect of exenatide (Fig. 4*A*) and KCl (Fig. 4*B*). This effect was seen even with very short-term exposure to BIO (15 min, Fig. 3*D*), indicating that it was unlikely to be due to gene expression effects, and BIO treatment did not increase cyclin D2 protein levels, indicating that it did not cause increased β-catenin-dependent gene expression in the same time frame (data not shown). Further, 10 μM iCRT5, XAV-939, or KY-02111, which disrupt the interaction between TCF7L2

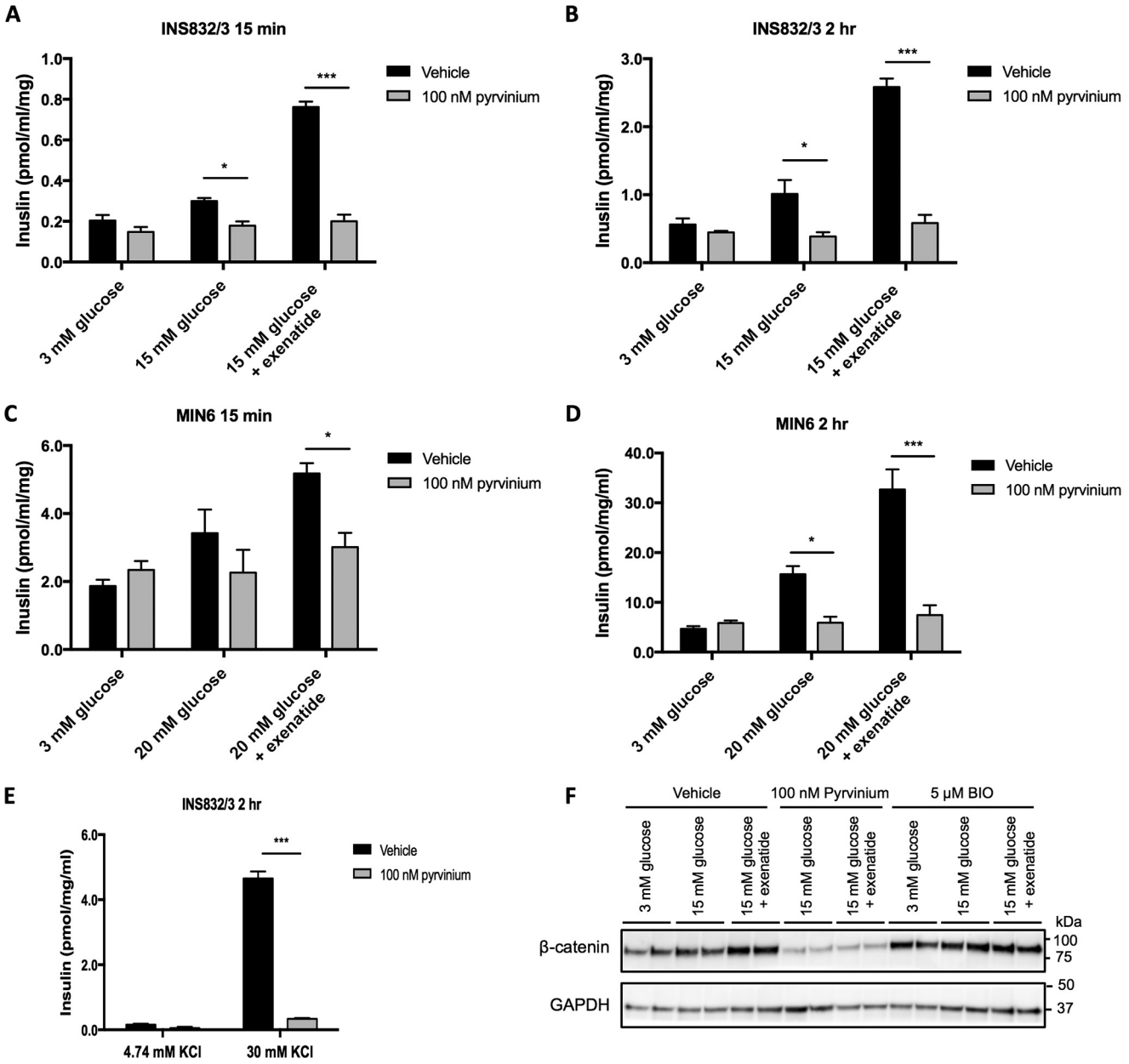


FIGURE 1. **Inhibiting β-catenin in INS832/3 and MIN6 β-cells prevents glucose- and exenatide-stimulated insulin secretion.** Cells were glucose-starved for 1 h and pre-treated for 30 min with pyrvinium (100 nM) or DMSO as a control. *A* and *B*, INS832/3 cells were treated with 3 mM glucose, 15 mM glucose, or 15 mM glucose + 10 nM exenatide. *C* and *D*, MIN6 cells were treated with 3 mM glucose, 20 mM glucose, or 20 mM glucose + 10 nM exenatide. *E*, cells were treated with 4.74 mM KCl or 30 mM KCl. Treatments contained pyrvinium (100 nM) where indicated, and cells were incubated for 15 min (*A* and *C*) or 2 h (*B*, *D*, and *E*). Medium was collected, and insulin was concentration determined using the AlphaLISA insulin kit. *, $p < 0.05$ and ***, $p < 0.001$ as compared with the untreated cells of the same glucose condition, as assessed by one-way ANOVA with Tukey's post hoc test. Similar results were obtained in at least three independent experiments. *F*, cell lysates of inhibitor-treated cells were used for Western blotting analysis to visualize the effect on β-catenin protein level relative to GAPDH loading control.

and β-catenin, and hence attenuate their combined effect on gene expression, did not reduce GSIS in our hands (data not shown).

To verify that the synthetic GLP-1 agonist exenatide was representative of physiological incretins, we compared exenatide with the GLP-1(7–36) amide peptide, which is the principle active form of GLP-1 *in vivo*. Treating INS832/3 cells with 10 nM GLP-1(7–36) amide potentiated GSIS to a similar level as 10 nM exenatide (Fig. 5*A*), and the effect of both was attenuated to a comparable degree by pyrvinium treatment. In INS832/3 cells transfected with siRNA specific to β-catenin, the effect of GLP-1(7–36) amide on GSIS was reduced in a manner that was comparable with that observed for exenatide (Fig. 5, *B* and *C*).

