Impaired cAMP generation contributes to defective glucose-stimulated insulin secretion after long-term exposure to palmitate

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ABSTRACT

Chronic palmitate exposure impairs glucose-stimulated insulin secretion and other aspects of β-cell function but the underlying mechanisms are not known. Using various live-cell fluorescence imaging approaches we show here that long-term palmitate treatment influences cAMP signaling in pancreatic β-cells. Glucose stimulation of mouse and human β-cells induced oscillations of the sub-plasma-membrane cAMP concentration but after 48 h exposure to palmitate, most β-cells failed to increase cAMP in response to glucose. In contrast, GLP-1-triggered cAMP formation and glucose- and depolarization-induced increases in cytoplasmic \( \text{Ca}^{2+} \) concentration were unaffected by the fatty acid treatment. Insulin secretion from control β-cells was pulsatile but the response deteriorated after long-term palmitate exposure. Palmitate-treated mouse islets showed reduced expression of adenyl cyclase 9 and knockdown of this protein in insulinoma cells reduced the glucose-stimulated cAMP response and insulin secretion. We conclude that impaired glucose-induced generation of cAMP is an important determinant of defective insulin secretion after chronic palmitate exposure.

INTRODUCTION

There is a strong link between type 2 diabetes and obesity and the disease is often associated with increased plasma free fatty acid (FFA) concentrations (1,2). While acute exposure of insulin-secreting β-cells to saturated fatty acids, such as palmitate, stimulates insulin secretion (3,4), long-term palmitate exposure is associated with β-cell dysfunction, including reduction of glucose-stimulated insulin secretion (5-9). The mechanisms by which chronic palmitate exposure impairs insulin secretion are incompletely understood but probably involve multiple effects at different levels (9). For example, palmitate has been reported to cause alterations in β-cell metabolism (10) and ion channel activity (11). The lipid has also been found to reduce insulin
biosynthesis and to induce mitochondrial uncoupling, ceramide synthesis and ER stress, eventually leading to apoptotic cell death (7,12-15). However, reduced insulin secretion after long-term exposure to fatty acids has been reported to occur before apparent signs of increased β-cell death, alterations of insulin synthesis, glucose metabolism, ATP-sensitive K+ channel (K\textsubscript{ATP} channel) regulation or Ca\textsuperscript{2+} signaling, suggesting that the secretion defect occurs at a late step in the exocytosis process (16), not as a result of decreased release competence of the secretory vesicles, but because they become uncoupled from the sites of Ca\textsuperscript{2+} entry (17).

The depolarization-triggered entry of Ca\textsuperscript{2+} through L-type voltage-dependent Ca\textsuperscript{2+} channels is the final step in a chain of events by which glucose triggers insulin secretion (18,19). Apart from the triggering role of Ca\textsuperscript{2+}, insulin secretion is markedly amplified by cAMP. While cAMP is known to mediate the insulinotropic effects of e.g. glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide (20), accumulating evidence indicate that cAMP elevation is also required for a normal insulin secretory response to glucose alone (21,22). In the present study we used various live-cell imaging techniques to investigate whether the reduced insulin response to glucose after chronic exposure to palmitate is associated with changes of β-cell cAMP signaling.

**RESEARCH DESIGN AND METHODS**

**Materials**

Adrenaline, 8-Br-cAMP, forskolin, glucagon-like peptide-1 (7-36) amide (GLP-1), HEPES, 2-mercaptoethanol, poly-L-lysine, palmitate, 2',5'-dideoxyadenosine, and insulin were from Sigma (St Louis, MO, USA) and Lipofectamine 2000, DMEM, trypsin, penicillin, streptomycin, glutamine and fetal calf serum from Life
Technologies (Carlsbad, CA, USA). Plasmids and adenoviral vectors encoding a translocation biosensor for the cAMP concentration beneath the plasma membrane ([cAMP]_{pm}) have previously been described (23). The sensor consists of a truncated and membrane-anchored PKA regulatory RIIβ subunit tagged with CFP and a PKA catalytic Cα subunit tagged with YFP (24). Insulin secretion dynamics was monitored using the PtdIns(3,4,5)P$_3$ translocation reporter GFP$_4$-Grp1 (21).

**Islet and cell culture, transfection and palmitate treatment**

Islets of Langerhans were isolated from C57Bl6J female mice as previously described (23). All animal handling procedures were approved by the local animal ethical committee. Human pancreatic islets from nine normoglycemic cadaveric donors (aged 47–76 years) were generously provided by the Nordic Network for Clinical Islet Transplantation. All experiments with human islets were approved by the Uppsala human ethical committee. The islets were isolated with semi-automated digestion filtration, purified on a continuous density gradient in a refrigerated cell processor (COBE 2991; COBE Blood Component Technology, Lakewood, CO)(25) and kept for 2–5 days at 37°C in an atmosphere of 5% CO$_2$ in CMRL 1066 culture medium (Mediatech, Herndon, VA) containing 5.5 mmol/L glucose and supplemented with 10 mmol/L nicotinamide, 10 mmol/L HEPES, 0.25 µg/ml Fungizone, 50 µg/ml gentamicin, 2 mmol/L glutamine, 10 g/ml ciprofloxacin, and 10% fetal calf serum. Following isolation and purification both mouse and human islets were cultured for 1–4 days in RPMI 1640 medium containing 5.5 mmol/L glucose and supplemented with 10% fetal calf serum, 100 µg/ml penicillin, 100 µg/ml streptomycin and 1% BSA. Palmitate (0.5 mmol/L) was present for 1 h or 48 h prior to the microscopy recordings. A 100 mmol/L stock solution of palmitate was prepared in 50% ethanol.
This solution was subsequently diluted in culture medium to a final concentration of 0.5 mmol/L and then allowed to complex with fatty acid-free BSA at 37°C. Control groups were exposed for the same period of time to media without palmitate, but otherwise with identical composition, including ethanol.

