Membrane cholesterol modulates the ability of glucose to stimulate insulin secretion from pancreatic β-cells. The molecular mechanism by which this occurs is not understood. Here, we show that in cultured β-cells, cholesterol acts through phosphatidylinositol 4,5-bisphosphate (PIP₂) to regulate actin dynamics, plasma membrane potential, and glucose-stimulated insulin secretion. Cholesterol-overloaded β-cells exhibited decreased PIP₂ hydrolysis, with diminished glucose-induced actin reorganization, membrane depolarization, and insulin secretion. The converse findings were observed in cholesterol-depleted cells. These results support a model in which cholesterol depletion is coupled through PIP₂ to enhance both plasma membrane Ca²⁺ influx from the extracellular space, as well as inositol 1,4,5-triphosphate-stimulated Ca²⁺ efflux from intracellular stores. The inability to increase cytosolic Ca²⁺ may be the main underlying factor to account for impaired glucose-stimulated insulin secretion in cholesterol-overloaded β-cells.

In pancreatic β-cells, glucose-stimulated insulin secretion (GSIS) is mediated by effects on both membrane potential and cortical actin (1). F-actin exists as a dense web beneath the β-cell plasma membrane (PM), which inhibits the recruitment and sustained exocytosis of insulin secretory granules (2, 3). Disruption of the cortical F-actin network permits access of insulin granules to the cell periphery and enhances GSIS (4). In both primary and cultured β-cells, glucose induces F-actin remodeling to mobilize insulin granules for docking and fusion at the PM (5, 6).

The exocytosis of peripheral insulin granules is controlled by ATP-sensitive K⁺ (Kₐₐₜ) channels. In β-cells, Kₐₐₜ channels consist of four inwardly rectifying K⁺ (Kir6.2) subunits and four SUR1 (sulfonylurea receptor 1) subunits (7). Kₐₐₜ channels are highly sensitive to changes in the ATP/ADP ratio, coupling glucose metabolism to membrane potential. Glucose stimulation increases the ATP/ADP ratio, inhibiting Kₐₐₜ channel activity to cause membrane depolarization, voltage-gated Ca²⁺ influx, and initiation of insulin secretion (8).

Impaired insulin secretion from pancreatic β-cells contributes to the progression of type 2 diabetes. Yet a full understanding of how GSIS normally occurs and how it is defective in diabetes has remained elusive (9). Recently, it has been suggested that alterations in cellular cholesterol levels may play a critical role to modulate GSIS. The idea that excess cholesterol inhibits GSIS is supported by data from mice lacking the ABCA1 cholesterol transporter specifically in β-cells (10). Islets from these mice have increased cholesterol and impaired GSIS. Similarly, elevated islet cholesterol levels in ApoE-deficient mice inhibit GSIS (11). Direct manipulation of membrane cholesterol in cultured β-cells further shows that excess cholesterol impairs GSIS, whereas cholesterol depletion enhances GSIS (11). Together, these data suggest that membrane cholesterol content contributes to the regulation of GSIS. The mechanisms by which cholesterol influences GSIS are not known.

In addition to the ATP/ADP ratio, phosphatidylinositol 4,5-bisphosphate (PIP₂) modulates the activity of Kₐₐₜ channels in β-cells (12). PIP₂ is a minor phospholipid that regulates cytoskeletal organization, membrane trafficking, the generation of second messengers, and the function of ion channels (13). PIP₂ is suggested to be important to GSIS, yet little is known about how this lipid is regulated in pancreatic β-cells. It has been shown in other cells that cholesterol controls PIP₂ distribution in membranes and regulates its downstream effects in a cell type-dependent manner (14).

In the present work, we tested the hypothesis that cholesterol regulates GSIS through PIP₂. In cultured β-cells, we found that depleting cholesterol stimulates PIP₂ hydrolysis, whereas overloading cholesterol enhances PM PIP₂ accumulation. Changes in PM PIP₂ in turn affect actin dynamics at the cell periphery and the extent of glucose-stimulated membrane depolarization in β-cells. Together with other data, we propose a model in which cholesterol acts through PIP₂ to regulate both the actin cytoskeleton and plasma membrane potential, thus impacting multiple aspects of GSIS.