Inhibition of β-Catenin Disrupts Insulin Vesicle Density near the Plasma Membrane—Given that β-catenin is known to play a role in synaptic vesicle exocytosis in neurons (28), we hypothesized that β-catenin may play a similar role in localizing insulin vesicles near the plasma membrane or their fusion with the membrane in β-cells. To address this, we used TIRF microscopy to investigate whether β-catenin plays a role in regulating insulin vesicle density near the cell surface. Under basal conditions, fluorescently labeled insulin can be detected in β-cells by

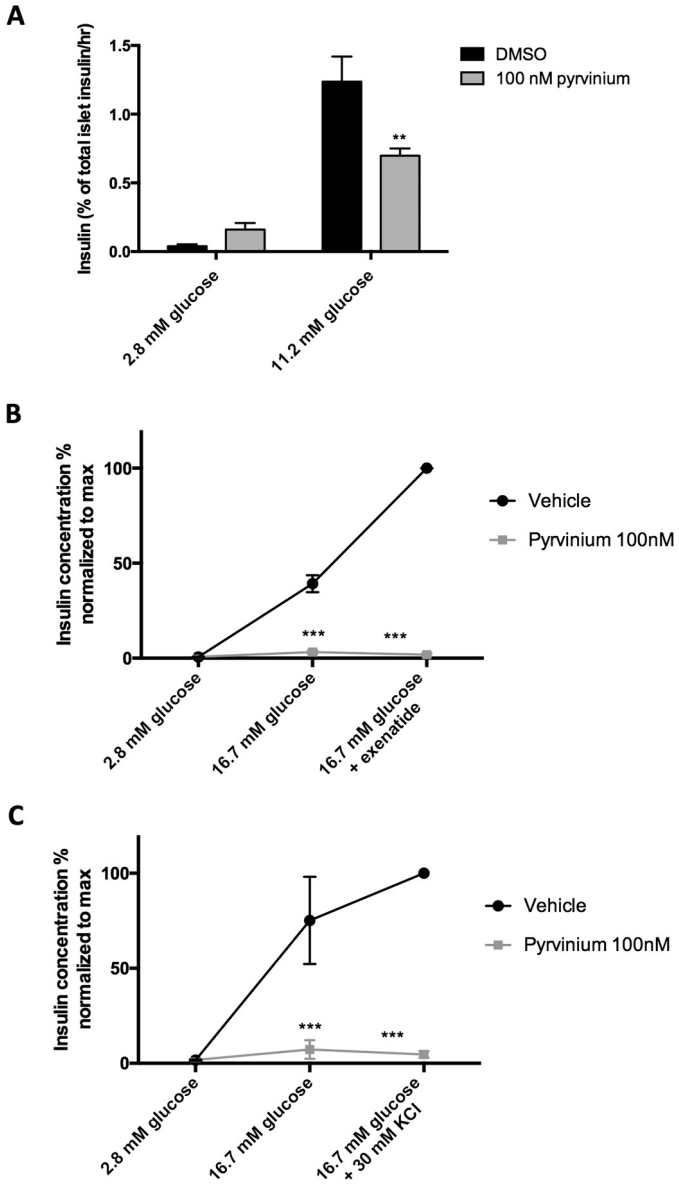


FIGURE 2. **Pyrvinium inhibits glucose-stimulated insulin secretion in isolated mouse islets.** *A, in vitro* static insulin secretion assay was performed using isolated islets (10 islets per group) receiving control (DMSO) or pyrvinium treatment with low (2.8 mM) or high (11.2 mM) glucose. Accumulated insulin in supernatant was normalized to islet insulin content. Data are presented as mean ± S.E. from at least three independent experiments. *B* and *C*, islet insulin secretion assay was performed by serial 1-h incubations at 37 °C using KRBH with 0.2% (w/v) BSA and either low (2.8 mM) or high (16.7 mM) glucose with or without 100 nM pyrvinium and secretagogues (exenatide (*B*) and KCl (*C*)). Medium was collected following each treatment, and insulin concentration was determined. Results are from three or four separate experiments with insulin concentration (mg/ml) normalized to the average maximum concentration of untreated islets at the highest stimulation within each experiment to account for batch-to-batch differences in total secretory capacity. **, $p < 0.01$, ***, $p < 0.001$ as compared with the untreated cells of the same glucose condition, as assessed by ANOVA with Tukey's post hoc test.

TIRF microscopy, indicating that insulin vesicles are present in close proximity to the plasma membrane. As expected, high glucose (15 and 20 mM for INS832/3 and MIN6 cells, respectively) and exenatide treatment depleted insulin vesicle density within the 100-nm region close to the plasma membrane in both INS832/3 (Fig. 6, *A* and *B*) and MIN6 cells (Fig. 6, *C* and *D*), which is consistent with these conditions inducing the exocytosis of insulin vesicles. Inhibiting β-catenin using pyrvinium prevented the glucose and exenatide depletion of insulin vesicle density at the cell periphery in both cell lines (Fig. 5). This was further confirmed by MIN6 cells transfected with β-catenin siRNA. In these cells, neither glucose nor exenatide treatment decreased insulin vesicle density from basal in the β-catenin siRNA transfected cells (Fig. 7). Together these findings provide strong evidence that β-catenin is required for appropriate fusion of insulin-containing granules with the membrane in response to glucose and incretins.

Overexpression of the β-Catenin Transcriptional Co-activator TCF7L2 Attenuates Insulin Secretion—To investigate the importance of β-catenin's transcriptional role in insulin secretion and to assess the potential impact of *TCF7L2* variants that are associated with increased *TCF7L2* expression (and type-2 diabetes), we transfected INS832/3 cells with a *TCF7L2* expression plasmid and assessed the effect on insulin secretion. As TCF7L2 is the transcriptional co-activator for β-catenin in the nucleus, we speculated that an abundance of TCF7L2 would sequester β-catenin from any potential cytoplasmic role in insulin secretion. In cells transfected with *TCF7L2*, plasmid TCF7L2 protein was overexpressed as compared with cells transfected with GFP control plasmid, and there was no alteration to the total level of β-catenin protein (Fig. 8*A*). In cells overexpressing TCF7L2, glucose- and exenatide-stimulated insulin secretion was reduced as compared with control transfected cells by 46 and 54%, respectively (Fig. 8*B*). The reduced insulin secretion in cells overexpressing TCF7L2 supports that the role for β-catenin in insulin secretion is not limited to its transcriptional effects and may help explain how certain *TCF7L2* variants, which are associated with overexpression, are linked to defective insulin secretion and type-2 diabetes.

Depletion of β-Catenin Alters the Intracellular Actin Cytoskeleton—Given that intracellular actin remodeling is required for insulin secretion, we investigated whether this a potential mechanism by which β-catenin modulates insulin secretion. As compared with control siRNA transfected cells, β-catenin siRNA transfected cells had a lower overall percentage of F-actin (36.1% *versus* 9.9%, respectively, Fig. 9*A*). As expected, latrunculin treatment was associated with a lower percentage of F-actin (16.7%), and phalloidin treatment was associated with a higher percentage of F-actin (81.7%). When imaged by confocal microscopy, cells transfected with β-catenin siRNA and treated with glucose displayed more intense actin staining at the cell periphery as compared with glucose-treated control siRNA transfected cells (Fig. 9*B*), indicating that loss of β-catenin disrupts intracellular actin remodeling, which is required for insulin secretion.