The islets were infected with adenovirus encoding GFP\textsubscript{4-Grp1} or the cAMP biosensor using $10^5$ fluorescent forming units/islet in culture medium as previously described (23). After 1 hour incubation at 37 °C, the inoculum was removed and the islets were washed and further cultured for 20-24 h. Before microscopy recordings, the islets were incubated for 30 min at 37°C in experimental buffer containing 125 mmol/L NaCl, 4.8 mmol/L KCl, 1.3 mmol/L CaCl\textsubscript{2}, 1.2 mmol/L MgCl\textsubscript{2}, and 25 mmol/L HEPES with pH adjusted to 7.40 with NaOH. They were then allowed to attach to the centre of poly-lysine-coated round 25-mm glass coverslips for 5 min. β-cells were identified based on their large size and negative response to adrenaline (23).

Rat insulinoma INS-1E cells (26) were used between passages 65-90 and cultured in a humidified atmosphere containing 5% CO\textsubscript{2} in RPMI 1640 medium containing 11 mmol/L glucose and supplemented with 10 mmol/L HEPES, 10% (v/v) heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mmol/L sodium pyruvate and 50 µmol/L 2-mercaptoethanol. For imaging experiments, cells were seeded onto poly-lysine coated 25-mm coverslips and transfections were performed with 0.2-0.5 µg of plasmid and 0.5-1 µg Lipofectamine\textsuperscript{TM} 2000 in 100 µL Opti-MEM®-I per coverslip. Cells were then further cultured in RPMI 1640 medium for 48 h in the absence or presence of 0.5 mmol/L palmitate in 1% (3:1) or 0.5% (6:1) of BSA.

Insulin-secreting MIN6 β-cells of passages 17-30 (27) were cultured in DMEM
containing 25 mmol/L glucose and supplemented with 15% fetal calf serum, 2 mmol/L glutamine, 70 μmol/L 2-mercaptoethanol, 100 U/mL penicillin and 100 μg/mL streptomycin. Where indicated, MIN6-cells were transfected with 0.5 μmol/L Lipofectamine™ 2000 and 100 nmol/L siRNA against adenylyl cyclase (AC) 9, 48 h prior to continued experimental handling.

**Assessment of cell viability**

Apoptosis in INSK1E cells was assayed by the Cell Death Detection ELISA PLUS kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. This photometric enzyme immunoassay measures cytoplasmic mono- and oligonucleosomes that increase after apoptosis-associated DNA degradation. The apoptosis measurements were related to the DNA content obtained in separate experiments and compared to the average value obtained from control cells.

The viability of cells within intact mouse islets was assessed by incubating islets for 15 min in experimental buffer supplemented with 10 μg/mL propidium iodide (Sigma) and 2 μmol/L calcein acetoxymethyl ester (Life Technologies) followed by imaging with a confocal microscope (described in (28)). The area of propidium iodide stained nuclei in relation to the calcein-stained viable cells were calculated with ImageJ (29).

**Batch measurements of insulin secretion**

Control and palmitate-treated islets were preincubated in experimental buffer for 30 min at 37 °C. Groups of 10 islets were then transferred to the different test media and incubated for 30 min. After collection of supernatants the islets were sonicated for 10 s in acid ethanol to extract total insulin. The sample contents of insulin were
determined by an electrochemiluminescence immunoassay (Meso Scale Diagnostics, Gaithersburg, MD.