**EXPERIMENTAL PROCEDURES**

**Cells and Materials**—832/13 INS-1 β-cells were a kind gift from Dr. Christopher B. Newgard (Duke University, Durham, NC). The cells were cultured in RPMI 1640 with 11.1 mM d-glucose.
Cholesterol Regulates GSIS through PIP₂

cose supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM Heps, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol at 37 °C and 5% CO₂ in a humidified atmosphere (15). The pleckstrin homology (PH) domain of PLCδ fused to GFP (PHPLCδ-GFP) was a gift from Dr. Pietro De Camilli (Yale University). The cells were transiently transfected with PHPLCδ-GFP and Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol and cultured for 48 h prior to microscopy. Stable cells overexpressing SCD1 were generated by transfecting INS-1 cells with pcDNA3.1/FLAG-Hygro-mSCD1 (a kind gift from Dr. Alan Tall (Columbia University, New York)) (16). All microscopy experiments as well as insulin secretion assays were performed in Heps balanced salt solution (HBSS) (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.16 mM MgSO₄, 20 mM Heps, 2.5 mM CaCl₂, 25.5 mM NaHCO₃, and 0.2% bovine serum albumin (essentially fatty acid-free), pH 7.2) (15).

Ultrasensitive insulin enzyme immunoassay kits were from Alpco Diagnostics (Salem, NH). Jasplakinolide, Alexa Fluor 488 DNase I, Alexa Fluor 546 phalloidin, the Amplex Red cholesterol assay kit, bis-(1,3-diethylthiobarbituric acid)trimethine oxonol (DiSBAC₂(3)), and Vybrant DiI cell-labeling solution were obtained from Invitrogen. PIP₂ diC₁₆ and its carrier (histone H1 and histone H1-TMR) were from Echelon (Salt Lake City, UT). MBCD, water-soluble cholesterol (1 g of water-soluble cholesterol contains ~40 mg of free cholesterol), filipin, sphiungomyelinase (SMase) from Streptomyces, cytochalasin D, U73122, neomycin, and all other chemicals were from Sigma.

Carrier-mediated Delivery of PIP₂—Carrier-mediated application of PIP₂ diC₁₆ to β-cells was performed as previously described (17). Briefly, PIP₂ diC₁₆ was combined with histone carrier H₁ to final concentrations of 300 and 100 μM, respectively, to form the carrier-phospholipid complex at room temperature for 10 min, followed by a 10-fold dilution. Cells were incubated with this PIP₂-histone complex in the presence or absence of MBCD or Chol for 30 min and then fixed and stained with phalloidin. To monitor the delivery of this PIP₂-histone complex, fluorescently labeled histone carrier H₁ (histone H₁-TMR) was used. All cells displayed rhodamine fluorescence using the procedure described.

Fluorescence Microscopy—Confocal microscopy was performed using an Axiosvert 100M inverted microscope equipped with an LSM 510 laser-scanning unit and a 63×1.3 numerical aperture plan Achromat objective (Carl Zeiss, Inc.). 488- and 543-nm laser lines were used to excite GFP/Alexa Fluor 488 and Dil/Alexa Fluor 546, respectively. Emitted light was passed through a 505–530-nm band pass filter for collection of GFP/Alexa Fluor 488 and a long pass filter (560 nm) for Dil/Alexa Fluor 546. Wide field microscopy was carried out using a Zeiss Axiosvert microscope (Carl Zeiss) equipped with a cooled CCD camera driven by AxioVision imaging software (Carl Zeiss). All images were acquired using a Zeiss plan-neofluar ×40/1.3 oil objective. Alexa Fluor 546 and DiSBAC₂(3) were imaged using a rhodamine filter set, Alexa Fluor 488 using a fluorescein isothiocyanate filter set, and filipin using a 4’,6-diamidino-2-phenylindole filter set. Identical image acquisition and display settings were used for all images from the same experiment.

