Original Article

Long-Term Exposure to Glucose and Lipids Inhibits Glucose-Induced Insulin Secretion Downstream of Granule Fusion With Plasma Membrane

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Mouse β-cells cultured at 15 mmol/l glucose for 72 h had reduced ATP-sensitive K^+ (K_{ATP}) channel activity (−30%), increased voltage-gated Ca^{2+} currents, higher intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$; +160%), more exocytosis (monitored by capacitance measurements, +100%), and greater insulin content (+230%) than those cultured at 4.5 mmol/l glucose. However, they released 20% less insulin when challenged with 20 mmol/l glucose. Glucose-induced (20 mmol/l) insulin secretion was reduced by 60–90% in islets cocultured at 4.5 or 15 mmol/l glucose and either oleate or palmitate (0.5 mmol/l). Free fatty acid (FFA)-induced inhibition of secretion was not associated with any major changes in $[Ca^{2+}]_i$ or islet ATP content. Palmitate stimulated exocytosis by twofold or more but reduced K^+-induced secretion by up to 60%. Basal (1 mmol/l glucose) K_{ATP} channel activity was 40% lower in islets cultured at 4.5 mmol/l glucose plus palmitate and 60% lower in islets cultured at 15 mmol/l glucose plus either of the FFAs. Insulin content decreased by 75% in islets exposed to FFAs in the presence of high (15 mmol/l), but not low (4.5 mmol/l), glucose concentrations, but the number of secretory granules was unchanged. FFA-induced inhibition of insulin secretion was not associated with increased transcript levels of the apoptosis markers Bax (*BclII*-associated X protein) and caspase-3. We conclude that glucose and FFAs reduce insulin secretion by interference with the exit of insulin via the fusion pore. *Diabetes* 56:1888–1897, 2007

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$[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; Cpt, carnitin palmitoyl transferase; FFA, free fatty acid; K_{ATP} channel, ATP-sensitive K^+ channel; UCP, uncoupling protein.

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Type 2 diabetes develops as a result of impaired β-cell function and is closely associated with increased plasma free fatty acid (FFA) concentrations (1,2). Whereas FFAs enhance the β-cell response on acute application (3–7), long-term exposure exerts lipotoxic effects and leads to blunted glucose-stimulated insulin secretion and decreased cell viability (1,8–11). Moderate increases in plasma glucose have also been reported to have adverse effects on β-cell function ("glucotoxicity") (12). The combination of FFAs and high glucose has been suggested to be particularly deleterious (13,14), and the term "glucolipotoxicity" has been coined to describe the phenomenon (15).

Pancreatic β-cells are electrically excitable, and changes in membrane potential link variations of the blood glucose concentration to increases or decreases in insulin secretion. A consensus model for the regulation of insulin secretion by glucose postulates that ATP-sensitive K^+ (K_{ATP}) channel activity maintains a negative (approximately −60 mV) β-cell membrane potential at substimulatory glucose concentrations and so prevents electrical activity and insulin secretion. Elevation of glucose stimulates β-cell glucose uptake and metabolism. The resultant increase in ATP and decrease in MgADP closes K_{ATP} channels. This leads to membrane depolarization, opening of voltage-gated Ca^{2+} channels, elevation of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), and ultimately exocytosis of insulin-containing secretory granules (16).

The exact mechanisms by which long-term exposure to lipids impairs insulin secretion have not been fully established. Previous work indicates that FFAs and their metabolites have multiple effects on β-cell function. These include changes in gene expression (1), metabolism (17), mitochondrial function (18) (perhaps via activation and/or increased expression of the uncoupling protein 2 [UCP-2]) (19–21), channel activity (22–24), and exocytosis (25). Here, we have used a combination of insulin release measurements, electron microscopy, $[Ca^{2+}]_i$ recordings, electrophysiology, and quantitative PCR to establish which step in β-cell stimulus-secretion coupling is impaired during long-term exposure to lipids.

RESEARCH DESIGN AND METHODS

Pancreatic islets were isolated from NMRI mice by collagenase digestion as previously reported (7). Palmitate and oleate were prepared in solutions bound to fatty acid–free BSA (7). Islets were cultured in RPMI 1640 containing 4.5 or 15 mmol/l glucose supplemented, or not, with 0.5 mmol/l palmitate or

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TABLE 1
Effects of long-term culture in high glucose and lipids on insulin content and secretion

Culture condition (72 h)	Insulin content (ng/islet)		Insulin secretion (ng/islet per h) during 1-h incubation							
			1 mmol/l glucose (A)		20 mmol/l glucose (B)		B–A		20 mmol/l glucose + 0.1 mmol/l tolbutamide (C)	
	Means	n	Means	n	Means	n	Means	n	Means	n
4.5 mmol/l glucose	59 ± 6	4	0.28 ± 0.05	4	2.98 ± 0.30*	4	2.70 ± 0.15	4	ND	—
4.5 mmol/l glucose + 0.5 mmol/l oleate	56 ± 9	4	0.55 ± 0.05†	4	0.91 ± 0.14‡	4	0.36 ± 0.07§	4	ND	—
4.5 mmol/l glucose + 0.5 mmol/l palmitate	70 ± 12	4	0.51 ± 0.02†	4	0.80 ± 0.09‡	4	0.29 ± 0.05§	4	ND	—
15 mmol/l glucose	198 ± 38	4†	0.49 ± 0.03	8†	2.36 ± 0.26*	8	1.89 ± 0.09§	8	3.13 ± 0.29‖	8
15 mmol/l glucose + oleate	59 ± 4	4¶	0.50 ± 0.03	8	0.99 ± 0.07*	8	0.49 ± 0.03#	8	1.18 ± 0.16	8
15 mmol/l glucose + palmitate	48 ± 8	4¶	0.52 ± 0.03	8	1.12 ± 0.11*	8	0.60 ± 0.04#	8	1.22 ± 0.17	8

*$P < 0.001$ vs. 1 mmol/l glucose; †$P < 0.001$ vs. line 1; ‡$P < 0.05$ vs. 1 mmol/l glucose; §$P < 0.001$ vs. line 1 same column; ‖$P < 0.001$ vs. line 4 same column; ¶$P < 0.001$ vs. line 4 same column; #$P < 0.001$ vs. line 4 same column. ND, not determined.

oleate for ~72 h. BSA was present at a concentration of 1% in all culture media. Using the stepwise equilibrium method (26), the free concentrations of oleate and palmitate were estimated to be 44 and 26 nmol/l, respectively. Insulin release, ATP content, mRNA levels, electron microscopy, and $[Ca^{2+}]_i$ were measured in intact islets, whereas single cells were used for the electrophysiological measurements. In the latter experiments, islets were first cultured for 48 h as outlined above. They were then dissociated into single cells, and the cell suspension was plated in Petri dishes and incubated for another 24 h in the same medium as that used before. Some experiments were performed on islets isolated from mice fed control and high-fat diets (5 and 40% fat, respectively).