Discussion

Altering the expression of β-catenin results in developmental changes in β-cells, so it has been widely assumed that gene expression is the major way that β-catenin is involved in β-cell function (16, 17, 31). However, we show here that alterations in β-catenin levels in pancreatic β-cell lines and isolated pancreatic islets have very rapid effects on insulin secretion mediated by glucose, exenatide, and GLP-1(7–36) amide and KCl. Altering β-catenin levels using the drug pyrvinium caused a dra-

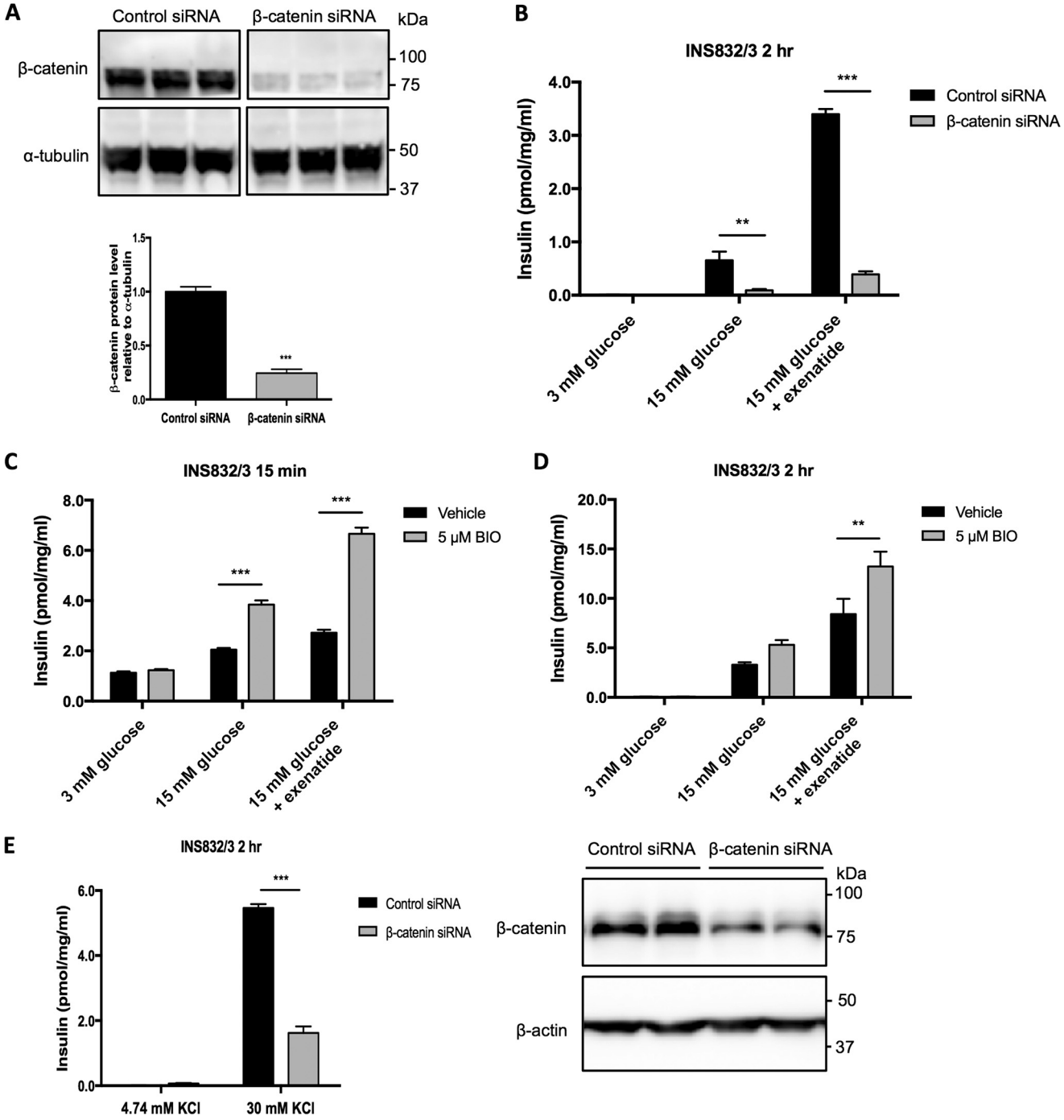


FIGURE 3. **siRNA knockdown of β-catenin attenuates glucose- and exenatide-stimulated insulin secretion, and inhibition of β-catenin degradation potentiates insulin secretion.** INS832/3 cells were transfected with siRNA specific to β-catenin or a scrambled control, and 48 h after transfection, cells were glucose-starved for 1 h and then incubated in 3 mM glucose, 15 mM glucose, or 15 mM glucose + 10 nM exenatide for 2 h. *A*, cell lysates were collected and used for Western blotting analysis to quantify total β-catenin protein level relative to α-tubulin as a loading control. *B*, medium was collected for determination of insulin concentration using the AlphaLISA kit. *C*, INS832/3 cells were transfected with siRNA and stimulated with 4.74 or 30 mM KCl for 2 h, and the concentration of insulin in the medium was determined. *D* and *E*, INS832/3 cells were glucose-starved for 1 h, and then pre-treated for 30 min with BIO (5 μM) or DMSO as a control. Glucose treatments contained BIO (5 μM) where indicated, and then cells were incubated for 15 min or 2 h, and insulin concentration was determined using the AlphaLISA insulin kit. **, $p < 0.01$ and ***, $p < 0.001$ as compared with scrambled control transfected cells of the same glucose condition, as assessed by ANOVA with Tukey's post hoc test. Similar results were obtained in at least three independent experiments.

matic reduction in insulin secretion. This is consistent with an effect mediated by reductions in β-catenin as we tested the specificity of this drug against >300 unrelated kinases (data not shown). Other possible explanations for pyrvinium's actions such as changes in cellular ATP/ADP levels or calcium transients cannot be ruled out, but because we observed similar results using siRNA to reduce β-catenin levels, we conclude that changes in β-catenin are providing a mechanism for the β-cells to regulate the levels of Ca^{2+}-dependent insulin secretion. Our results suggest that β-catenin is required for regulation of insulin vesicle dynamics near the cell membrane independently of its effects normally mediated by gene expression