Cold Triton Extractability—Cold Triton X-100 extractability of Dil incorporated into the PM of cells after various treatments was measured as described (18). Briefly, cells were washed with HBSS, labeled with 5 μM Dil (Vybrant Dil cell-labeling Solution) in HBSS at 37 °C for 20 s, washed with ice-cold HBSS, and extracted with 1% Triton X-100 on ice for 30 min. For quantification, cells were treated as described above, with the exception of replacing 1% Triton X-100 with HBSS for intact cells. Images were first background-corrected (19) and then quantified using MetaMorph analysis software (Molecular Devices, Sunnyvale, CA) by manually outlining each cell and taking the average fluorescence power associated with the cells. The ratio of average intensities obtained from the extracted and intact cells of each condition was determined as the percentage remaining cell-associated.

Insulin Secretion Assay and Cholesterol Measurement—For starvation, cells were switched from growth medium to 5 mM glucose for 24 h, followed by incubation for 2 h in basal HBSS containing 2 mM glucose. They were then exposed to different conditions (5 mM MβCD, 10 mM Chol, 50 milliunits/ml SMase) for 30 min, whereas control groups were maintained in basal glucose. Subsequently, all groups were washed and then switched to HBSS containing 2 or 20 mM glucose or 30 mM KCl for 60 min at 37 °C, after which HBSS was collected for insulin measurement by ultrasensitive insulin enzyme immunoassay kits. Cellular protein content was measured using the Micro BCA protein assay (Pierce) after 1% Triton X-100 extraction. Insulin secretion was normalized to protein content. Cholesterol was measured using the Amplex Red cholesterol assay kit, as described previously (11).

Live Cell Confocal Microscopy of PHPLCδ-GFP-expressing Cells—Sequential images of cells expressing PHPLCδ-GFP were taken for 10–30 min at 5–15-s intervals. A minimum laser power that would give a useful fluorescence signal was used, and the laser intensity was kept the same for all cells. Midcell sections were used to examine PHPLCδ-GFP translocation, and images focused on the PM plane were used to look at cell morphological changes. Cells were first imaged to establish a base line. A 10× concentrated stock of stimulus was then added to the cells on the microscope stage. Final concentrations of 20 mM glucose and 5 mM MβCD for translocation, 10 mM MβCD for morphological changes, and 10 mM Chol were used.

Actin Staining—Cells were washed, fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 5 min, and stained with Alexa 546-phalloidin either alone or in combination with Alexa 488-DNase I or filipin for 30 min, all at room temperature. For Fig. 6, A–C, PHPLCδ-GFP-expressing cells were fixed with 1% paraformaldehyde for 2 min at 37 °C, followed by 15 min in Alexa 546-phalloidin at room temperature. Under this condition, all cells were fixed, and most cells were permeabilized (20) to allow actin staining.

Measurement of Membrane Potential—For Fig. 7, A and B, starved cells were treated with MβCD or Chol for 30 min at 37 °C, during the last 10 min of which a 300 nM concentration of the fluorescent membrane potential probe, DiSBAC₂(3), was added. The imaging solution contained the same concentration of DiSBAC₂(3). 20 mM glucose was added to the solution (in the form of 10× concentrated stock) before the time lapse imaging.
For Fig. 7C, starved cells were incubated with DiSBAC$_2$(3) in 2 or 20 mM glucose and imaged in a solution containing the same concentrations of DiSBAC$_2$(3) and glucose with MβCD added as the stimulant. To correct for photobleaching, DiSBAC$_2$(3)-labeled cells were rapidly imaged repeatedly in the imaging solution with basal glucose.

**RESULTS**

**Reduced Membrane Microdomains Enhance GSIS**—Cholesterol is enriched in membrane microdomains, which are thought to adopt a liquid-ordered structure. Because of their higher sensitivity to cholesterol extraction relative to other membrane regions, microdomains can be disrupted using methyl-β-cyclodextrin (MβCD), a cholesterol chelator. We studied the effect of this perturbation by MβCD on GSIS in pancreatic islets and cultured β-cells (11). However, because non-microdomain cholesterol is also affected by MβCD, further work is required to define conclusively the role of membrane microdomains in GSIS. To examine if membrane fluidity regulates GSIS, independent of cholesterol abundance, two approaches were taken. First, SMase was used to partially digest membrane sphingomyelin, the majority of which is found in cholesterol-rich membrane microdomains (21). Second, β-cells overexpressing SCD1 (stearoyl-CoA desaturase 1) were generated. SCD1 is the rate-limiting enzyme in the biosynthesis of monounsaturated fatty acids and is a critical regulator of membrane biophysical properties. SCD1 overexpression in CHO cells results in dramatically altered lipid composition, without effects on total cholesterol, free cholesterol, cholesterol esters, or phosphatidylcholine (16). As shown in Fig. 1A, whereas cholesterol extraction using MβCD decreased cholesterol content, neither SMase treatment nor SCD1 overexpression significantly altered total cholesterol abundance. Control immunoblots confirmed SCD1 overexpression (Fig. 1B).