Ca^{2+} currents and cell capacitance were recorded using a standard whole-cell patch-clamp technique and Cs-filled electrodes containing 50 μmol/l EGTA (27). Changes in resting conductance and membrane potential were monitored using the perforated patch whole-cell configuration with K_2SO_4-filled electrodes (28). $[Ca^{2+}]_i$ was measured by microfluorimetry as outlined previously (7); confocal imaging confirmed that the whole-islet glucose-induced increases in $[Ca^{2+}]_i$ reflect the behavior of the β-cells with minimal contribution by non–β-cells. Insulin secretion was measured in Krebs-Ringer bicarbonate solution using in-house assays (7). For electron microscopy, islets were processed and analyzed as detailed previously (28). ATP was measured as previously described (29). Complete methods, including a description of the procedures for RNA isolation and quantitative RT-PCR, are provided in the supplementary material, which can be found in an online appendix (available at http://dx.doi.org/10.2337/db06–1150). All data are the means ± SE for the indicated (n) number of experiments. Statistical significances were evaluated using Student's t test or two-way ANOVA followed by a Bonferroni test.

RESULTS

Long-term exposure to lipids reduces insulin content and secretion. Islets cultured at 4.5 mmol/l glucose for 72 h exhibited low basal (1 mmol/l glucose) insulin secretion and responded with a >11-fold stimulation when challenged with 20 mmol/l glucose (Table 1). Inclusion of oleate or palmitate in the culture medium doubled basal insulin secretion but inhibited that evoked by 20 mmol/l glucose by >85%.

Islets cultured in the presence of 15 mmol/l glucose had more than twofold higher basal secretion than those cultured at 4.5 mmol/l glucose (Table 1). Stimulation with 20 mmol/l glucose produced a fivefold enhancement of secretion in islets cultured in 15 mmol/l glucose alone, significantly less than that found for islets cultured in low glucose. The secretory response to 20 mmol/l glucose was reduced by ~65–75% in islets cultured in 15 mmol/l glucose plus oleate or palmitate. Although the K_{ATP} channel blocker tolbutamide (100 μmol/l) enhanced glucose-induced secretion by ~30% in islets cultured at 15 mmol/l glucose alone, it failed to stimulate insulin secretion beyond that evoked by 20 mmol/l glucose alone in islets cultured with 15 mmol/l glucose and either of the FFAs.

Islet insulin content was 3.4-fold greater in islets cultured at 15 mmol/l glucose than at 4.5 mmol/l glucose. Insulin content was reduced by 60–70% when oleate or palmitate was included in the high-glucose culture medium, whereas it was only marginally affected when the FFAs were added to the low-glucose culture medium.

Insulin secretion in the presence of 20 mmol/l glucose expressed as a percentage of insulin content amounted to 5.1 ± 0.6, 1.6 ± 0.3, and 1.1 ± 0.2% in islets cultured at 4.5 mmol/l glucose with no lipid, oleate, and palmitate, respectively. Corresponding values for islets cultured at 15 mmol/l glucose were 1.2 ± 0.2, 1.7 ± 0.1, and 2.3 ± 0.3%.

FFAs elevate basal $[Ca^{2+}]_i$ but only marginally affect glucose-stimulated $[Ca^{2+}]_i$ increases. In islets cultured at 4.5 mmol/l glucose (Fig. 1*A*), increasing glucose from 1 to 5 mmol/l and then 15 mmol/l had dual effects on $[Ca^{2+}]_i$: 5 mmol/l produced a transient reduction in $[Ca^{2+}]_i$, whereas 15 mmol/l produced an initial peak in $[Ca^{2+}]_i$ followed by a series of oscillations. Tolbutamide (100 μmol/l) induced a rapid peak in $[Ca^{2+}]_i$ followed by a maintained plateau of a magnitude greater than the $[Ca^{2+}]_i$ peak produced by 15 mmol/l glucose alone. Responses to glucose and tolbutamide were identical in islets cultured at 4.5 mmol/l glucose and palmitate (Fig. 1*B*) or oleate (not shown).

Islet β-cells cultured in 15 mmol/l glucose alone exhibited an enhanced responsiveness to glucose. Basal $[Ca^{2+}]_i$, measured in 1 mmol/l glucose, was ~35 nmol/l higher than that seen in islets cultured at 4.5 mmol/l glucose. Elevation of glucose to 5 mmol/l initiated fast $[Ca^{2+}]_i$ oscillations (Fig. 1*C*). When glucose was increased to 15 mmol/l, $[Ca^{2+}]_i$ increased to a stable (nonoscillating) plateau, and inclusion of tolbutamide in the perfusion medium had only a marginal (10%) additional effect.

In islets cultured in high glucose and palmitate for 72 h, basal $[Ca^{2+}]_i$ was elevated by >100 nmol/l compared with that measured in islets cultured at high glucose alone (Fig. 1*D*), but the responses to glucose (5 and 15 mmol/l) and tolbutamide were similar to those observed in islets cultured at 15 mmol/l glucose alone. The observed increase in basal $[Ca^{2+}]_i$ was promptly reversed by diazoxide (not