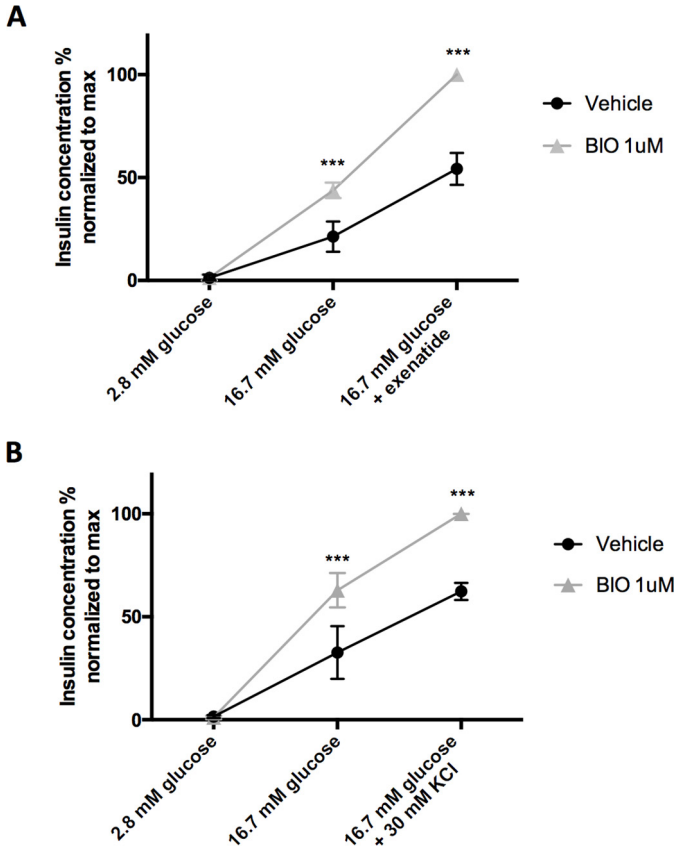


FIGURE 4. **Islet insulin secretion assay.** *A* and *B*, assays were performed by serial 1-h incubations at 37 °C using KRBH with 0.2% (w/v) BSA and either low (2.8 mM) or high (16.7 mM) glucose with or without 1 μM BIO and secretagogues (exenatide (*A*) and KCl (*B*)). Medium was collected following each treatment, and insulin concentration was determined. Results are from three or four separate experiments with insulin concentration (mg/ml) normalized to the average maximum concentration of untreated islets at the highest stimulation within each experiment to account for batch-to-batch differences in total secretory capacity. ***, $p < 0.001$ as compared with the untreated cells of the same glucose condition, as assessed by ANOVA with Tukey's post hoc test.

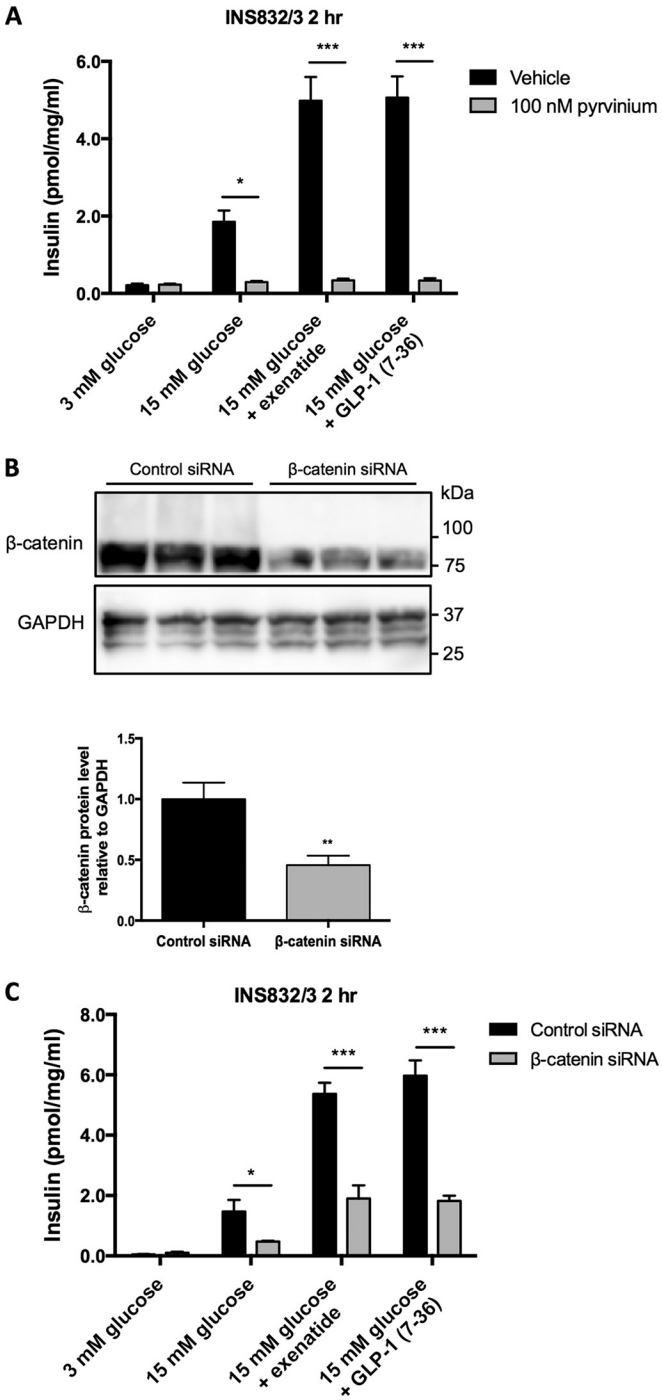
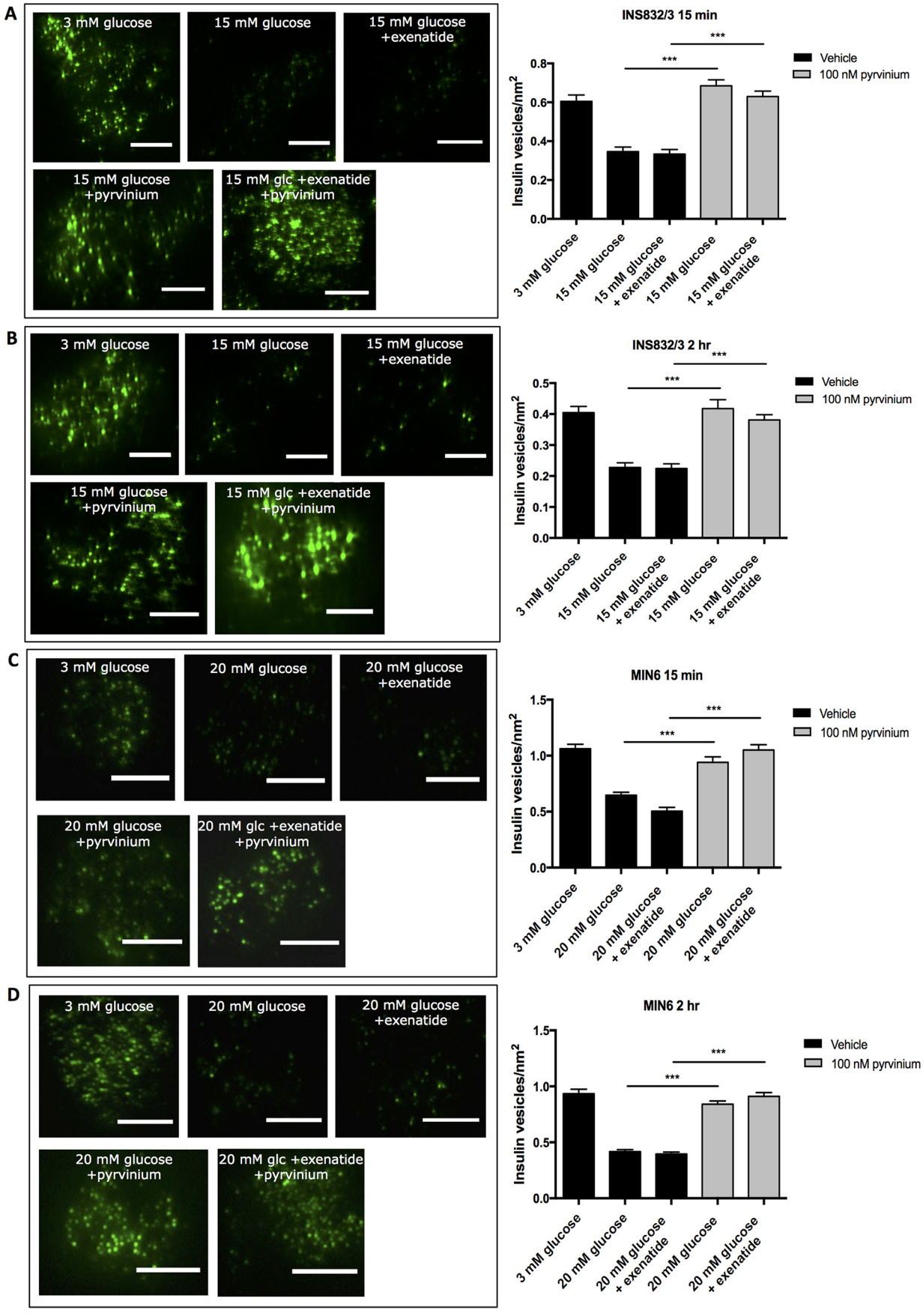