GSIS was enhanced by both SMase treatment and SCD1 overexpression, similar to MβCD treatment (Fig. 1C). By contrast, overloaded the cells with Chol inhibited GSIS (Fig. 1C), as previously reported (11). These data suggest that a more fluid lipid environment promotes GSIS. The result from SCD1-overexpressing cells is particularly interesting, because it suggests that increasing overall membrane fluidity is as effective at enhancing GSIS as removing cholesterol or sphingomyelin.

To determine the effects of these interventions on overall PM organization, we utilized the fact that cholesterol-rich membrane microdomains are insoluble in cold Triton X-100 (22). A change in detergent resistance, although not always conclusive, has been used as an indication of altered overall membrane fluidity (23). The presence of liquid-ordered domains in cells used in Fig. 1, A–C, was assessed by confocal microscopy using a technique involving detergent extraction of cells whose PM was labeled by a fluorescent lipid probe with preference for the ordered domains (DiI) (18). In control cells, treatment with cold Triton X-100 caused the appearance of a small number of holes in the DiI-labeled PM, indicating that the major portion of the membrane was resistant to Triton X-100 (Fig. 1D), as reported previously (18). In cells treated with MβCD or SMase and in cells overexpressing SCD1, there was an increase in the detergent-soluble regions, suggesting a decrease in liquid-ordered membrane microdomains (Fig. 1E). This is consistent with the idea that cholesterol and sphingomyelin are integral components of some types of microdomains. Because SCD1 regulates lipid saturation and saturated acyl chains confer detergent resistance on membranes (24, 25), our study shows that a change in SCD1 expression level leads to altered membrane properties in INS-1 cells.

**Membrane Lipids Affect the Actin Cytoskeleton**—Proteins and lipids localized to membrane microdomains regulate the...
changes in the cortical actin structure. Under basal conditions, as single confocal sections, provide the resolution to examine F-actin staining (Fig. 2A). By contrast, cholesterol-overloaded cells had increased G-actin abundance and F-actin content, cholesterol-overloaded cells, differential interference contrast images revealed thin lamellae, driven by F-actin assembly at the cell edge, where G-actin was completely absent (supplemental Fig. S1). To characterize the relationship between cellular cholesterol abundance and F-actin content, cholesterol-overloaded β-cells were stained with both phalloidin and a cholesterol probe, filipin (29). As shown in Fig. 3A, cells brightly labeled with filipin also displayed high phalloidin fluorescence (arrowheads), suggesting a correlation between cholesterol content and the presence of polymerized actin. Quantification of filipin and phalloidin fluorescence in 66 cells generated a Pearson correlation coefficient of $R^2 = 0.3662 (p = 0.009)$ (Fig. 3B). Colocalization of cholesterol and F-actin was difficult to observe, because a large portion of cholesterol was present in membranes of intracellular organelles, similar to that observed in several other cell types (20, 30–35). Yet, in cells with heterogeneous PM filipin staining, regions enriched in cholesterol consistently contained concentrated F-actin (Fig. 3C).

We next examined how changes in the actin cytoskeleton affected GSIS. Disruption of F-actin by cytochalasin D enhanced GSIS, whereas stabilization of F-actin by jasplakolide inhibited GSIS (supplemental Fig. S2). These results are consistent with data showing that actin rearrangement is required for insulin secretion (4, 36, 37). Together, the data suggest that disruption of membrane microdomains and increased membrane fluidity (Fig. 1, D and E) promote GSIS (Fig. 1C) by reducing cortical actin (Fig. 2B), consistent with the idea that an actin web may present a barrier to insulin exocytosis (2). Conversely, stabilizing the cells with cholesterol stabilizes and expands the F-actin network at the cell periphery (Fig. 2 and supplemental Fig. S1), leading to inhibition of GSIS (Fig. 1C).