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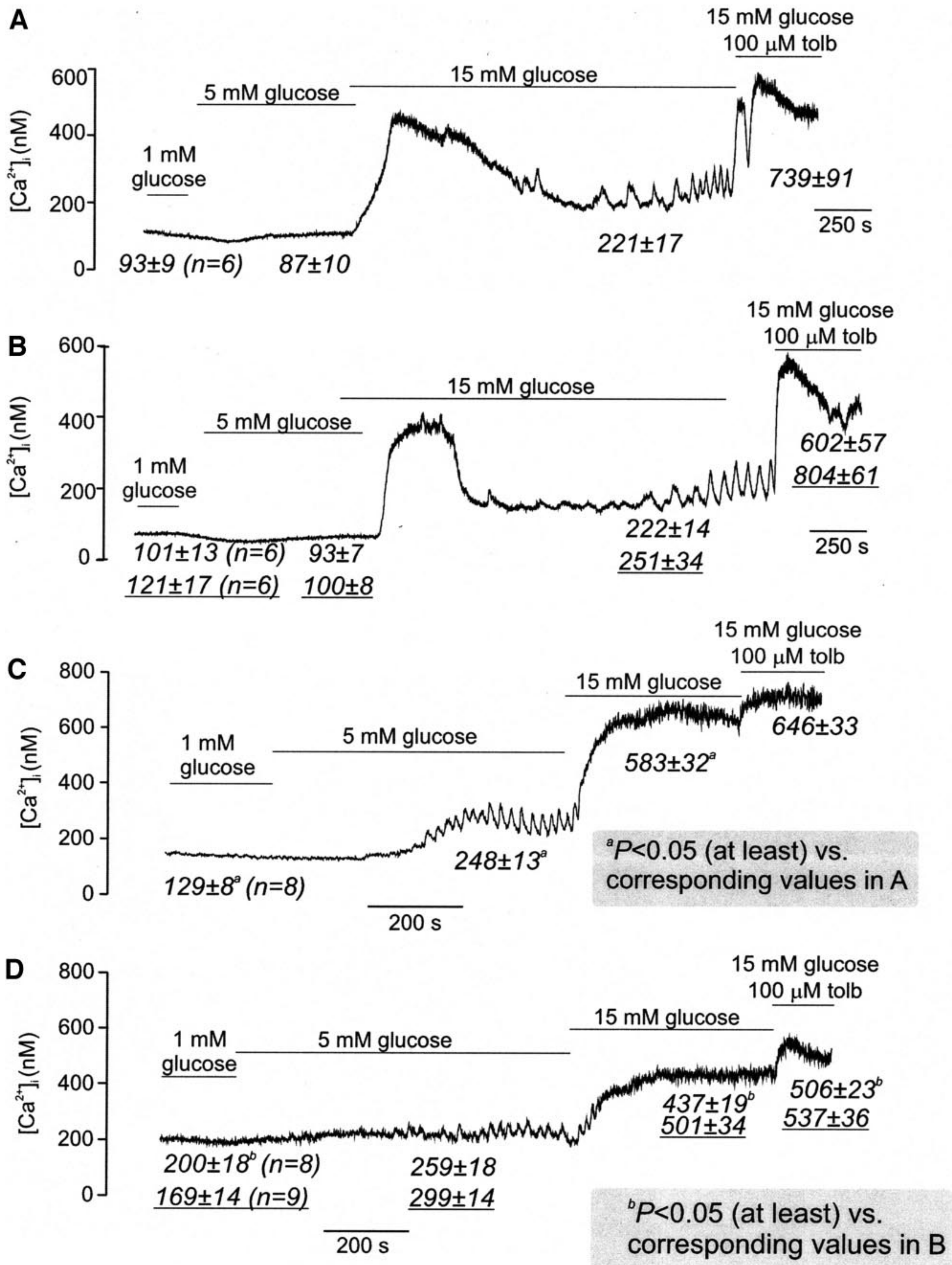

FIG. 1. ***A–D*: Changes in $[Ca^{2+}]_i$ on addition of glucose (5–15 mmol/l) or 100 μmol/l tolbutamide (tolb) in islets cultured as indicated (underlined). *A*: 4.5 mmol/l glucose. *B*: 4.5 mmol/l glucose plus 0.5 mmol/l palmitate. *C*: 15 mmol/l glucose. *D*: 15 mmol/l glucose plus 0.5 mmol/l palmitate. Average $[Ca^{2+}]_i$ levels are given above/below representative traces. Underlined values in *B* and *D* were obtained with oleate instead of palmitate. Statistical significances are indicated in gray areas.**

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shown). Long-term culture in high glucose and oleate produced similar effects (not shown).

Effects of high-fat feeding on insulin secretion, insulin content, and $[Ca^{2+}]_i$. Mice fed a high-fat diet for 15 weeks exhibited fasting hyperglycemia (9.9 vs. 4.5 mmol/l for mice fed a control diet, $P < 0.001$). Insulin content in islets isolated from mice fed the high-fat diet was 99 ± 16% (n = 5) of that found in mice fed the control diet. Nevertheless, insulin secretion evoked by glucose (20 mmol/l) or tolbutamide (0.1 mmol/l) was reduced by 64 ± 7 and 50 ± 11% (n = 5), respectively. Although islets from control mice exhibited $[Ca^{2+}]_i$ oscillations in the presence of 20 mmol/l glucose, islets from mice fed the high-fat diet responded to this glucose concentration with a more sustained elevation (S.C., R. Ramracheya, A.A. Toye, J. Fearnside, K. Pinnick, D. Gauguier, A. Clark, P.R., unpublished data).

Whole-cell K_{ATP} channel conductance is decreased in FFA-pretreated β-cells. Perforated patch whole-cell patch-clamp recording, which preserves cell metabolism, was used to examine the effects of long-term exposure to lipids on K_{ATP} channel activity. The membrane potential was ramped between −110 and 0 mV to estimate the slope conductance (G) of the β-cell; G was measured over the linear part of the current-voltage relationship between −100 and −50 mV (Fig. 2*A*) and normalized to cell capacitance (C_m) to correct for differences in cell size. In the presence of 1 mmol/l glucose, G/C_m averaged ~0.35 nS/pF for β-cells cultured at 15 mmol/l glucose (Fig. 2*A* and *C*) but was 60–75% lower for β-cells cultured in 15 mmol/l glucose plus either palmitate (Fig. 2*B* and *C*) or oleate (not shown). Elevation of extracellular glucose to 20 mmol/l reduced G/C_m in all three groups of cells ($P < 0.001$), and no further reduction was detected when 100 μmol/l tolbutamide was added to 20 mmol/l glucose solution. In cells cultured at 4.5 mmol/l glucose, the resting conductance at 1 mmol/l glucose averaged 0.45 nS/pF, which decreased by ~40% when the islets were cultured in 4.5 mmol/l glucose plus palmitate. The ability of 20 mmol/l glucose or tolbutamide to block K_{ATP} channel activity was unchanged by palmitate (Fig. 2*D*).

Whole-cell K_{ATP} conductance was measured in the standard whole-cell configuration with intracellular solutions containing 0.3 mmol/l ADP and 0.3 mmol/l ATP to maximally activate the K_{ATP} channel. G/C_m averaged 1.3 ± 0.1, 1.1 ± 0.2, and 1.6 ± 0.2 nS/pF (n = >8) in β-cells cultured in 15 mmol/l glucose alone or in combination with oleate or palmitate, respectively.

Effects of glucose and FFAs on islet ATP content. Glucose stimulation produced a concentration-dependent increase in ATP in islets cultured at 4.5 mmol/l glucose (Fig. 3*A*). Inclusion of palmitate and oleate in the 4.5 mmol/l glucose culture medium doubled the ATP content measured at 1 mmol/l glucose. Glucose (5–20 mmol/l) increased ATP content in both control islets and those exposed to oleate, whereas no further increase was seen in islets exposed to palmitate.

In islets cultured at 15 mmol/l glucose with or without oleate, ATP levels measured at 1 mmol/l glucose were ~100% higher than those observed in islets cultured at 4.5 mmol/l glucose, and there was no further increase at higher glucose concentrations (Fig. 3*B*). Culture in 15 mmol/l glucose plus palmitate lowered basal ATP content (measured at 1 mmol/l glucose). However, there was no difference in ATP content at 5 and 20 mmol/l glucose compared with control islets.