**RNA isolation and real-time PCR**

Total RNA was extracted from islets using the RNEasy micro kit (Qiagen, Hilden, Germany). Real-time PCR was performed using the Lightcycler instrument (Roche) and the Quanti Tect SYBR(R) Green RT-PCR kit (Qiagen, Hilden, Germany) using the following primers: mouse AC1, forward 5’-ctctactacagtctacte-3’, reverse 5’-cttagagagtctgtgtcct-3’; human AC1, forward 5’-cagtaagcatctggggaaac-3’, reverse 5’-agtcaacagtgctgccctg-3’; mouse AC3, forward 5’-tgaggagagactaatccac-3’, reverse 5’-tgggtgactcttgaagctg-3’; human AC3, forward 5’-tgcacactcatggcttttag-3’, reverse 5’-cagggcacaagaggaagtag-3’; mouse AC5, forward 5’-aacgactccacatgac-3’, reverse 5’-aatgactccagactagaagtag-3’; human AC5, forward 5’-aggtgtgttccctcatttc-3’, reverse 5’-ccagcagagcactccctata-3’; mouse AC6, forward 5’-tatgccgtccttcctcct-3’, reverse 5’-aggcagagatgaacacaag-3’; human AC6, forward 5’-ggccatcaggtactcattta-3’, reverse 5’-caccaagtagtctctctcact-3’; mouse AC8, forward 5’-gtcaggaagcacaactc-3’, reverse 5’-cttaggtggcaggaagagt-3’; human AC8, forward 5’-agtactttgtctcaggggg-3’, reverse 5’-gcaatcagactacagcact-3’; mouse AC9, forward 5’-catacagaagcagccagtag-3’, reverse 5’-ccgaacaggtcatggtag-3’; human AC9, forward 5’-tgcggctacactgtctgc-3’, reverse 5’-gccttgggttgtttatgc-3’; mouse PDE8B, forward, 5’-gactgtgaagaagagag-3’; reverse, 5’-atgtctgtgaatgtcaagt-3’; PDE1C, forward, 5’-aagccagcagagcttcct-3’; reverse, 5’-ggcaaggtatcgcattc-3’; PDE3B, forward, 5’-ccttacgtctgttctccta-3’; reverse, 5’-tgaatcagatgtcgtctgtgaa-3’; PDE4A, forward, 5’-catcactgtctcagcata-3’; reverse, 5’-taagcctgcttcctca-3’; mouse β-actin: forward 5’-gttacaggaagctctcacc-3’, reverse 5’-gggacacaaagcctctcata-3’; human
β-actin, forward 5’-gggcatgggctagaaggatt-3’, reverse 5’-tcgatgggtactctagggt-3’; human glyceraldehyde phosphate dehydrogenase (GAPDH), forward 5’-aattccatggcaccgtcaag-3’, reverse 5’-gatctcgctcctggaagatgg-3’. Mouse PCR products were normalized to β-actin and expression levels are given relative to control according to the formula: fold change=2\(^{-\Delta\Delta Ct}\), where \(\Delta\Delta Ct=Ct_{AC/PDE\ test} - Ct_{\beta\text{-actin\ test}}\) - \((Ct_{AC/PDE\ control} - Ct_{\beta\text{-actin\ control}})\). For human ACs, PCR efficiency was calculated from a standard curve generated from experiments on total RNA from EndoC-βH1-cells (generous gift from Professor Nils Welsh, Uppsala University). The relative expression is given according to the formula: fold change = \((E_{AC})^{\Delta Ct_{AC}}/(E_{\text{actin}})^{\Delta Ct_{\beta\text{-actin}}},\) where E=efficiency from standard curve; \(\Delta Ct_{AC} = Ct_{AC\ control} – Ct_{AC\ test}\); and \(\Delta Ct_{\beta\text{-actin}} = Ct_{\beta\text{-actin\ control}} – Ct_{\beta\text{-actin\ test}}\).

**Recordings of \([\text{cAMP}]_{pm}\) and plasma membrane PtdIns(3,4,5)P\textsubscript{3}**

Measurements of fluorescence from the CFP/YFP-based \([\text{cAMP}]_{pm}\) reporter or the GFP-based PtdIns(3,4,5)P\textsubscript{3} sensor were performed with total internal reflection fluorescence (TIRF) microscopy using either a custom-built prism-based system (22) or an objective-based setup (23). The prism-type TIRF setup was built around an E600FN upright microscope (Nikon Corp, Tokyo, Japan). A helium-cadmium laser (Kimmon, Tokyo, Japan) provided 442 nm light for excitation of CFP and the 514 nm line of an argon laser (ALC 60X, Creative Laser production, Munich, Germany) was used to excite YFP. Interference filters (Semrock, Rochester, NY, USA) mounted in a filter wheel (Sutter Instruments, Novato, CA, USA) were used to select the appropriate wavelength. The merged laser beam was homogenized and expanded by a rotating light shaping diffuser (Physical Optics Corp, Torrance, CA, USA) and refocused through a modified quartz dove prism (Axicon, Minsk, Belarus) with a 70°
angle to achieve total internal reflection. The experimental chamber with cells was mounted on the custom-built stage of the microscope such that the cover slip was maintained in contact with the dove prism by a layer of immersion oil. Fluorescence light was collected through a 40x, 0.8-NA water immersion objective (Nikon). The objective-based system consisted of an Eclipse Ti microscope (Nikon) with a TIRF illuminator (Nikon) and a 60x, 1.45-NA objective. The 458-, 488- and 514-nm lines of an argon laser (ALC60X, Creative Laser Production) were used to excite CFP, GFP and YFP, respectively. The beam was coupled to the TIRF illuminator through an optical fibre (Oz Optics, Ottawa, Canada). In both evanescent wave microscope setups, fluorescence was detected with back-illuminated EMCCD cameras (iXON DU-897, Andor Technology, Belfast, Northern Ireland) under MetaFluor (Molecular Devices Corp, Downington, PA) software control. Emission wavelengths were selected with filters (485 nm/25 nm half-bandwidth for CFP, 527/27 nm for GFP and 560/40 nm for YFP (Semrock Rochester, NY)) mounted in a filter wheel (Sutter Instruments). For time lapse recordings images or image pairs were acquired every 5 s. To minimize exposure of the cells to the potentially harmful laser light, the beam was blocked by a mechanical shutter (Sutter Instruments) between image captures.