**Cholesterol Content Modulates PIP$_2$ Hydrolysis**—PIP$_2$, which relies on cholesterol for its localization and function, is an important regulator of actin polymerization (14, 38). Whereas some but not other hormone-stimulated PIP$_2$ turnover is cholesterol-dependent, how cholesterol abundance alone may regulate PIP$_2$ metabolism is not well understood (39, 40). To assess PIP$_2$ turnover, a reporter containing GFP fused to the PH domain of PLCδ (PH$^\text{PLCδ}$-GFP) was used. The PH$^\text{PLCδ}$ domain binds PIP$_2$ specifically and with high affinity, and PH$^\text{PLCδ}$-GFP...
translocation to the cytosol has been used previously to monitor PIP2 metabolism (41, 42).

Quantification of fluorescence intensities from mid–β-cell confocal images revealed that adding MβCD decreased PHPLC–GFP at the PM and increased its abundance in the cytosol (Fig. 4A). Since PHPLC–GFP also binds inositol 1,4,5-triphosphate (IP3) in the cytosol, this translocation of PHPLC–GFP is interpreted as hydrolysis of PM PIP2 to generate cytosolic IP3, as previously reported (28). Because prolonged incubation with high concentrations of MβCD (>10 mM) could cause cells to retract, an initial concern was that the increase in intracellular PHPLC–GFP may be due to volume change. However, the fact that PHPLC–GFP translocation upon MβCD addition was extremely rapid (<1 min) and occurred with as little as 1 mM MβCD argued against that artifact, because under these conditions there was no apparent morphological change in β-cells. This conclusion is also strengthened by the finding that no significant change was observed in PHPLC–GFP distribution in fibroblasts that retracted as a result of epidermal growth factor addition (43).

When soluble cholesterol was added, cells produced PM ruffles enriched in PHPLC–GFP, and cytosolic PHPLC–GFP fluorescence was slightly decreased (Fig. 4B). The cells also extended membrane protrusions (Fig. 4B, inset) similar to those in actin-labeled cholesterol-overloaded cells (Fig. 2B and supplemental Fig. S1). PIP2 turnover at the PM could result from both depletion (by hydrolysis or conversion to other phosphoinositides) and resynthesis. Thus, the enrichment of PM PIP2 upon cholesterol overloading could result from blunted PIP2 hydrolysis (since cytosolic IP3 was not increased) and unaffected (or accelerated) synthesis at the PM.

Glucose stimulated PIP2 hydrolysis, as previously reported (44), yet this response was slower and less marked than that induced by cholesterol depletion (Fig. 4C). When cells were treated first with glucose and then with MβCD (added long after the glucose-induced cytosolic PHPLC–GFP fluorescence had peaked), there was again a rapid and dramatic translocation of PHPLC–GFP from PM to cytosol (Fig. 4C). When cells were treated first with MβCD and then glucose, PHPLC–GFP translocation was observed (not shown). However, when cells were treated first with soluble cholesterol (Fig. 4D, second image), glucose was unable to stimulate PIP2 hydrolysis, as indicated by a lack of IP3 increase in the cytosol (Fig. 4D, third image). This was the case even after prolonged stimulation (>40 min). When MβCD was subsequently added, translocation of PHPLC–GFP was again observed (Fig. 4D, last image). Together, these data support the notion that excess cholesterol inhibits glucose-stimulated PIP2 hydrolysis, and this effect can be relieved by cholesterol depletion.

**Actin Reorganization Is Linked to PIP2 Dynamics**—PIP2 regulates actin by interacting directly with actin-binding proteins (45). In particular, PIP2 binds profilin to make G-actin available for incorporation into filaments (46). PIP2 is therefore an ideal candidate to mediate the effect of cholesterol on the actin cytoskeleton, connecting the results in Figs. 2–4. We performed time lapse confocal microscopy to study effects of cholesterol modulation on cell morphology, which may reflect changes in the actin cytoskeleton. The PM of PHPLC–GFP-expressing β-cells was imaged to link actin reorganization to PIP2 dynamics. Images taken before and after the MβCD addition are shown in red and green, respectively, in Fig. 5, A–C. Dramatic retraction of β-cells was observed after a high dose of MβCD treatment (Fig. 5A). When cells were first treated with neomycin, which binds and sequesters PIP2 (47), MβCD-induced retraction was greatly reduced (Fig. 5B). These data suggest that acute effects of cholesterol depletion on cell morphology are mediated, at least in part, by PIP2.