TABLE 2
Fold changes in islet mRNA levels after long-term incubation with glucose and lipids

Culture condition	Snap25a		Chgb		Cpt1		GLUT2		Ins1		Syt4l/Slp4	
	Means	n	Means	n	Means	n	Means	n	Means	n	Means	n
15 mmol/l glucose	1.8 ± 0.24*	4	4.6 ± 0.88*	4	0.91 ± 0.14	5	8.7 ± 1.62*	4	20.5 ± 5.05*	5	4.0 ± 0.6*	4
0.5 mmol/l palmitate + 15 mmol/l glucose	1.0 ± 0.08	5	1.4 ± 0.14*	5	2.5 ± 0.19†	6	0.68 ± 0.05†	6	0.77 ± 0.04‡	5	0.82 ± 0.04*	4
0.5 mmol/l oleate + 15 mmol/l glucose	0.88 ± 0.12	4	1.5 ± 0.06*	4	3.2 ± 0.32‡	4	0.49 ± 0.14*	6	0.79 ± 0.06*	6	0.93 ± 0.20	4
0.5 palmitate + 4.5 mmol/l glucose	0.93 ± 0.06	6	1.1 ± 0.1	6	2.6 ± 0.11†	5	0.87 ± 0.14	5	1.8 ± 0.26*	5	1.1 ± 0.05	6
0.5 mmol/l oleate + 4.5 mmol/l glucose	1.0 ± 0.09	6	1.2 ± 0.12	6	2.6 ± 0.24‡	5	0.67 ± 0.2	5	1.6 ± 0.21	4	1.1 ± 0.1	6

Data are the ratios of mRNA levels relative those measured at the respective glucose concentration. The effects of high glucose are normalized to expression in islets cultured at 4.5 mmol/l glucose. *$P < 0.05$, ‡$P < 0.01$, and †$P < 0.001$ vs. FFA-free medium containing 4.5 or 15 mmol/l glucose. Chgb, granule protein chromogranin B; Cpt1, carnitin palmitoyl transferase-1; Ins1, insulin gene 1; Snap25a, synaptosomal-associated protein 25, isoform a; Syt4l/Slp4 synaptotagmin-like protein 4.

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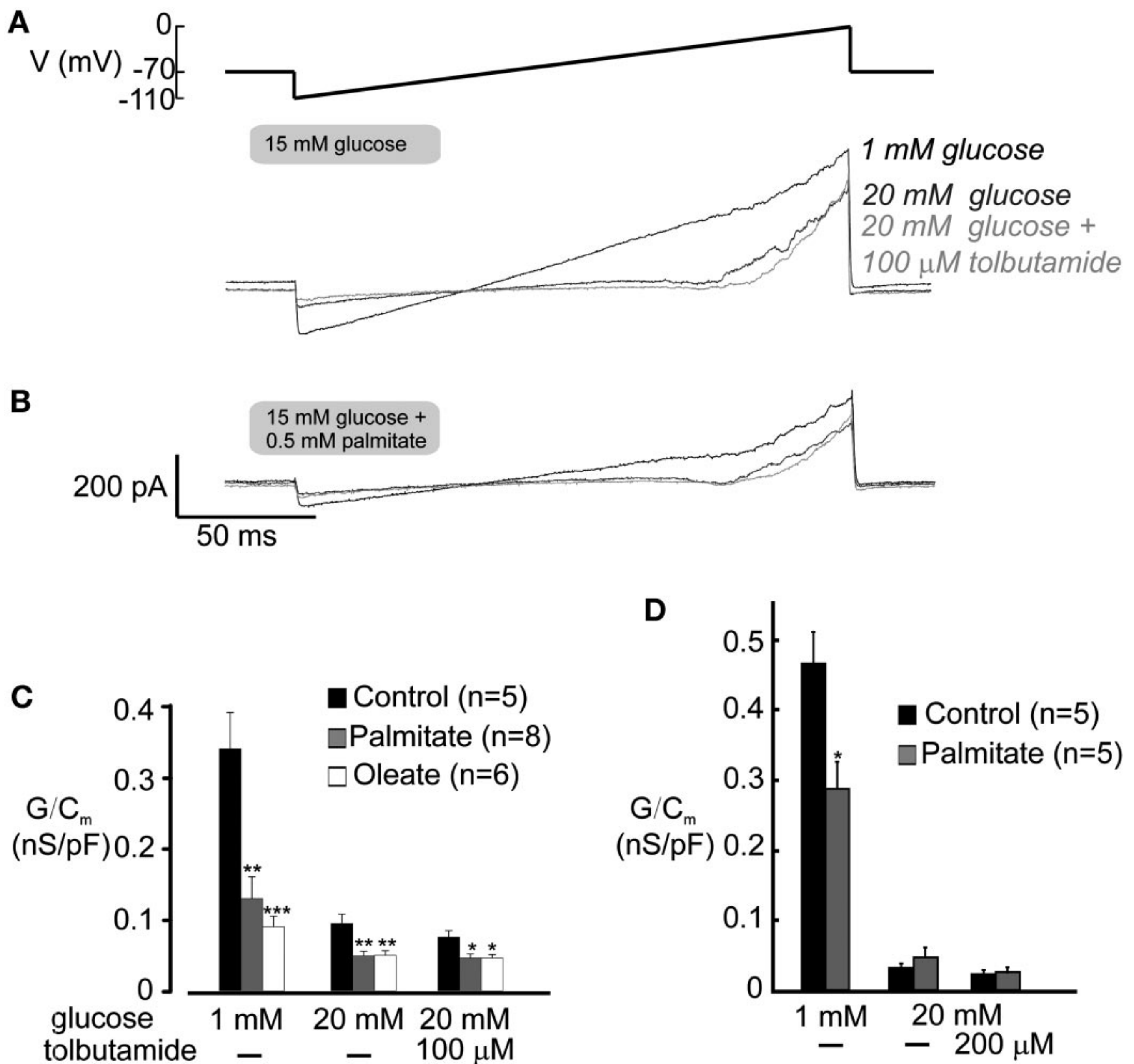

FIG. 2. *A* and *B*: Current ramps recorded from isolated β-cells cultured in 15 mmol/l glucose (*A*) or 15 mmol/l glucose plus 0.5 palmitate (*B*) and then exposed to the experimental conditions indicated. *C–D*: Slope conductance (G) normalized to cell capacitance (C_m) under the indicated experimental in islets cultured at 15 mmol/l (*C*) or 4.5 mmol/l glucose. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

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Effects of palmitate and oleate on membrane potential. In the presence of 1 mmol/l glucose, the membrane potential averaged −66 ± 2 mV (n = 13), −56 ± 5 mV (n = 9), and −56 ± 6 mV (n = 12) for cells cultured at 15 mmol/l glucose alone or in combination with palmitate or oleate, respectively. One-third of cells (7 of 21) that had been exposed to FFAs generated action potentials at 1 mmol/l glucose (Fig. 4*B* and *C*), a feature never observed in control cells (n = 13) (Fig. 4*A*). Cells from all three groups invariably fired action potentials when exposed to 20 mmol/l glucose. In islets cultured at 4.5 mmol/l glucose, or 4.5 mmol/l glucose plus 0.5 mmol/l palmitate, the membrane potential measured in 1 mmol/l glucose averaged −58 ± 4 mV (n = 5) and −60 ± 2 mV (n = 5), respectively (not shown).