**Recordings of the cytoplasmic Ca\(^{2+}\) concentration**

For measurements of the cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), cells and islets were pre-incubated during 30-40 min in the presence of 1 µmol/L of the acetoxymethyl esters of the Ca\(^{2+}\) indicators Fura-PE3 or Fluo-5F. Imaging of the Fura-PE3-loaded cells was performed with an inverted microscope (Diaphot, Nikon) equipped with a 40x 1.3-NA objective and an epifluorescence illuminator (Cairn Research Ltd, Faversham, UK) connected through a 5-mm diameter liquid light guide.
to an Optoscan monochromator (Cairn Research Ltd) with a 150-W xenon arc lamp. The monochromator provided excitation light at 340 and 380 nm that was reflected by a 400-nm dichroic beam splitter, and emission was measured at 510 nm/40 nm half-bandwidth using a Cascade (Photometrics, Tucson, AZ, USA) or iXON DU-897 (Andor Technology) EM-CCD camera. The Metafluor software (Molecular Devices) controlled the monochromator and the camera, acquiring image pairs every 2 s with 100-400 ms integration at each wavelength and <1 ms for changing wavelength and slits. To minimize bleaching and photodamage, the monochromator slits were closed until the start of the next acquisition cycle. Ratio images (340/380 nm) were obtained after subtraction of background and $[\text{Ca}^{2+}]_i$ values calculated as previously described (30). Fluo-5F-loaded islets were imaged using the objective-based TIRF system described above for the PtdIns(3,4,5)P$_3$ measurements.

**Data analysis**

Image analysis was made using MetaFluor (Molecular Devices). $[\text{cAMP}]_{pm}$ was expressed either as the ratio of CFP over YFP fluorescence after background subtraction with the basal ratio normalized to 1 to compensate for variability in expression levels, or as the Cα-YFP fluorescence intensity (F) in relation to the initial fluorescence intensity ($F_0$) after subtraction of background. Sub-plasma-membrane $[\text{Ca}^{2+}]_i$, and the membrane PtdIns(3,4,5)P$_3$ concentration were evaluated as relative changes of the background-corrected Fluo-5F and GFP$_4$-Grp1 fluorescence intensities, respectively ($F/F_0$). Time-average levels of the second messengers were calculated by measuring the area under curve followed by normalization for the elapsed time. Data are presented as means ± SEM. The primary cell data were obtained from at least 3 independent islet preparations. The apoptosis data was
statistically analyzed with one-way ANOVA followed by Tukey’s multiple comparison test and the analyses of fraction responsive cells were made with the chi square test. In all other cases statistical significances were evaluated using Student’s t-test.

RESULTS

Long-term exposure to palmitate suppresses glucose-induced cAMP signaling in β-cells

[cAMP]ₚₘ was monitored in INS1E β-cells as well as in superficially located β-cells within intact mouse and human islets. As previously reported for MIN6- and primary mouse β-cells (21,23), INS1E-cells responded to an increase in the glucose concentration with pronounced [cAMP]ₚₘ oscillations (Fig. 1A). Subsequent addition of 100 nmol/L GLP-1 caused stable [cAMP]ₚₘ elevation. After 48 h exposure to 0.5 mmol/L palmitate in 0.5 or 1% BSA (fatty acid:BSA molar ratio of 6:1 and 3:1, respectively) the glucose-induced [cAMP]ₚₘ response was significantly delayed and the amplitude suppressed, whereas the amplitude and duration of GLP-1-triggered [cAMP]ₚₘ elevation was unaffected (Fig. 1B-F). The palmitate treatments caused modest, but significant (P<0.01 at 3:1; P<0.001 at 6:1) increases of INS1E-cell apoptosis (Fig. 1G).

Similarly, mouse β-cells in intact islets responded to an increase of the glucose concentration from 3 to 20 mmol/L with shortly delayed and pronounced [cAMP]ₚₘ elevation, often with oscillations with frequencies in the 0.09-0.27/min range (Fig. 2A). Addition of 100 nmol/L GLP-1 triggered stable [cAMP]ₚₘ elevation, which was counteracted by 5 µmol/L adrenaline used to confirm the β-cell identity (23) (Fig. 2A). After 48 h exposure to 0.5 mmol/L palmitate in 1% BSA, the glucose-induced
[cAMP]_{pm} elevation, but not that triggered by GLP-1, was significantly suppressed (Fig. 2B and E, P<0.001). GLP-1 was also tested at 100 pmol/L, and also at this concentration, there was no difference in [cAMP]_{pm} between palmitate-treated islets and control (time-average CFP/YFP 1.79±0.35 vs 1.98±0.22, n=35 and 13 cells, respectively, from 5 islets in 3 independent experiments). The poor glucose response was not due to initial cAMP elevation by the palmitate treatment since there was no cAMP-lowering effect of the AC inhibitor 2’,5’-dideoxyadenosine (100 µmol/L; data not shown). The impaired glucose response was nor due to reduced islet viability. Accordingly, the ratio of propidium iodide to calcein labelled cell areas were similar in control (0.0027±0.0005, n=36 islets) and palmitate-treated islets (0.0036±0.0011, n=30 islets) and hundred-fold lower than in heat-shocked control islets incubated for 5 min at 50 °C (0.2668±0.0297, n=50 islets).