FIGURE 5. **Inhibiting β-catenin prevents the effect of the incretin GLP-1(7–36) amide on insulin secretion.** *A*, INS832/3 cells were glucose-starved for 1 h and pre-treated for 30 min with pyrvinium (100 nM) or DMSO as a control and then treated with 3 mM glucose, 15 mM glucose, 15 mM glucose + 10 nM exenatide, or 15 mM glucose + 10 nM GLP-1(7–36) amide. Glucose treatments contained pyrvinium (100 nM) where indicated, and cells were incubated for 2 h. Medium was collected for determination of insulin concentration using the AlphaLISA insulin kit. *B* and *C*, INS832/3 cells were transfected with siRNA specific to β-catenin or a scrambled control, and 48 h after transfection, cells were glucose-starved for 1 h and then incubated in 3 mM glucose, 15 mM glucose, or 15 mM glucose + 10 nM exenatide for 2 h. *B*, cell lysates were collected and used for Western blotting analysis to quantify total β-catenin protein level relative to α-tubulin as a loading control. *C*, medium was collected, and insulin concentration was determined using the AlphaLISA insulin kit. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ as compared with the untreated cells of the same glucose condition, as assessed by ANOVA with Tukey's post hoc test. Similar results were obtained in at least three independent experiments.

via an effect on the actin cytoskeleton. This would be consistent with an often overlooked finding that there is no evidence for active signaling via β-catenin to gene expression in adult mouse islets although β-catenin is present in the islets (32).

Our data show an involvement of β-catenin in the later stages of insulin release. Many of these mechanisms need to be localized at the plasma membrane, so a role for β-catenin in this localization is appealing as a substantial number of these proteins are localized at the plasma membrane as part of the adherens junctions (20–22), and it is known these proteins are required for proper regulation of insulin secretion (23). This possibility is supported by the observation that insulin secretory vesicles have many similarities to neuronal synaptic vesicles and β-catenin is known to have an important role in localizing the reserve pool of synaptic vesicles (28). β-Catenin modulates the localization of synaptic vesicles at the plasma membrane as part of a complex with N-cadherin, scribble, and β-pix that modulates actin-mediated recruitment of synaptic vesicles (28, 33, 34).

β-Catenin's N-terminal region interacts with α-catenin, which can regulate F-actin (35). The role of β-catenin in regu-



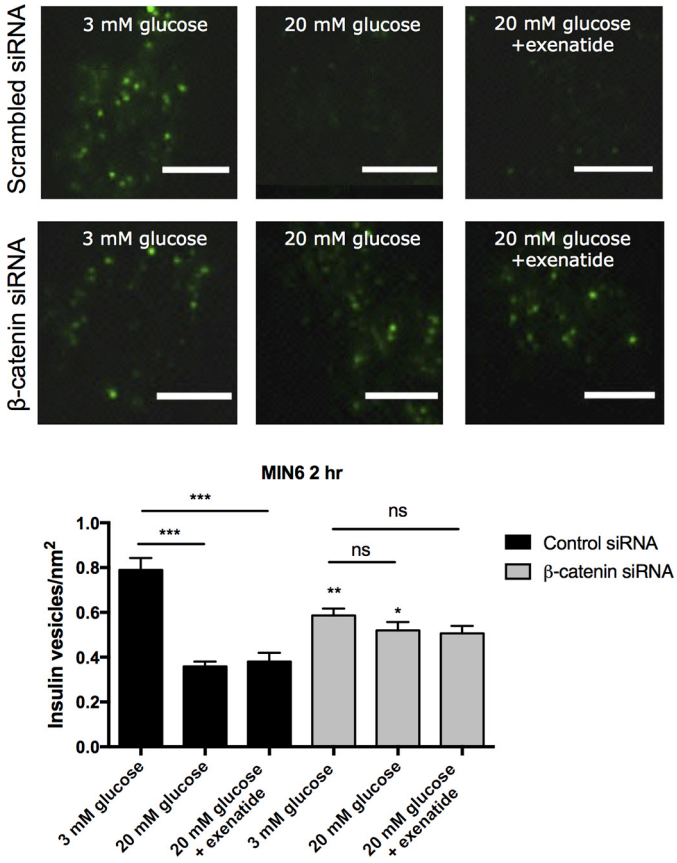


FIGURE 7. **Loss of β-catenin perturbs insulin vesicle distribution near the plasma membrane.** MIN6 cells were transfected with siRNA specific to β-catenin or a scrambled control and a GFP plasmid to identify transfected cells. 48 h after transfection, cells were glucose-starved for 1 h and incubated in 3 mM glucose, 20 mM glucose, or 20 mM glucose + 10 nM exenatide for 2 h. Cells were fixed, and insulin vesicles were stained with anti-insulin antibody and Alexa Fluor 555®-conjugated secondary antibody. Fluorescence within 100 nM of the plasma membrane was visualized using TIRF microscopy, and the density of insulin vesicles was quantified using Imaris software. *Scale bars* are 5 μm. ***, $p < 0.001$ as compared with scrambled control transfected cells of the same glucose condition, as assessed by one-way ANOVA with Tukey's post hoc test. Images are representative of ~20 taken for each treatment, and the vesicle density was calculated from at least 15 images. Similar results were obtained in at least three independent experiments. *ns*, not significant.

lating actin has not been studied in the context of insulin secretion, and indeed we find that loss of β-catenin alters F-actin in β-cells. These results provide mechanistic insight into how β-catenin affects insulin secretion. F-actin can have both positive and negative effects on insulin secretion, and the overall effect can be dependent on the nature of F-actin remodeling (5). Cortical F-actin acts as a physical barrier impeding insulin secretion, and glucose stimulation induces localized depolymerization of this actin, allowing access of insulin granules to the cell periphery for exocytosis (3, 36–38). Glucose also stimulates the polymerization of F-actin that acts as a cytoskeletal track for insulin granule trafficking in more central parts of the cell (39, 40). Here we observe a reduced F-actin fraction in cells where β-catenin had been knocked down and a denser actin structure near the cell periphery that is absent in control siRNA transfected cells. This is consistent with our other data showing that inhibition or depletion of β-catenin restricts insulin granule release from the cell. Similar effects have been reported for other proteins regulating insulin secretion via modulation of the actin cytoskeleton. For instance, secretagogin knockdown also decreases F-actin and increases the density of the actin structure at the cell periphery (41). Although we observed an effect of β-catenin on actin remodeling, we have not identified the exact mechanism for how this occurs, and we cannot exclude a direct effect on other processes involved in insulin secretion, particularly given the other known roles and interactions of β-catenin.