Ca^{2+} currents and exocytosis are unperturbed by culture with FFAs. Long-term exposure to palmitate in the presence of 4.5 or 15 mmol/l glucose also interfered with insulin secretion evoked by 75 mmol/l extracellular K^+ (Fig. 5*A*). Palmitate-induced inhibition of secretion ranged between 30% in islets cultured at 15 mmol/l glucose to 60% in islets cultured at low glucose. High K^+ was an approximately threefold stronger stimulus of insulin secretion in islets cultured at 15 mmol/l glucose than in those maintained at 4.5 mmol/l.

The peak Ca^{2+} current in β-cells from islets cultured at 4.5 and 15 mmol/l glucose averaged 45 ± 9 pA (n = 8) and 105 ± 13 pA (n = 13; $P < 0.001$), respectively. Inclusion of oleate in the culture medium was without effect at both glucose concentrations (not shown), but the peak current

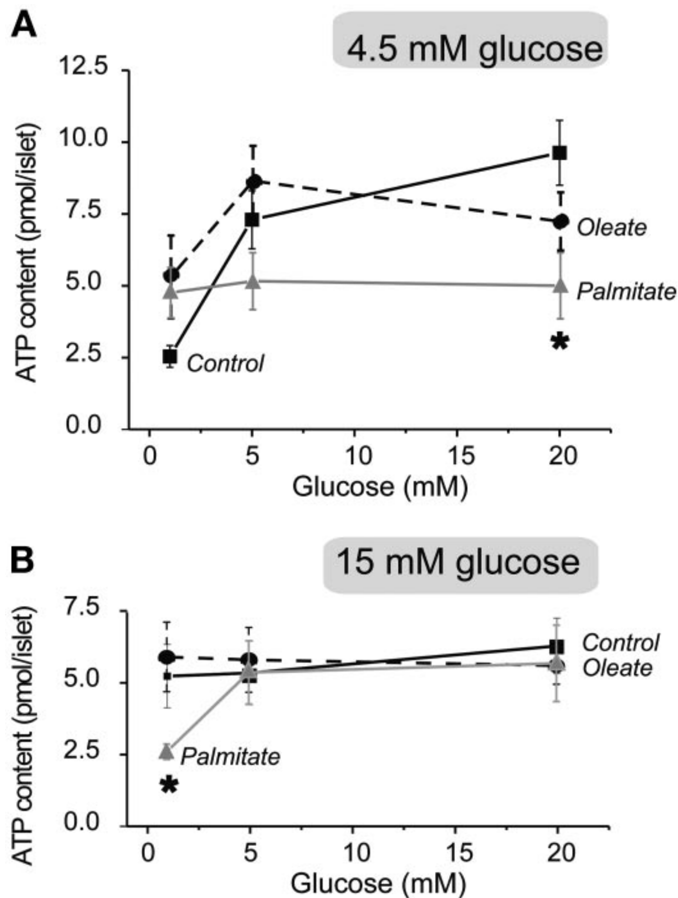

FIG. 3. ***A*** **and** ***B*****: Islet ATP content in islets cultured in the absence (■) or presence of 0.5 mmol/l oleate (●) or palmitate (△) at 4.5 mmol/l (*A*) or 15 mmol/l glucose (*B*) after a 1-h incubation in the presence of 1, 5, and 20 mmol/l glucose. *P < 0.05 and **P < 0.01 vs. control (same glucose concentration).**

increased to 109 ± 22 pA (n = 13, $P < 0.02$ vs. 4.5 mmol/l glucose alone) and 155 ± 19 pA (n = 12, $P < 0.05$ vs. 15 mmol/l glucose alone) when palmitate was added (Fig. 5*B*). Neither glucose nor palmitate affected the voltage dependence of the current (not shown).

We also studied the effects of oleate and palmitate on transmembrane Ca^{2+} fluxes by measuring the $[Ca^{2+}]_i$ increases elicited by 75 mmol/l extracellular K^+, which depolarizes the β-cell to approximately −10 mV (28). Long-term culture with palmitate in the presence of 15 mmol/l glucose (Fig. 5*C*) or oleate (not shown) had no effect on the depolarization-evoked transient $[Ca^{2+}]_i$.

Capacitance measurements were used to study the effects of the FFAs on exocytosis. The stimulus consisted of trains of 10 500-ms depolarizing pulses to 0 mV from −70 mV (Fig. 5*D*). In cells cultured at 4.5 mmol/l glucose, the increase in capacitance evoked by the train averaged ~230 fF (Fig. 5*E*); this increased 2.2-fold when the islets were cultured at 15 mmol/l glucose. Long-term culture in oleate and either 4.5 or 15 mmol/l glucose was without effect on exocytosis (not shown). By contrast, palmitate stimulated exocytosis 2.7-fold ($P < 0.05$) and 2.1-fold ($P < 0.05$) over that seen in the presence of 4.5 or 15 mmol/l glucose alone (Fig. 5*E–F*).

Ultrastructural changes in β-cells cultured at high glucose with or without palmitate or oleate. Figure 6*A*–C shows electron micrographs of β-cells in intact pancreatic islets after culture at 15 mmol/l glucose in the absence or presence of palmitate or oleate. Large lipid droplets were observed in the cytoplasm of β-cells exposed to oleate. In cells cultured with palmitate, crescent-like structures, reminiscent of the angular vacuoles documented in Ins-1 cells (30), were occasionally seen.

Islets cultured at 4.5 mmol/l glucose contained ~15,000 secretory granules, of which 1,100 were docked with the plasma membrane (Fig. 6*D*). Both the docked and the total number of granules were reduced by ~20% by culture at 15 mmol/l glucose, and the FFAs had no additional effect. Glucose and FFAs were also without effect on granule diameter. The diameter of the central (insulin-containing) dense core averaged ~55% in all groups except in β-cells exposed to palmitate, where it fell to 45% (not shown).

Effects of glucose and FFAs on islet mRNA content. The amounts of mRNA encoding key proteins were evaluated by quantitative RT-PCR (Table 2). Long-term exposure to 15 mmol/l glucose increased the mRNAs for Snap25a (synaptosomal-associated protein 25, isoform a; a protein involved in exocytosis), Chgb (the granule protein chromogranin B) (31), GLUT2, Ins1 (insulin gene 1), and granuphilin (Syt4l/Slp4 [synaptotagmin-like protein 4], a protein involved in exocytosis) 1.8- to >20-fold relative to that seen in islets exposed to 4.5 mmol/l glucose. The effects on Ins1, Syt4l/Slp4, and GLUT2 transcription were partially antagonized by oleate and/or palmitate, whereas high glucose and the FFAs acted synergistically on the transcription of Chgb. The FFAs also increased transcription of carnitin palmitoyl transferase-1 (Cpt-1) at both low and high glucose. Transcript levels for the genes encoding hexokinase-1, UCP-2, Kir6.2, SUR1, Cacna1c (α1C Ca^{2+} channel subunit), syntaxin 1a, Bax (*Bcl*II-associated X protein), and caspase-3 (the latter two being markers of