Human islet β-cells behaved essentially as mouse β-cells. At 3 mmol/L glucose, [cAMP]_{pm} was low and stable in 57% of the cells (n=14) and in the remaining cells there were modest [cAMP]_{pm} fluctuations (not shown). When the glucose concentration was increased to 20 mmol/L, [cAMP]_{pm} increased and oscillated (frequency 0.08-0.24/min; amplitude 0.16±0.03 ratio units; n=37 oscillations from 8 cells) often from an elevated level (Fig. 2C). GLP-1 (100 nmol/L) induced sustained [cAMP]_{pm} elevation, which was reversed by 5 µmol/L adrenaline (Fig. 2C). Like in INS1EK and mouse β-cells, 48 h exposure to palmitate selectively suppressed the glucose-induced [cAMP]_{pm} elevation (P<0.001) (Fig. 2D-E).

**Short palmitate treatment does not affect glucose- and GLP-1-induced [cAMP]_{pm} signaling**

Acute exposure of mouse islet β-cells to 0.5 mmol/L palmitate (1% BSA) in the
presence of 3 mmol/L glucose sometimes resulted in transient \([cAMP]_{pm}\) elevation (7 out of 15 cells; Fig. 3A). However, there was no effect when palmitate was added in the presence of 20 mmol/L glucose or 100 nmol/L GLP-1 (Fig 3B-C). Likewise, there were no differences in glucose- or GLP-1-induced cAMP signaling after 1 h pre-incubation with palmitate (Fig. 3D-F). The mechanism behind the modest \([cAMP]_{pm}\) elevation triggered by acute palmitate treatment is not known, but may be secondary to the elevation of the cytoplasmic \(\text{Ca}^{2+}\) concentration ([Ca\(^{2+}\)]\text{\(_i\)}) by fatty acid-mediated activation of GPR40 receptors (31).

**Palmitate does not impair glucose-induced [Ca\(^{2+}\)]\text{\(_i\)} signaling**

Since glucose-induced cAMP signaling is amplified by Ca\(^{2+}\) (23) we next investigated whether [Ca\(^{2+}\)]\text{\(_i\)} is affected by long-term exposure to palmitate. In both INS1E- and mouse islet \(\beta\)-cells loaded with Fura-PE3, the basal [Ca\(^{2+}\)]\text{\(_i\)} at sub-stimulatory glucose concentrations was slightly, but significantly (P<0.05), increased after exposure to palmitate, but the fatty acid neither influenced the ability of 20 mmol/L glucose to induce [Ca\(^{2+}\)]\text{\(_i\)} oscillations, nor the [Ca\(^{2+}\)]\text{\(_i\)} elevation induced by K\(^+\) depolarization (Fig. 4A-D, G, H) or the synchronization of oscillations among neighboring \(\beta\)-cells (not shown). TIRF recordings of sub-plasma membrane [Ca\(^{2+}\)]\text{\(_i\)} with the low-affinity indicator Fluo-5F also did not reveal any influence of palmitate on [Ca\(^{2+}\)]\text{\(_i\)} (Fig. 4E-F, I).

**Palmitate impairs glucose-induced pulsatile insulin secretion**

To investigate if the compromised \([cAMP]_{pm}\) signaling was associated with impaired insulin secretion, we monitored the time-course of insulin secretion from individual cells within intact islets by detecting the lipid PtdIns(3,4,5)P\(_3\), which is formed in the
plasma membrane following autocrine insulin receptor activation (21). Control mouse and human islet β-cells responded to glucose with pronounced PtdIns(3,4,5)P$_3$ oscillations reflecting pulsatile insulin secretion (Fig. 5A-B). The glucose response was almost abolished after 48 h palmitate treatment, and the cells showed only a transient increase or minute stable PtdIns(3,4,5)P$_3$ elevation without oscillations (Fig. 5C-E). The reduced response was not due to impaired insulin signaling, since exogenously added insulin evoked PtdIns(3,4,5)P$_3$ elevation (Fig. 5C-E). Consistent with deficient cAMP production underlying the impaired secretory response to glucose the fraction of cells responding with increased PtdIns(3,4,5)P$_3$ was doubled and the response amplitude several-fold increased when 20 mmol/L glucose was added in the presence of the AC activator forskolin (Fig. 5E-F). The palmitate-induced suppression of glucose-induced insulin secretion was confirmed by measurements of insulin release from batch-incubated islets using a conventional immunoassay (Fig 5G). The results also demonstrate that 1 nmol/L GLP-1 restores insulin secretion to similar levels as in the control (Fig 5G).

Reduction of AC expression underlies palmitate impairment of glucose-induced cAMP production

Next, the expression of ACs and phosphodiesterases (PDEs), synthesizing and degrading cAMP, respectively, was evaluated by real-time PCR. After 48 h palmitate treatment of mouse islets, AC9 mRNA was reduced to 70% (P<0.01) but there were no changes in expression of AC1, AC3, AC5, AC6, AC8 (Fig. 6A) or any of the tested PDEs (PDE1C, -3B, -4A and -8B; Fig. 6B). In human islets, AC5 was reduced and AC8 was increased, while other ACs did not show significant alterations in mRNA expression (Fig. 6C). The importance of AC9 for glucose-induced [cAMP]$_{pm}$
signaling in mouse β-cells was investigated by siRNA-mediated knock-down in MIN6 cells. After 48 h treatment with siRNA, AC9 mRNA was reduced to 50%, and only 21% of the cells responded to glucose with [cAMP]_{pm} oscillations as compared to 91% in control-siRNA-treated cells (Fig. 6D-E). The average glucose-induced [cAMP]_{pm} response was thus significantly suppressed after AC9 knockdown (Fig. 6F; P<0.001). Also the GLP-1-induced [cAMP]_{pm} elevation was reduced, but not to the same extent (Fig. 6F; P<0.05). The reduced AC9 expression was accompanied by impaired insulin secretion. When evaluating glucose-induced PtdIns(3,4,5)P_3 formation, the initial 10 min of the glucose response was unaffected, whereas the subsequent sustained response was significantly reduced in siRNA-treated cells (Fig 6G-H, K). The lower insulin secretion most likely reflected the deficient cAMP production, since the response was restored by addition of 1 mmol/L 8-Br-cAMP (Fig 6I-K).