When soluble cholesterol was added to the cells, three distinct morphological changes were observed: formation of thin membrane protrusions reminiscent of lamellipodia (Figs. 2B and 4B and supplemental Fig. S1), flattening and spreading of overall cell shape (Fig. 5C), and extensive ruffling (Figs. 4B and 5D and supplemental video). The supplemental video recorded dramatic membrane reorganization as the β-cell underwent cholesterol modulation. All of these responses require actin
polymerization (48, 49). These processes were reversed by the addition of MβCD (Fig. 5D and supplemental video). Similar results were obtained when membrane-targeted GFPs were used in place of PH\(_{\text{PLC}_\beta}\)-GFP (data not shown). Strikingly, as cholesterol became enriched in the PM, distinct PIP\(_2\)-rich clusters formed on a previously uniform background (Fig. 5C, later time points). These data are in agreement with the idea that cholesterol-dependent recruitment of PIP\(_2\)-binding proteins promotes the assembly of membrane domains enriched in both cholesterol and PIP\(_2\) (50). In summary, the morphological changes, including retraction, expansion, ruffling, and protrusion, all reflect a significant effect of cholesterol on cellular actin network in β-cells.

To further examine the relationship among cholesterol, PIP\(_2\), and actin, we looked at the distribution of PM PIP\(_2\) and cortical actin in β-cells. Except for obvious surface deformations, such as invaginations and ruffles, PIP\(_2\) was present uniformly (as assessed by PH\(_{\text{PLC}_\beta}\)-GFP) on the PM of control β-cells (e.g. Fig. 5, A–C, first time point). This is consistent with findings in other cell types (50, 51). We therefore induced PIP\(_2\) clusters by overloading β-cells with soluble cholesterol (Figs. 5C, later time points, and 6B). To reduce artifacts that could coalesce PIP\(_2\) microdomains (50), minimal fixation and no permeabilization were used. Under these conditions, all of the cells were fixed, and most cells were permeabilized (20) to allow staining of actin with phalloidin in PH\(_{\text{PLC}_\beta}\)-GFP-expressing cells. Furthermore, imaging with PH\(_{\text{PLC}_\beta}\)-GFP was shown to yield closely comparable labeling patterns before and after fixation (17). In midcell confocal sections, excellent agreement of actin enrichment and PIP\(_2\) clusters was observed in cholesterol-overloaded cells (Fig. 6, A–C, arrowheads).

To test further the involvement of PIP\(_2\) in cholesterol-regulated actin dynamics, we utilized neomycin to sequester PIP\(_2\), U73122 to inhibit PLC-dependent PIP\(_2\) hydrolysis (50), and carrier-mediated delivery of long chain synthetic PIP\(_2\) diC\(_{16}\), to increase PM PIP\(_2\) concentration (17). All three treatments caused actin polymerization at the cell periphery (Fig. 6, D–F). Moreover, the effects of MβCD and soluble cholesterol on actin rearrangement were masked by the presence of neomycin, U73122, or carrier-PIP\(_2\). For example, Fig. 6, G–I, shows that pretreatment of β-cells with neomycin (Fig. 6H) or U73122 (Fig. 6I) completely blocked the ability of MβCD to reduce cortical actin (Figs. 2 and 6G).

**Cholesterol Modulation Regulates Membrane Potential**—In addition to regulating the actin cytoskeleton, PIP\(_2\) activates K\(_{\text{ATP}}\) channels. PIP\(_2\) increases the open probability and reduces the ATP sensitivity of the K\(_{\text{ATP}}\) channel by interacting with Kir6.2, the pore-forming subunit (52, 53). Because the above data implicate membrane cholesterol as a regulator of PM PIP\(_2\), we examined the effect of cholesterol to modulate membrane potential in β-cells. We used the potential-sensitive dye, DiSBC2(3), which enters cells and fluoresces in a manner that correlates with the degree of membrane depolarization (54). In β-cells treated with MβCD, glucose-stimulated membrane depolarization was enhanced (Fig. 7, A and B). By contrast, in β-cells overloaded with cholesterol, glucose was unable to stimulate membrane depolarization. Together with the data above indicating that cholesterol enrichment leads to PM PIP\(_2\) accumulation (Fig. 4B), these observations support and extend previous work showing that PIP\(_2\) inhibits the ability of ATP
to close $K_{\text{ATP}}$ channels (52). Adding MβCD to β-cells caused membrane depolarization of cells maintained in both 2 and 20 mM glucose, although this depolarization was less than that induced by glucose (Fig. 7C). Thus, glucose-stimulated ATP production exerts a much greater effect to inhibit $K_{\text{ATP}}$ channels than does reduced PIP$_2$ from cholesterol depletion.