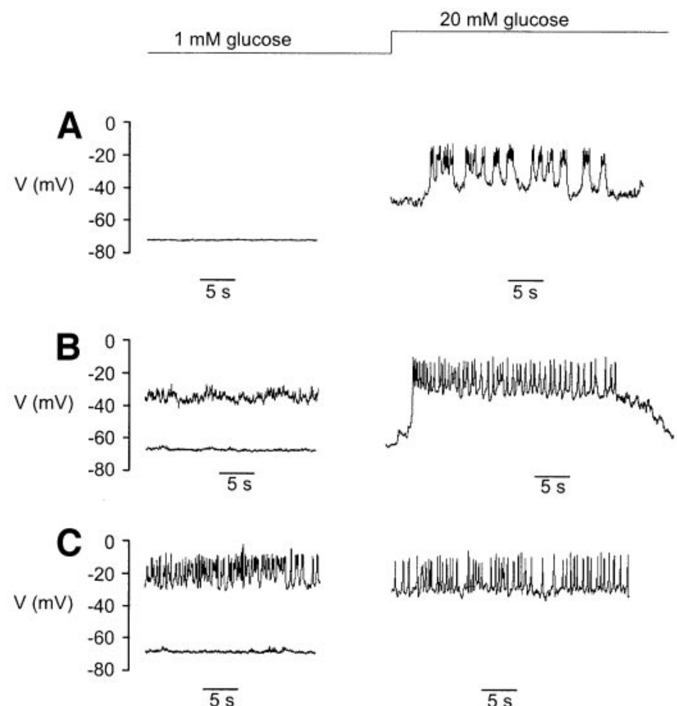

FIG. 4. ***A–C*****: Membrane potential in β-cells cultured in 15 mmol/l glucose (*A*), 15 mmol/l glucose plus 0.5 mmol/l palmitate (*B*), or 15 mmol/l glucose plus 0.5 mmol/l oleate (*C*) when exposed to 1 or 20 mmol/l glucose as indicated. For the FFA groups, examples of both electrically silent cells (*lower traces*) and cells generating action potentials (*upper traces*) at 1 mmol/l glucose are shown.**

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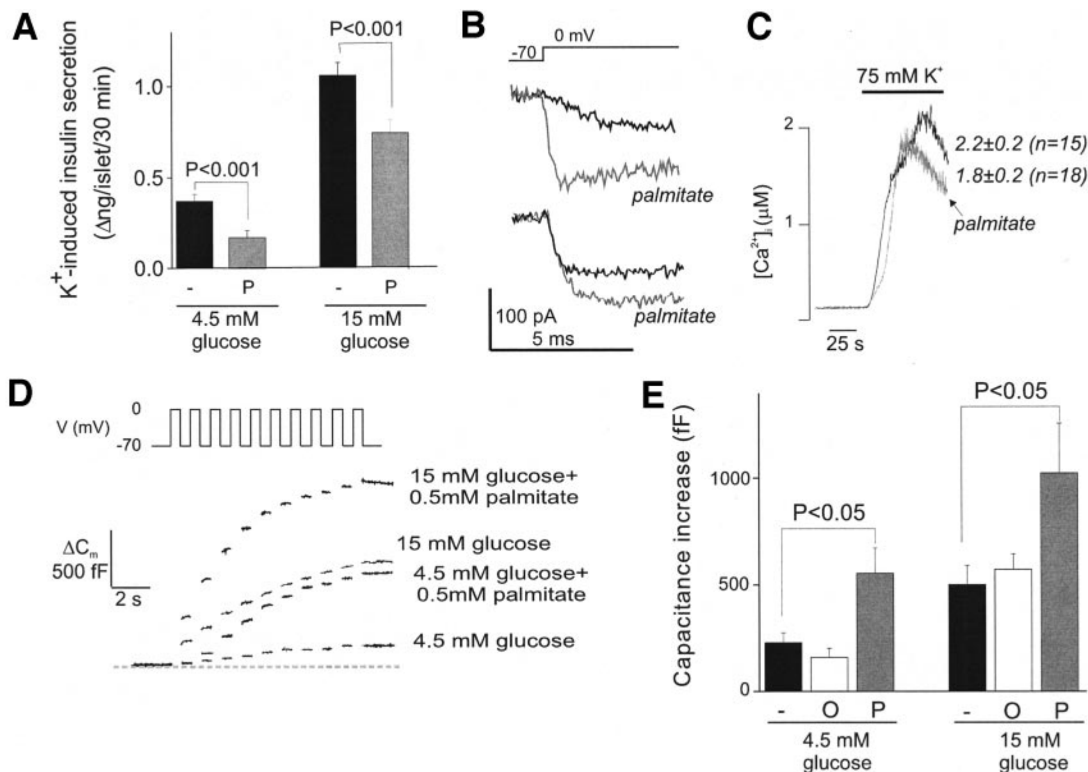

FIG. 5. *A*: Net K^+-induced insulin secretion observed after culture at 4.5 and 15 mmol/l glucose and in the absence (-) and presence of palmitate (P) as indicated ($n = 10$). *B*: Voltage-gated Ca^{2+} currents measured in cells cultured in the absence and presence of palmitate (P) at 4.5 mmol/l (top) or 15 mmol/l (bottom) glucose. *C*: $[Ca^{2+}]_i$ in intact islets cultured as indicated before and after depolarization with 75 mmol/l K^+. *D*: Depolarization-elicited capacitance increases (ΔC_m) evoked by a train of depolarization (V; indicated schematically above capacitance trace) in β-cells cultured as stated to the right. *E*: Total increases in cell capacitance evoked by trains of depolarization (see panel *E*) after culture at 4.5 and 15 mmol/l glucose and in the absence (-) and presence of oleate (O) and palmitate (P) as indicated ($n = 4$–15).

apoptosis) were unaffected by glucose and the FFAs (not shown).

DISCUSSION

We have investigated the changes in β-cell function that develop during long-term exposure of islets to high glucose or lipids to determine why glucose-induced insulin secretion is suppressed.

Effects of high glucose. Islets cultured at 4.5 mmol/l glucose had low basal insulin secretion and responded with an 11-fold enhancement of secretion when stimulated with 15 mmol/l glucose. β-Cells cultured at 4.5 mmol/l glucose were well granulated with many docked granules. Increasing glucose from 5 to 15 mmol/l evoked a biphasic increase in $[Ca^{2+}]_i$ similar to that observed in freshly isolated islets (28).