β-Catenin has no catalytic activity. However, it is a scaffold protein that interacts with a number of proteins involved in regulating vesicle trafficking; for instance, the C-terminal of β-catenin binds to a number of PDZ domain-containing proteins involved in vesicle trafficking and cytoskeleton organization (29), including the scaffold protein Pdzd2, which is known to be involved in insulin secretion (42). The PDZ-binding domain also links β-catenin to proteins that modulate the cytoskeleton via Rho GTPases (43, 44). For instance, β-catenin has recently been shown to spatially organize the activation of the Rac-GEF (GTP exchange factor) Tiam2 via its PDZ-binding domain (29). Downstream of Rho GTPase family members, activation of p21-associated kinase (PAK1) is essential for glucose regulation of F-actin and insulin exocytosis (45), and PAK1 itself can interact with and regulate β-catenin (46, 47). Additionally, β-catenin interacts with the PDZ domain of Synip (29), which regulates the function of syntaxin-4, which in turn is the most important t-SNARE regulating insulin secretion (38). In this way, β-catenin may influence the activity of syntaxin-4 and insulin vesicle localization or fusion. Another way that β-catenin may regulate insulin secretion is via regulation of ion channel localization, as β-catenin is known to interact with and regulate the surface expression of Ca^{2+}-activated potassium channels such as HSlo (48, 49). In β-cells, Ca^{2+}-activated potassium channels are involved in membrane repolarization (50, 51). How these interactions might regulate insulin vesicle trafficking in β-cells remains to be determined.

Although the exact mechanism by which β-catenin regulates insulin vesicle localization is not yet known, a role for β-catenin as part of the insulin secretion machinery and associated actin remodeling may also explain a way in which the diabetes susceptibility gene *TCF7L2* influences GSIS. TCF7L2 and E-cadherin both bind to the same region of β-catenin; because they bind with similar affinity they will compete with each other for binding, and this will be affected by relative expression levels of

FIGURE 6. **Inhibiting β-catenin prevents glucose- and exenatide-induced depletion of insulin vesicles near the plasma membrane.** Cells were glucose-starved for 1 h and pre-treated for 30 min with pyrvinium (100 nM) or DMSO as a control. *A* and *B*, INS832/3 cells were treated with 3 mM glucose (*glc*), 15 mM glucose, or 15 mM glucose + 10 nM exenatide. *C* and *D*, MIN6 cells were treated with 3 mM glucose, 20 mM glucose, or 20 mM glucose + 10 nM exenatide. Glucose treatments contained pyrvinium (100 nM) where indicated, and cells were incubated for 15 min (*A* and *C*) or 2 h (*B* and *D*). Cells were fixed, and insulin vesicles were stained with anti-insulin antibody and Alexa Fluor 555®-conjugated secondary antibody. Fluorescence within 100 nm of the plasma membrane was visualized using TIRF microscopy, and the density of insulin vesicles was quantified using Imaris software. *Scale bars* are 5 μm. ***, $p < 0.001$ as compared with the untreated cells of the same glucose condition, as assessed by ANOVA with Tukey's post hoc test. Images are representative of ~25 taken for each treatment, and the vesicle density was calculated using at least 20 images. Similar results were obtained in at least three independent experiments.

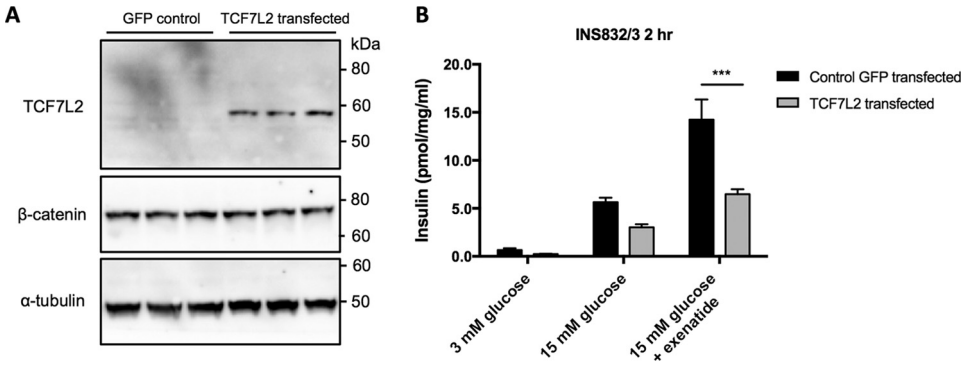


FIGURE 8. **Overexpressing TCF7L2 in INS832/3 β-cells impairs glucose- and exenatide-stimulated insulin secretion.** INS832/3 cells were transfected with *TCF7L2* plasmid or a GFP control plasmid, and 48 h after transfection, cells were glucose-starved for 1 h and then incubated in 3 mM glucose, 15 mM glucose, or 15 mM glucose + 10 nM exenatide for 2 h. *A*, cell lysates were collected and used for Western blotting analysis to quantify total TCF7L2 protein level relative to α-tubulin as a loading control. *B*, medium was collected for determination of insulin concentration using the AlphaLISA kit. ***, $p < 0.001$ as compared with the control GFP transfected cells of the same glucose condition, as assessed by ANOVA with Tukey's post hoc test. Similar results were obtained in at least three independent experiments.