**DISCUSSION**

Deleterious effects of FFAs on β-cell function are well documented (5-8), but the mechanisms underlying the defective insulin secretion are not well understood. In the present study we show that inhibition of glucose-induced cAMP production in β-cells contributes to impaired insulin secretion after long-term exposure to palmitate.

With the advent of techniques to monitor cAMP dynamics in single-cells, increasing evidence indicates that intracellular cAMP elevation is an integral part of glucose stimulus-secretion coupling in β-cells (21,32,33). Measurements in the sub-plasma membrane space of both clonal and primary mouse β-cells have shown that cAMP often oscillates and contribute to pulsatile release of insulin (21-23). The present data show that glucose triggers cAMP oscillations also in human β-cells.
Increases of cAMP amplify insulin secretion both via protein kinase A (PKA) and the guanine nucleotide exchange factor Epac. The cAMP effectors act at multiple levels to influence the activity of various ion channels and other proteins involved in signaling and exocytosis (34-40). It is therefore not surprising that deteriorated glucose-induced cAMP generation results in impaired insulin secretion.

The observation that glucose-induced cAMP signaling is suppressed in palmitate-treated cells is consistent with previous findings that the secretory defect involves a late step in stimulus-secretion coupling (16). The poor cAMP response was not secondary to changes in $[\text{Ca}^{2+}]_i$, which was essentially unaffected by palmitate exposure, except for slight elevation of basal $[\text{Ca}^{2+}]_i$, also observed in previous studies (16). The upstream events with metabolic generation of ATP, membrane depolarization and $\text{Ca}^{2+}$ influx therefore seem unaffected, which is consistent with palmitate lacking effect on glycolytic flux and ATP synthesis in MIN6 and mouse islet $\beta$-cells (16,41).

It has been suggested that palmitate impairs insulin secretion via PKA-mediated induction of the cAMP early repressor (ICER-1γ) and suppression of connexin-36 expression (42), and lipotoxicity has indeed been associated with impaired coordination of $\text{Ca}^{2+}$ signals in human islets (43). The increased PKA activity suggested to underlie the altered connexin-36 expression is difficult to reconcile with the present observation of reduced cAMP production. Moreover, we did not observe any striking deterioration of the synchronization of glucose-induced $[\text{Ca}^{2+}]_i$ oscillations among neighboring $\beta$-cells. The impaired insulin secretion after chronic palmitate exposure has also been attributed to dissociation of the secretory vesicles from the $\text{Ca}^{2+}$ entry sites (17). Our data is more easily reconciled with such a mechanism. Accordingly, the protein complex responsible for tethering of secretory
vesicles to Ca\textsuperscript{2+} channels includes components that are directly regulated by cAMP. For example, the SNARE proteins syntaxin and SNAP25 are both regulated by PKA, and Epac interacts with the Rab-binding protein RIM2, which in turn interacts with the L-type voltage-dependent Ca\textsuperscript{2+} channel (37,44,45). Defective cAMP generation might thus affect the interaction between Ca\textsuperscript{2+} channels and the secretory granules.

Reduced expression of ACs provides a plausible explanation for the defective glucose-induced cAMP responses. In human islets, AC5 was reduced by palmitate treatment. Interestingly, this enzyme was recently demonstrated to be important for glucose-induced insulin secretion in human β-cells (46). In the mouse, AC9 seems to be more important. Although the activity of this AC typically is low (47), its functional importance was verified in siRNA knockdown experiments in MIN6 cells. Further studies are warranted to clarify the detailed involvement in insulin secretion of the many ACs expressed in mouse and human islets. Moreover, it cannot be excluded that additional factors contribute to the palmitate-induced impairment of cAMP signaling, such as altered expression of additional cAMP-regulating proteins or direct modulation of protein function via e.g. acylation or palmitoylation (48,49).

β-cell dysfunction induced by long-term exposure to palmitate typically culminates in apoptotic cell death (7,12). However, our relatively mild treatment regime had little effects on cell viability, indicating that the observed signaling defect represents an early event in the lipotoxic process causing β-cell failure. Importantly, the deficient glucose-induced cAMP production did not reflect a general deterioration of the cAMP signaling system since the cells responded normally to GLP-1. cAMP has been found to protect from apoptosis in many cell types, including palmitate-induced apoptosis in insulin-secreting cells (50). Impaired glucose-induced cAMP generation might
therefore not only explain defective insulin secretion but also contribute to the general β-cell dysfunction after chronic exposure to palmitate.