Long-term culture at high glucose (15 mmol/l) had multiple effects. First, the resting K_{ATP} conductance was reduced by ~30% relative to that in islets cultured at 4.5 mmol/l glucose (Fig. 2*D*). Second, basal $[Ca^{2+}]_i$ (i.e., that measured at 1 mmol/l glucose) was slightly elevated and basal insulin secretion approximately doubled. Unlike islets cultured at 4.5 mmol/l glucose, those cultured at 15 mmol/l glucose subsequently responded to 5 mmol/l glucose with $[Ca^{2+}]_i$ oscillations of the type observed at glucose concentrations of ≥10 mmol/l in freshly isolated islets. Furthermore, the steady-state $[Ca^{2+}]_i$ measured at 15 mmol/l glucose was 2.6-fold greater than that seen for islets cultured at 4.5 mmol/l glucose (580 vs. 220 nmol/l) (Fig. 4*A* and *B*). Collectively, these findings are consistent with the idea, as previously reported for rat islets (32,33), that long-term exposure of β-cells shifts the threshold for insulin secretion and increased $[Ca^{2+}]_i$ to lower glucose concentrations. Our data suggests that these effects principally occur via reduced K_{ATP} channel activity. The increased magnitude of the voltage-gated Ca^{2+} current might also contribute to an increased excitability. The latter effect was not correlated with increased Cacna1c mRNA levels, indicating the effect involves metabolic regulation rather than increased channel density (34).

Measurements of intracellular ATP indicated that basal ATP levels (measured at 1 mmol/l glucose) were twofold higher in islets cultured at 15 mmol/l glucose than in islets maintained at 4.5 mmol/l glucose. That no further increase in ATP content was observed in response to stimulation with 20 mmol/l glucose also suggests that basal metabolism under these conditions is close to the maximal rate. It may seem surprising that ATP levels measured in islets cultured at 15 mmol/l glucose and subsequently exposed to 20 mmol/l glucose were lower than in islets cultured at 4.5 mmol/l glucose. However, it has been shown that total ATP levels are influenced both by the culture condition and by the amount of insulin (and thus ATP) secreted (35). This is likely because insulin granules contain high concentrations of ATP. Thus, it may be difficult to compare the absolute ATP content obtained after culture in different media.

Although insulin gene transcription was enhanced 20-fold when the glucose concentration of the culture medium was increased from 4.5 to 15 mmol/l, the effect on insulin content was limited to a fourfold increase. This discrepancy is most likely caused by enhanced insulin

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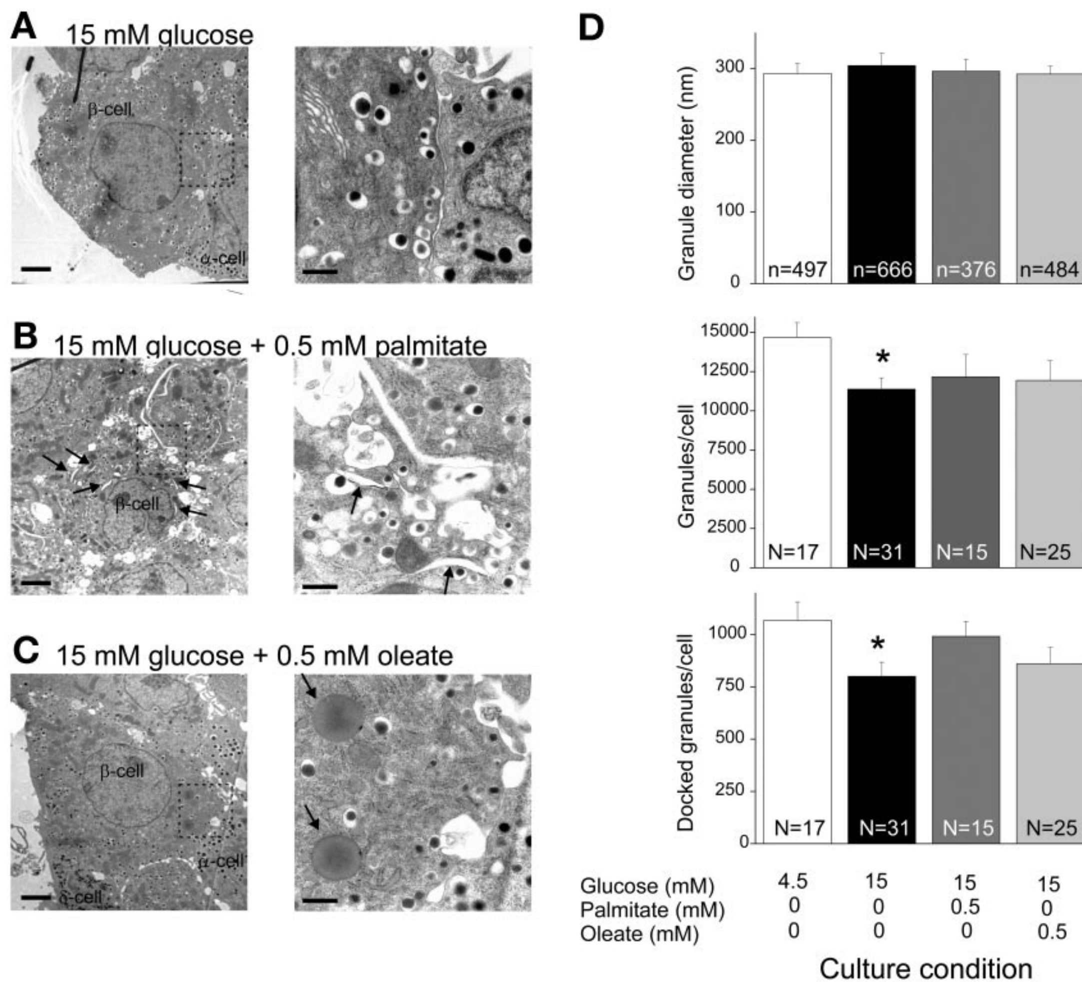

FIG. 6. ***A–C*****: Electron micrographs of β-cells in islets cultured 15 mmol/l glucose (*****A*****), 15 mmol/l glucose plus 0.5 mmol/l palmitate (*****B*****), or 15 mmol/l glucose plus 0.5 mmol/l oleate (*****C*****). Arrows in *****B***** and *****C***** highlight lipid accumulation. The areas indicated to the left are shown at a greater magnification to the right. Scale bars are 2 μm (*****left*****) and 500 nm (*****right*****).** ***D*****: Summary of effects of glucose and FFAs on granule diameter and granule number (total and docked) as indicated. The indicated number of granules (n) or cells (N) in 4–7 islets from two animals were analyzed. *$P < 0.05$ vs. 4.5 mmol/l glucose.**

release into the culture medium. Tonic stimulation of insulin secretion during the 72-h culture period can also explain the 20% decrease in the total number of granules. Nevertheless, exocytosis (measured as increases in cell capacitance or insulin secretion evoked by stimulation with high K^+) was 2.2- to 2.8-fold higher in islets cultured at high glucose than in islets maintained at the lower glucose concentration. Both the increased magnitude of the voltage-gated Ca^{2+} current and increased expression of some (e.g., Snap25a) exocytotic proteins may contribute to this effect. That K^+-evoked secretion was higher in islets cultured at 15 mmol/l glucose may be a result of elevated basal ATP levels (36) (Fig. 2*E*).