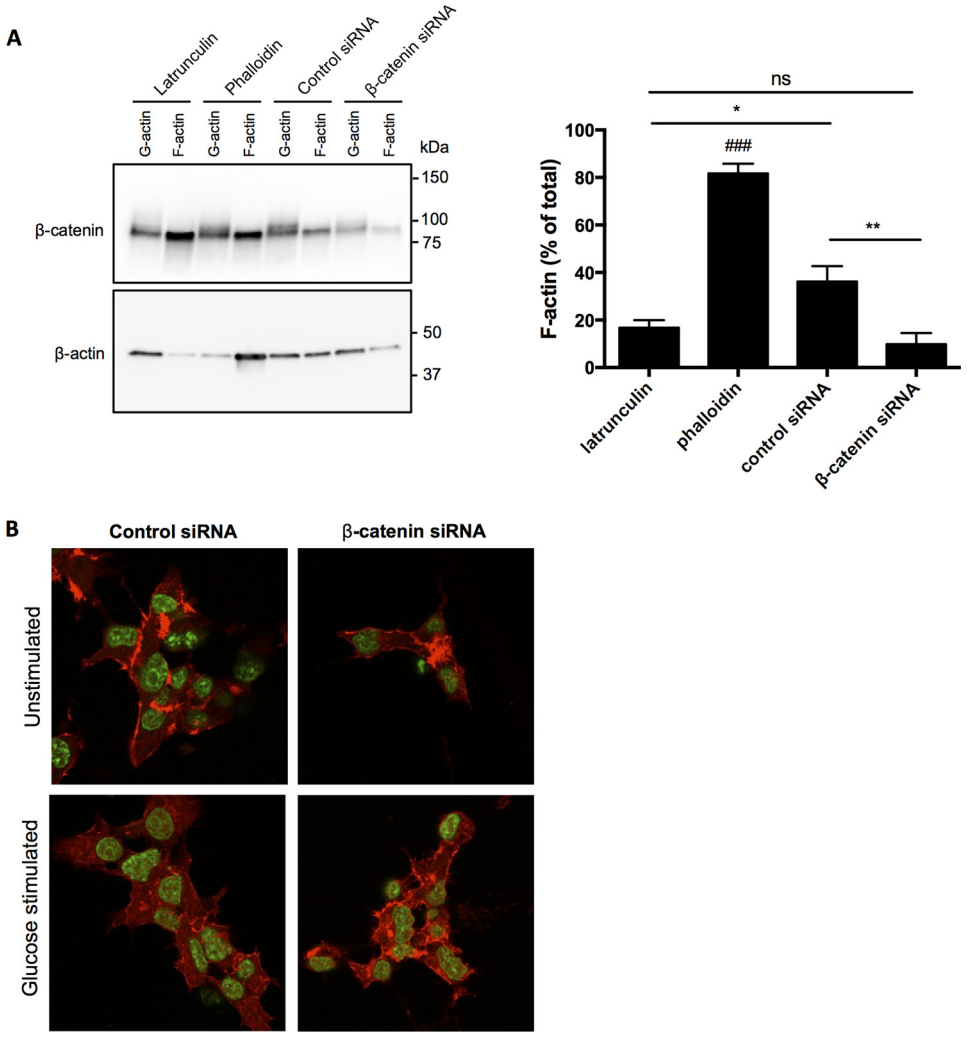


FIGURE 9. **Depletion of β-catenin affects intracellular actin remodeling.** *A*, F-actin and G-actin fractions were isolated from INS832/3 cells transfected with control siRNA or β-catenin siRNA, and then used for Western blotting analysis with anti-β-actin antibody and anti-β-catenin antibody to confirm siRNA knockdown. Latrunculin and phalloidin were used as control treatments for decreasing and increasing F-actin, respectively. The percentage of F-actin was quantified by densitometry. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ as compared with the control GFP transfected cells of the same glucose condition, as assessed by ANOVA with Tukey's post hoc test. *B*, transfected cells were seeded on glass coverslips, starved in low glucose, and treated with high glucose for 2 h. Cells were fixed and stained for F-actin using Alexa Fluor® 633 phalloidin (*red*), and the nucleus was visualized with DAPI (*green*).

each (52). Variants in *TCF7L2* that cause overexpression disrupt insulin secretion from β-cells (12). A recent study has provided support to the hypothesis that the effect of *TCF7L2* is mediated via β-cell resistance to incretin hormones and alteration in the expression of GLP-1 receptor (13). However, the exact mechanism by which TCF7L2 regulates insulin secretion

is unknown. The major binding partner of TCF7L2 is β-catenin, and together they act in the nucleus to activate gene transcription. Here we show that the overexpression of TCF7L2 impairs insulin secretion, which could be explained by the fact that the localization of β-catenin can be regulated by TCF7L2; hence an abundance of TCF7L2 may impair insulin secretion by sequestering β-catenin in the nucleus. This effect on β-catenin localization would make it unavailable to bind cadherins or participate in interactions that regulate actin cytoskeletal rearrangement and insulin vesicle trafficking to the plasma membrane. Similarly, when we pharmacologically inhibit the β-catenin/TCF7L2 interaction, which is the transcriptional effector unit, we observe no decrease in insulin secretion. This suggests a transcription-independent effect of β-catenin on insulin secretory processes but does not exclude a transcription-independent effect on the level of GLP-1 receptor.

In summary, this study shows that β-catenin is required for Ca^{2+}-dependent insulin secretion in β-cell lines and isolated pancreatic islets and that β-catenin regulates insulin vesicle localization near the plasma membrane independently of its gene regulatory effects. Further, we show that β-catenin is involved in regulating the actin cytoskeleton, which provides a mechanistic context for the role of β-catenin in insulin secretion. Further studies will be required to understand exactly β-catenin's role in these processes, but here we confirm that it plays a critical role in regulating insulin vesicle trafficking to the plasma membrane.

Experimental Procedures

Cell Culture—INS832/3 β-cells (kindly provided by Professor Christopher B. Newgard, Duke University) were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from Gibco, Life Technologies), 1 mM sodium pyruvate, 10 mM HEPES, and 50 μM 2-mercaptoethanol (all from Sigma-Aldrich). MIN6 cells (used at passages 30–41) were maintained in DMEM containing 25 mM glucose, 10% (v/v) fetal bovine serum, 10 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin and were switched low glucose (5.5 mM) culture medium for 48 h prior to experimentation. Insulin secretion experiments were performed on confluent cells in 12-well culture plates and TIRF microscopy was performed on cells that were 60–80% confluent in a glass bottom 35-mm Fluorodish cell culture dishes (World Precision Instruments).

Cell Transfection—All transfections and subsequent experiments were performed using medium lacking antibiotics. To knock down β-catenin, cells at 70–80% confluence were transfected with validated siRNA specific to β-catenin or with control scrambled siRNA (Stealth™ RNAi siRNA Negative Control, Med GC) at a final concentration of 30 nM using Lipofectamine® 2000 transfection reagent (INS832/3 cells) or Lipofectamine® LTX transfection reagent (MIN6 cells). To overexpress TCF7L2, INS832/3 cells were transfected at 70–80% confluence with *TCF7L2* plasmid pcDNA/Myc TCF4, which was a gift from Bert Vogelstein (Addgene plasmid 16512 (53)), or control GFP plasmid (pEGFP-C1; Clontech PT3028-5, kindly provided by Professor Tao Xu, Institute of Biophysics, Chinese Academy of Sciences) using Lipofectamine 3000® transfection reagent at a ratio of 3:1 reagent (μl) to DNA (μg). Following transfection, medium was replaced after 24 h, and experiments were performed 48 h after transfection. Cells to be analyzed by TIRF microscopy were co-transfected with a plasmid expressing GFP to allow for the identification of transfected cells. All reagents for siRNA experiments were from Life Technologies and used according to the manufacturer's instructions.

Insulin Secretion Assay—Prior to insulin secretion experiments, cells were starved in modified Krebs-Ringer bicarbonate HEPES (KRBH) buffer (119 mM NaCl, 4.74 mM KCl, 1.19 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.19 mM KH_2PO_4, 2.54 mM $CaCl_2$, and 20 mM HEPES), pH 7.4, containing 0.2% (w/v) BSA for 1 h. When required, cells were pre-treated with inhibitor during the final 30 min of starvation. Following starvation, the medium was replaced with KRBH containing low glucose (3 mM), high glucose (15 mM for INS832/3 cells and 20 mM for MIN6 cells), high glucose + 10 nM exenatide (Byetta®), or high glucose + 10 nM GLP-1(7–36) amide (GenScript). Cells were treated with 100 nM pyrvinium pamoate (Sigma-Aldrich) or 5 μM BIO (Sigma-Aldrich) as indicated. iCRT5 was obtained from Axon Medchem. Following incubation for the indicated times, an aliquot of medium was collected and diluted appropriately, and the insulin content was determined using the AlphaLISA Insulin Assay Kit (PerkinElmer). Cells were rinsed twice with ice-cold PBS, and cellular lysates were harvested in buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM vanadate, 100 mM NaF, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AESBF), 4 μg/ml aprotinin, 0.4 μg/ml pepstatin, 4 μg/ml leupeptin, and 30 μM *N*-Acetyl-Leu-Leu-Norleu-al (ALLN). Lysates were collected and centrifuged at 16,100 × *g* for 10 min, and cleared supernatants were analyzed by polyacrylamide gel electrophoresis for Western blotting.