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REFERENCES


5. Bollheimer LC, Skelly RH, Chester MW, McGarry JD, Rhodes CJ: Chronic exposure to free fatty acid reduces pancreatic beta cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation. J Clin Invest. 1998; 101:1094-1101


12. Maestre I, Jordan J, Calvo S, Reig JA, Cena V, Soria B, Prentki M, Roche E: Mitochondrial dysfunction is involved in apoptosis induced by serum withdrawal and fatty acids in the β-cell line INS-1. Endocrinology. 2003; 144:335-345


46. Hodson DJ, Mitchell RK, Marselli L, Pullen TJ, Brias SG, Semplici F, Everett KL, Cooper DM, Bugliani M, Marchetti P, Lavallard V, Bosco D, Piemonti L,
Johnson PR, Hughes SJ, Li D, Li WH, Shapiro AM, Rutter GA: ADCY5 couples glucose to insulin secretion in human islets. Diabetes. 2014;

47. Cooper DM: Regulation and organization of adenylyl cyclases and cAMP. Biochem J. 2003; 375:517-529


LEGENDS TO FIGURES

FIG. 1. \([\text{cAMP}]_{\text{pm}}\) signaling and apoptosis in INS1E \(\beta\)-cells after long-term exposure to palmitate.

A-C: TIRF microscopy recordings of \([\text{cAMP}]_{\text{pm}}\) in individual INS1E \(\beta\)-cells stimulated by an increase in the glucose concentration from 2 to 20 mmol/L and by addition of 100 nmol/L GLP-1 under control conditions (A; \(n=19\)) and after 48 h treatment with 0.5 mmol/L palmitate in 3:1 (B; \(n=34\)) or 6:1 (C; \(n=11\)) molar ratio with BSA. D: Time-average \([\text{cAMP}]_{\text{pm}}\) in the presence of 20 mmol/L glucose in cells pre-treated or not with palmitate. Means±SEM. ***, \(P<0.001\) for difference from control. E: Means±SEM for the time delay between glucose stimulation and the first \([\text{cAMP}]_{\text{pm}}\) elevation. ***, \(P<0.001\) for difference from control. F: Time-average \([\text{cAMP}]_{\text{pm}}\) after stimulation with GLP-1 in the presence of 20 mmol/L glucose. Means±SEM. G: Apoptosis in INS1E \(\beta\)-cells pre-treated or not with palmitate. Means±SEM for the percentage apoptosis determined by a photometric immunoassay for cytoplasmic oligonucleosomes with the data normalized to the 3:1 control from 4 independent experiments. **, \(P<0.01\) and ***, \(P<0.001\) for difference from control.

FIG. 2. Impaired glucose-induced \([\text{cAMP}]_{\text{pm}}\) signaling in long-term palmitate-treated mouse and human \(\beta\)-cells.

TIRF microscopy recordings of \([\text{cAMP}]_{\text{pm}}\) in mouse and human islet \(\beta\)-cells maintained in buffer containing 3 mmol/L glucose. A: Mouse \(\beta\)-cell showing oscillations of \([\text{cAMP}]_{\text{pm}}\) in response to 20 mmol/L glucose. GLP-1 (100 nmol/L) induced stable \([\text{cAMP}]_{\text{pm}}\) elevation which is inhibited by 5 \(\mu\)mol/L adrenaline (adr). Representative for 12 cells. B: Impaired glucose-induced \([\text{cAMP}]_{\text{pm}}\) elevation in a mouse islet \(\beta\)-cell pre-treated with palmitate for 48 h (\(n=14\)). C: \([\text{cAMP}]_{\text{pm}}\) responses
to 20 mmol/L glucose, 100 nmol/L GLP-1 and 5 µmol/L adrenaline (adr) in a control human islet β-cell. Representative for 8 cells in islets from 3 donors. D: [cAMP]_{pm} responses in a human islet β-cell exposed to palmitate for 48 h. n=8 cells in islets from 3 donors. E: Means±SEM for the time-average [cAMP]_{pm} during glucose and GLP-1 stimulation in control and palmitate-treated β-cells.

FIG. 3. [cAMP]_{pm} signaling in mouse islet β-cells following short exposure to palmitate.

TIRF microscopy recordings of [cAMP]_{pm} in mouse islet β-cells maintained in buffer containing 3 mmol/L glucose. A: Addition of 0.5 mmol/L palmitate in 1% BSA (3:1 palmitate:BSA molar ratio) or 100 nmol/L GLP-1 triggers transient [cAMP]_{pm} elevations. The GLP-1 effect is inhibited by 5 µmol/L adrenaline (adr). Representative for 7 out of 15 cells. B: Acute treatment with 0.5 mmol/L palmitate has no effect on glucose- or GLP-1-induced [cAMP]_{pm} elevation (n=9). C: Means±SEM for the time-average [cAMP]_{pm} in cells before (open bars) or after (grey bars) acute palmitate treatment. *, P<0.05 for difference from control. D, E: [cAMP]_{pm} responses triggered by elevation of glucose from 3 mmol/L to 20 mmol/L and additions of 100 nmol/L GLP-1 and 5 µmol/L adrenaline in control cells (D, n=7) and cells pre-treated for 1 h with palmitate (E, n=8). F: Means±SEM for the time-average [cAMP]_{pm} in cells treated or not for 1 h with palmitate.

FIG. 4. Glucose-induced [Ca^{2+}]_{i} signaling is not impaired by long-term palmitate exposure.