Given that so many critical parameters are enhanced in islets cultured at high glucose, it is surprising that the insulin secretory capacity of islets cultured at 15 mmol/l glucose was not much higher than that of islets cultured at 4.5 mmol/l glucose. Rather, their response to glucose was 20% smaller (Table 1), and insulin secretion normalized to insulin content fell from ~5 to 1%. This paradox can be explained by the recent report that the fraction of kiss-and-run exocytotic events (i.e., those that are not associated with release of the peptide cargo) is dramatically increased at the expense of full fusions in rat β-cells cultured in the presence of 30 mmol/l glucose for 48 h (33). The observed 25% reduction of glucose-induced insulin secretion is consistent with the idea that long-term exposure to elevated glucose results in a large fraction of the release events being aborted before the exit of insulin. In this context it may be relevant that glucose increased granuphilin (Syt4l/Slp4) transcript levels fourfold; overexpression of granuphilin has been reported to inhibit insulin secretion (37–39).

Effects of FFAs. Oleate and palmitate were strong inhibitors of glucose-induced insulin secretion, regardless of whether the islets were cultured at 4.5 and 15 mmol/l glucose. Long-chain acyl-CoAs are powerful modulators of K_{ATP} channel gating and increase channel activity in isolated membrane patches by reducing the inhibitory effect of ATP (23,40–42). If the inhibition of insulin secretion in islets cultured in the presence of lipids were secondary to intracellular accumulation of long-chain acyl-CoAs and activation of K_{ATP} channels, then it should result in an increased resting conductance and β-cell membrane hyperpolarization. No such increase in resting K_{ATP} channel activity was observed (if anything, it was reduced) and there was no impairment of the ability of glucose to reduce the resting K^+ conductance (Fig. 2*C–D*). We therefore conclude that lipid-induced overactivity of K_{ATP} channels does not explain the suppression of glucose-induced insu-

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lin secretion. The finding that $[Ca^{2+}]_i$ signaling was unperturbed by culture in the presence of the lipids (Fig. 1) rather argues that the defect lies downstream of K_{ATP} channel closure. This conclusion is underscored by the finding that tolbutamide- or high-K^+–evoked insulin secretion was also suppressed by the FFAs.

Exposure to FFAs for 72 h in vitro may seem a brief period compared with the situation in vivo, where the β-cells may be exposed to elevated levels of lipids for years. However, it should be noted that similar effects were obtained when feeding mice a high-fat diet for 15 weeks. This led to a 50% reduction of glucose- and tolbutamide-induced insulin secretion while not interfering with intracellular $[Ca^{2+}]_i$ signaling or lowering islet insulin content, reminiscent of the in vitro results. The observation that the ability of glucose to increase $[Ca^{2+}]_i$ was not affected (if anything, it was enhanced) indicates that the secretion defect arises at a level distal to glucose recognition or $[Ca^{2+}]_i$ handling.

Contrary to what has previously been reported (43) long-term exposure to lipids in vitro did not result in increased transcription of UCP-2 in mouse islets. We acknowledge that increased uncoupling can occur by activation of UCP-2 even in the absence of any changes in mRNA levels. However, our measurements of islet ATP content, K_{ATP} channel activity, and $[Ca^{2+}]_i$ are not indicative of compromised ATP generation. If anything, the opposite is implied. Even if uncoupling occurs, the overall effect of lipid exposure appears to be enhanced production of ATP. The lipid depots that result from protracted exposure to lipids (manifested as the lipid droplets seen in electron microscopy) (Fig. 6) represent a large amount of fuel for FFA oxidation and subsequent ATP production. Increased Cpt-1 levels (indicated by the PCR measurements) after FFA exposure provide a mechanism for enhanced transport of long-chain acyl-CoAs into the mitochondria with resulting augmentation of β-oxidation and increased ATP production. Some β-cells cultured in the presence of palmitate, but not oleate, contained crescent-like structures similar to the angular vacuoles documented in Ins-1 cells (30). However, they were much less prominent than in the clonal cells. It seems likely that the presence of endogenous lipids permits primary β-cells to store most of the palmitate as a mixed-composition triglyceride (44), thus preventing the formation of cytotoxic tripalmitin. Nevertheless, as a consequence of less efficient storage as triglycerides (44), some palmitate may remain in the cytosol as palmitoyl-CoA. There is some experimental evidence that palmitoyl-CoA enhances exocytosis (25), although our own measurements have failed to confirm this (7).

Increased expression of granuphilin has been reported to mediate the negative effects of FFAs on insulin secretion (39), but we were unable to confirm this (Table 2). The insulin content of islets cultured in the presence of 15 mmol/l glucose and 0.5 mmol/l oleate or palmitate was reduced by 70–75% compared with islets cultured at high glucose alone (Table 1). This effect correlated with a 25% reduction of insulin mRNA levels, consistent with what has previously been observed in rat islets (45,46). Although insulin content was strongly reduced in islets exposed to FFAs, the number of granules per β-cell was unaffected. Thus, the insulin content per granule is not constant but varies depending on the culture conditions. The granule diameter was not affected by glucose or the lipids, but the dense core–to–granule diameter ratio was reduced from ~0.55 to 0.45 after exposure to palmitate, which might be an indication of lowered granule insulin content (predicting a ~45% decrease in insulin content). A lowered insulin content per granule would certainly explain much of the observed inhibition of insulin secretion produced by oleate in the presence of high glucose. In the case of palmitate, the stimulation of exocytosis would partially compensate for the inhibition. This would account for the observation that insulin secretion expressed as a percentage of insulin content was doubled after culture in the presence of palmitate (from 1 to ~2% per h). The finding that 33% of the β-cells cultured in the simultaneous presence of glucose and lipids generated action potentials already at 1 mmol/l glucose probably explains why "basal" insulin secretion from these islets is as high as in islets cultured with high glucose alone, whereas insulin release evoked by glucose and high-K^+ was strongly reduced.

Contrary to what has been reported previously in mouse (43) and rat islets (8), we did not detect any FFA-induced decrease in insulin content in islets cultured at 4.5 mmol/l glucose. Thus, a lowered insulin content per granule cannot account for the inhibitory effects of the FFAs on insulin secretion we observed under these conditions. Nevertheless, glucose- and high-K^+–induced insulin secretion was strongly (60–80%) inhibited by the FFAs, although both exocytosis (increased cell capacitance) and $[Ca^{2+}]_i$ signaling were unaffected or even enhanced. This indicates that the FFAs may interfere with the emptying of secretory granules and cause a switch from full fusion to incomplete kiss-and-run fusion events in a way analogous to that produced by high-glucose culture (33). In this context it may be of relevance that an increase in cell capacitance is observable already when the fusion pore diameter has increased to ~1.5 nm, which is insufficient to allow the exit of insulin (47). It is tempting to speculate that high glucose and FFA interfere with the expansion of the fusion pore and that this restricts the exit of insulin into the islet capillaries.

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