Islet Isolation and Static Insulin Secretion—All experiments involving mice were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of A*STAR under IACUC Number 110683 or by the University of Auckland Animal Ethics Committee under Number 001732. Islets were isolated by collagenase digestion from the offspring of 129, C57BL/6, or CD1 mice (12 weeks of age and fed a regular diet), and then purified by hand-picking as described previously (54) or by a protocol devised by combining several published methods (55–57). For this method, islets were isolated by collagenase digestion using 0.9 mg/ml collagenase V (Sigma) in Isolation-KRBH (129 mM NaCl, 5 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM HEPES, 1 mM $CaCL_2$, 5.56 mM glucose, and 0.1% BSA plus 0.02 mg/ml DNase) for 19 min at 37 °C followed by wash steps and separation of islets, first using cell separation medium Histopaque (1.1 g/ml) and finally using a 40-μm cell strainer. Following isolation, islets were transferred into a non-tissue culture-treated Petri dish and cultured overnight at 11 mM glucose in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1% penicillin/streptomycin (v/v), 1 mM sodium pyruvate, 7.5 mM

HEPES, and 50 μM 2-mercaptoethanol, pH 7.4 (Invitrogen) at 27 °C for the first 48 h with a medium change at 12–24 h. Islets were then incubated at 37 °C, and medium was changed every 3–4 days. For incubations, a different batch of islets was used per experimental replicate.

Prior to treatment, islets were aliquoted into 24-well plates (8 per well) or microtubes (10–15 size-matched islets per tube) and fasted for 1 h in KRBH buffer supplemented with 0.2% BSA and 2.8 mM glucose. During the fasting period, islets were pretreated with 100 nM pyrvinium pamoate or DMSO (control). Following fasting, the medium was replaced with KRBH buffer containing low (2.8 mM) glucose, high (11.2 or 16.7 mM) glucose, or low glucose with 30 mM KCl. Islets were treated with 100 nM pyrvinium pamoate, 1 μM BIO, or control (DMSO) as indicated. After a 1-h treatment, supernatant was taken for insulin measurements (ELISA, Mercodia or AlphaLISA, PerkinElmer), and pelleted islets were taken in radioimmunoprecipitation assay buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0), and Complete protease and PhosSTOP inhibitor cocktails (Roche Applied Science) for insulin measurements in islets (ELISA, Mercodia).

Western Blotting Analysis—Western blotting analysis was carried out with antibodies specific against total β-catenin (1:2000; Symansis), α-tubulin (1:20,000; Sigma-Aldrich), β-actin (1:20,000; Sigma-Aldrich), and GAPDH (1:20,000; Abcam). Following overnight incubation in primary antibody, membranes were washed and incubated with anti-mouse (1:25,000; Sigma-Aldrich) or anti-sheep (1:15,000; Dako) IgG-horseradish peroxidase-conjugated antibody for 1 h at room temperature and developed using Clarity™ Western ECL substrate (Bio-Rad Laboratories).

Immunofluorescence Staining—Cells were incubated in KRBH buffer (136 mM NaCl, 4.7 mM KCl, 5 mM $NaHCO_3$, 1.3 mM $MgSO_4(7H_2O)$, 1.3 mM KH_2PO_4, 1 mM $CaCl_2$, 10 mM HEPES, 0.5% (w/v) BSA) for 1 h prior to stimulation with glucose, exenatide, or indicated treatments. When required, 100 nM pyrvinium was included for the final 30 min of starvation. Following treatment, cells were fixed for 15 min in 4% paraformaldehyde, permeabilized for 15 min in 0.2% Triton X-100, and blocked with 0.2% BSA for 1 h. Insulin vesicles were labeled by antibody staining with monoclonal anti-insulin antibody (1:500; Sigma-Aldrich) and anti-mouse Alexa Fluor 555®-conjugated antibody (1:500; Life Technologies), and then stored in PBS until imaging.

TIRF Microscopy—TIRF microscopy was performed using Leica AM TIRF MC equipment on a Leica DM6000B microscope controlled by LAS AF 3 software (Leica Microsystems). Cells suitable for imaging were identified by fluorescence microscopy using an HC PL APO 100×/1.47 oil objective, and TIRF images (603–678-nm filter) were obtained on a Leica DFC310Fx camera from samples excited with a 561-nm laser introduced into the excitation light path at an appropriate angle to image ~100 nm into the cell, as described previously (58, 59). Images were managed using ImageJ (National Institutes of Health) and insulin vesicle density was assessed in individual cells using ImageJ to quantify the cellular area and Imaris (version 7.2, Bitplane) to detect "spots" of ~0.5-nm diameter.

G/F-actin Measurement—Cells were rinsed with PBS and lysed in pre-warmed (at 37 °C) actin stabilization buffer (50 mM PIPES, pH 6.9, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 5% (v/v) glycerol, 0.1% Nonidet P-40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% 2-mercaptoethanol, 0.001% Antifoam A, 1 mM ATP, and protease inhibitors). Cells were scraped off the culture dish surface, homogenized by passing through a 31-gauge needle five times, and incubated for 10 min at 37 °C. Lysates were centrifuged for 5 min at 2000 × *g*, at 37 °C. The homogenate was then centrifuged for 1 h at 100,000 × *g* at 37 °C. The F-actin pellet was resuspended in Milli-Q water containing 10 μM cytochalasin D and analyzed by Western blotting using anti-β-actin and anti-β-catenin antibody alongside the G-actin-containing supernatant. Latrunculin and phalloidin were included as positive control treatments to decrease or increase the percentage of F-actin, respectively.

Statistical Analyses—Statistical analyses were performed using the statistical software package GraphPad Prism 6.0 (GraphPad Software Inc.). Results are presented as the mean ± S.E., and statistical differences were determined using one-way ANOVA with Tukey's post hoc test: *, $p < 0.05$, **, $p < 0.005$, ***, $p < 0.001$.

Author Contributions—B. S., E. C., K. L. L., W. C. D., W. H., W. E. H., and P. R. S. contributed to the experimental design. B. S., K. L. L., W. C. D., E. C., Y. F., and W. E. H. performed the experiments. B. S., E. C., K. L. L., W. C. D., W. E. H., and P. R. S. analyzed and interpreted the data. B. S. and P. R. S. wrote the paper.

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A Critical Role for β-Catenin in Modulating Levels of Insulin Secretion from β -Cells by Regulating Actin Cytoskeleton and Insulin Vesicle Localization

Brie Sorrenson, Emmanuelle Cognard, Kathryn L. Lee, Waruni C. Dissanayake, Yanyun Fu, Weiping Han, William E. Hughes and Peter R. Shepherd

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