A-D: Wide-field epifluorescence recordings of [Ca^{2+}]_{i} in individual INS1E-cells and mouse islets loaded with Fura-PE3. Elevation of glucose from 2 mmol/L to 20
mmol/L triggers [Ca\(^{2+}\)]\(_i\) oscillations in control (A, n=87) and palmitate-treated (B, n=65) INS1E-cells. Increase of the K\(^+\) concentration to 30 mmol/L evoked prompt and stable [Ca\(^{2+}\)]\(_i\) elevation. C-D: 20 mmol/L glucose induces [Ca\(^{2+}\)]\(_i\) oscillations in control (C, n=6) and palmitate-treated (D, n=6) mouse islets. The traces are quantifications from regions corresponding to the whole islet. E-F: TIRF recordings of sub-plasma membrane [Ca\(^{2+}\)]\(_i\) in single cells within intact Fluo-5F-loaded mouse islets, treated or not with palmitate, and stimulated with 20 mmol/L glucose and 30 mmol/L K\(^+\). G: Means±SEM for the basal [Ca\(^{2+}\)]\(_i\) in the presence of 2 mmol/L glucose and the amplitudes of the [Ca\(^{2+}\)]\(_i\) increases triggered by 20 mmol/L glucose or 30 mmol/L K\(^+\) in control and palmitate-treated INS1E-cells. *, P<0.05 for difference from control. H: Means±SEM for the time-average [Ca\(^{2+}\)]\(_i\) in the presence of 3 and 20 mmol/L glucose and the amplitudes of the [Ca\(^{2+}\)]\(_i\) increases triggered by 30 mmol/L K\(^+\) in control and palmitate-treated mouse islets. *, P<0.05 for difference from control. I: Means±SEM for the time-average Fluo-5F fluorescence during the initial peak and the subsequent (late) responses to glucose high K\(^+\).

FIG. 5. Long-term exposure to palmitate suppresses pulsatile insulin secretion from mouse and human islets.

TIRF microscopy recordings of insulin release from mouse and human islet cells expressing the fluorescent PtdIns(3,4,5)\(_P3\) reporter GFP\(_4\)-Grp1. A: Elevation of the glucose concentration from 3 to 20 mmol/L triggers PtdIns(3,4,5)\(_P3\) oscillations corresponding to pulsatile insulin secretion in a mouse islet cell. Addition of 100 nmol/L exogenous insulin causes prompt and stable PtdIns(3,4,5)\(_P3\) elevation. n=26 cells. B: Glucose-induced PtdIns(3,4,5)\(_P3\) oscillations in a human islet cell. Representative for 9 cells from 4 donors. C: Lack of glucose-, but not insulin-induced,
PtdIns(3,4,5)P₃ response in a mouse islet cell pre-treated with palmitate. n=27. D: Glucose- and insulin-induced PtdIns(3,4,5)P₃ responses in a human islet cell pre-treated with palmitate. n=8 cells from 4 donors. E: Means±SEM for the time-average PtdIns(3,4,5)P₃ responses induced by 20 mmol/L glucose, 5 µmol/L forskolin and 100 nmol/L insulin in control and palmitate-treated mouse and human islet cells. ***, P<0.001 for difference from control. †, P<0.05 compared to absence of forskolin. F: Fraction of cells responding with increased levels of PtdIns(3,4,5)P₃ after stimulation with 20 mmol/L glucose in the absence or presence of 5 µmol/L forskolin in control and palmitate-treated mouse islet cells. ***, P<0.001 compared to control; †, P<0.05 compared to absence of forskolin. G: Insulin release from batches of islets incubated in 3, 20, or 20 mmol/L glucose with 1 nmol/L GLP-1. N=4 experiments. *, P<0.05 for difference from control. †, P<0.05 for difference from 20 mmol/L glucose.


A-C: Real-time PCR analysis of the expression of mRNA for different ACs (A) and PDEs (B) in mouse islets (n=4 independent experiments) and ACs in human islets (C; n=4) after 48 h treatment with palmitate. The results are presented in relation to non-treated control after normalization to the housekeeping gene β-actin. Glyceraldehyde phosphate dehydrogenase (GAPDH) was included as an extra control. *, P<0.05; **, P<0.01 for difference induced by palmitate treatment. D-E: TIRF recordings of [cAMP]ₚm in single MIN6 β-cells stimulated with 20 mmol/L glucose and 100 nmol/L GLP-1 after treatment with non-target control siRNA (D, n=36) or siRNA against AC9 (E, n=40). F: Means±SEM for the time-average glucose- and GLP-1-induced
[cAMP]_{pm} responses in MIN6 β-cells treated with control or AC9-specific siRNA. *, P<0.05, ***, P<0.005 for difference from control. G-J: TIRF recordings of insulin release from MIN6 β-cells expressing the fluorescent PtdIns(3,4,5)P_{3} reporter GFP_{4-Grp1}. Cells exposed to non-target control (G, n=54; and I, n=34) or AC9-specific siRNA (H, n=47; and J, n=27) were stimulated with 20 mmol/L glucose, 1 mmol/L 8-Br-cAMP and 100 nmol/L insulin as indicated. K: Means±SEM for the time-average PtdIns(3,4,5)P_{3} responses during the initial 10 minutes of glucose-stimulation and a 10-min period 20 minutes later with or without added 8-Br-cAMP. **, P<0.01 for difference from control.
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