If GSIS is increased in cholesterol-depleted cells in part due to enhanced $K_{\text{ATP}}$-mediated membrane depolarization, then KCl-stimulated insulin secretion (KSIS) should be less affected by membrane cholesterol abundance. This is because the ATP sensitivity of the $K_{\text{ATP}}$ channel is bypassed in KSIS, so that the effect of PIP$_2$ to regulate $K_{\text{ATP}}$ channel activity will impact insulin secretion to a lesser degree than in GSIS. Consistent with this idea, cholesterol depletion and overload had less effect on KSIS than on GSIS (Fig. 8). There was still some regulation by cholesterol in KSIS (Fig. 8A). However, compared with control cells, cholesterol depletion resulted in 80 ± 25% higher GSIS versus 33 ± 14% higher KSIS; cholesterol overload led to 44 ± 7% lower GSIS versus 28 ± 8% lower KSIS (Fig. 8B). Because KCl stimulation did not entirely abolish the effect of cholesterol to modulate insulin secretion, there may be factors independent of $K_{\text{ATP}}$ channels that also contribute to cholesterol regulation of GSIS, such as cholesterol-dependent glucokinase activation (11).

**DISCUSSION**

Various reports have shown an important role of cholesterol to regulate secretory responses, particularly through cholesterol-dependent because PIP$_2$-binding proteins, known as PIP$_2$-modulins, localize to rafts (14). Myristoylated and palmitoylated raft proteins, such as GAP43, MARCKS, and CAP23/NAP22, sequester PIP$_2$ into clusters in a cholesterol-dependent manner (14, 50, 62–64). These proteins cycle between the cytoplasm and the inner leaflet of the PM, depending on their post-translational modification states and possibly membrane cholesterol content (65). As shown in Fig. 5C, cholesterol overloading may promote the accumulation of PIP$_2$-modulins at the PM, resulting in the formation of visible PIP$_2$ clusters, similar to the finding that overexpression of PIP$_2$-modulins accumulates PIP$_2$ at the PIP$_2$-enriched domains (50). Since free lateral diffusion is required for PIP$_2$ action (14), MARCKS-bound PIP$_2$ is apparently inaccessible to PLC for hydrolysis (66, 67). Thus, it may be that by disrupting the raft localization of PIP$_2$-modulins, cholesterol depletion promotes the local liberation of PIP$_2$ for hydrolysis and enhances PIP$_2$ availability as a signaling molecule. On the other hand, changes to PLC could affect PIP$_2$ hydrolysis. Under the experimental conditions used, a change in the PLC gene expression is unlikely to account for the observed results due to the immediate effect of cholesterol modulation on PIP$_2$ hydrolysis (<5 min; Fig. 4). Whether PLC activity is affected by cholesterol treatment will be examined in future studies.

Although cholesterol depletion reduces PM PIP$_2$ concentration, it acts differently from PIP$_2$-sequestering proteins/drugs that diminish the availability of PIP$_2$ for PLC-catalyzed hydrol-
ysis. Cholesterol effects also differ from experimental systems in which PIP₂ synthesis is blocked, either by pharmaceutical reagents or by knocking down PIP₂-producing kinases. Of note, in addition to stimulating PIP₂ hydrolysis, glucose also promotes PIP₂ synthesis (68). Such an increase in PM PIP₂ may be masked by the higher affinity of the PHPLC⁻/H9254-GFP probe for IP₃ over PIP₂ in our experiments (69, 70). There is no evidence that glucose-stimulated PIP₂ resynthesis is affected in cholesterol-depleted cells.

The data here link cholesterol regulation of PM PIP₂ with the role of PIP₂ to organize the actin cytoskeleton. They suggest that PIP₂ participates in cholesterol-induced actin reorganization in β-cells. Possibly by controlling local PIP₂ availability (Fig. 4), PM cholesterol regulates key actin activities (Figs. 2, 3, 5, and 6). Cholesterol depletion promotes PM PIP₂ hydrolysis and actin depolymerization at the cell periphery, whereas cholesterol overload sequesters PM PIP₂ and leads to the accumulation of cortical actin and formation of membrane structures. The model we propose is consistent with previous observations that increased PIP₂ promotes actin assembly and that PIP₂ hydrolysis leads to actin disassembly (45). In T lymphocytes, enrichment of raft-associated PIP₂ results in increased filopodia and cell spreading (71), not unlike the effects of cholesterol overloading we observed in β-cells (Fig. 5C).
The $K_{\text{ATP}}$ channel couples intracellular metabolism to membrane potential in pancreatic $\beta$-cells. Closure of $K_{\text{ATP}}$ channels upon a rise in the ATP/ADP ratio is state-dependent, in that ATP binds more readily to closed states of the $K_{\text{ATP}}$ channel (72, 73). Therefore, as the open probability decreases, due to PIP$_2$ hydrolysis upon cholesterol removal, $K_{\text{ATP}}$ channels will probably become more sensitive to ATP-mediated inhibition. Conversely, accumulation of PIP$_2$ in the setting of excess cholesterol will decrease ATP sensitivity by stabilizing open states of the channel and blunt $\beta$-cell membrane depolarization in response to glucose. This model is consistent with the finding that increased PIP$_2$ causes a reduction in GSIS by rendering $K_{\text{ATP}}$ channels insensitive to ATP (12).

Previous studies indicate that excess cholesterol inhibits GSIS (10, 11), yet the molecular mechanisms underlying this effect have not been studied extensively. Here, we show that cholesterol-overloaded $\beta$-cells are unresponsive to glucose stimulation in terms of PIP$_2$ hydrolysis, actin reorganization, and membrane depolarization. PIP$_2$ exerts profound effects on the sensitivity of $K_{\text{ATP}}$ channels to ATP, which can extend over orders of magnitude (53). Therefore, our results indicate how defective regulation of $K_{\text{ATP}}$ channel activity may result from excess cholesterol. This mechanism may be particularly important for the development of type 2 diabetes, because it suggests how increased cholesterol can act directly on $\beta$-cells to impair insulin secretion and disrupt glucose homeostasis.

Given the global presence and role of cholesterol in cellular functions, it is unlikely that any one mechanism is solely responsible for the observed effect in $\beta$-cells. However, it seems likely that altered intracellular Ca$^{2+}$ handling may be a final common pathway by which cholesterol modulates GSIS (11). Our data support the view that cholesterol depletion increases PIP$_2$ hydrolysis and IP$_3$ production, which leads to the release of Ca$^{2+}$ from intracellular stores. Simultaneously, reduced PM PIP$_2$ induces membrane depolarization by increasing the ATP sensitivity of $K_{\text{ATP}}$ channels, which then promote voltage-gated Ca$^{2+}$ influx. The elevation of [Ca$^{2+}$], is sufficient to trigger PLC activity, which hydrolyzes PIP$_2$ in pancreatic islets and insulinoma $\beta$-cells (74–76). The converse may occur in cholesterol-overloaded cells, lessening the intracellular Ca$^{2+}$ response to glucose stimulation (11).

Although the regulation of PIP$_2$ distribution and actin polymerization is cell type-dependent, the findings described here in $\beta$-cells may apply to other cells. The variability in the ATP sensitivity of $K_{\text{ATP}}$ channels in diverse tissues has been suggested to result from effects of PIP$_2$ (52). Our data further suggest that membrane cholesterol may modulate $K_{\text{ATP}}$ channel sensitivity (via PIP$_2$) in various tissues. In cardiomyocytes, for example, $K_{\text{ATP}}$ channels are activated despite the presence of inhibitory concentrations of ATP under physiological conditions (72). Whether membrane cholesterol regulates this response as well as similar responses in other cells will be the topic of future studies.

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