cAMP Mediators of Pulsatile Insulin Secretion from Glucose-stimulated Single β-Cells*

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Pulsatile insulin release from glucose-stimulated β-cells is driven by oscillations of the Ca2+ and cAMP concentrations in the subplasma membrane space ([Ca2+]pm and [cAMP]pm). To clarify mechanisms by which cAMP regulates insulin secretion, we performed parallel evanescent wave fluorescence imaging of [cAMP]pm, [Ca2+]pm, and phosphatidylinositol 3,4,5-trisphosphate (PIP3) in the plasma membrane. This lipid is formed by autocrine insulin receptor activation and was used to monitor insulin release kinetics from single MIN6 β-cells. Elevation of the glucose concentration from 3 to 11 mM induced, after a 2.7-min delay, coordinated oscillations of [Ca2+]pm, [cAMP]pm, and PIP3. Inhibitors of protein kinase A (PKA) markedly diminished the PIP3 response when applied before glucose stimulation, but did not affect already manifested PIP3 oscillations. The reduced PIP3 response could be attributed to accelerated depolarization causing early rise of [Ca2+]pm that preceded the elevation of [cAMP]pm. However, the amplitude of the PIP3 response after PKA inhibition was restored by a specific agonist to the cAMP-dependent guanine nucleotide exchange factor Epac. Suppression of cAMP formation with adenyl cyclase inhibitors reduced already established PIP3 oscillations in glucose-stimulated cells, and this effect was almost completely counteracted by the Epac agonist. In cells treated with small interfering RNA targeting Epac2, the amplitudes of the glucose-induced PIP3 oscillations were reduced, and the Epac agonist was without effect. The data indicate that temporal coordination of the triggering [Ca2+]pm and amplifying [cAMP]pm signals is important for glucose-induced pulsatile insulin release. Although both PKA and Epac2 partake in initiating insulin secretion, the cAMP dependence of established pulsatility is mediated by Epac2.

Pancreatic β-cells release insulin in response to glucose stimulation, and appropriate secretion is essential for glucose homeostasis. Signals derived from glucose metabolism lead to closure of ATP-sensitive K+ (KATP) channels and depolarization of the plasma membrane. This in turn activates voltage-dependent Ca2+ entry, and the resulting elevation of the cytoplasmic Ca2+ concentration ([Ca2+]i) triggers exocytosis of insulin granules (1). Studies of the detailed kinetics of glucose-stimulated insulin release have clarified that it consists of distinct regular pulses, the first of which is more pronounced (2, 3). It has been proposed that the initial and late secretion may reflect release of insulin from functionally distinct granule pools (4, 5). The first phase has been proposed to correspond to exocytosis of granules from a small readily releasable pool with rise of [Ca2+]i, as the only triggering signal, whereas later secretion supposedly involves recruitment of granules from a reserve pool by a series of ATP-dependent reactions (6). Glucose induces oscillations of [Ca2+]i in β-cells (7), and these oscillations seem to underlie pulsatile release of insulin from isolated islets (8, 9) and individual β-cells (10–12).

Ca2+-triggered exocytosis is potently amplified by cAMP (13), and this messenger has long been known to mediate the insulinoctropic action of glucagon and the incretin hormones glucagon-like peptide 1 and glucose-dependent insulinoctropic polypeptide (13–15). In contrast, the role of cAMP in glucose-induced insulin secretion has been uncertain. Early studies indicated that glucose alone only modestly elevates cAMP (16–18) but that the sugar could amplify hormone-stimulated formation of cAMP (13, 19). More recently, fluorometric recordings of cAMP in MIN6 cells indicated that glucose alone elevates the intracellular cAMP level (20). Using a new technique for single-cell measurements of cAMP, we recently found that glucose triggers pronounced oscillations of the cAMP concentration beneath the plasma membrane ([cAMP]pm) and that these changes are important for regulating the kinetics of insulin secretion (21). However, the precise mechanisms by which cAMP acts remain unclear.

Protein kinase A (PKA) and the cAMP-regulated guanine nucleotide exchange factor cAMP-GEFII, also known as “Exchange protein directly activated by cAMP 2” (Epac2), are the major cAMP effectors expressed in β-cells (22–24). Studies of insulin granule exocytosis based on cell membrane capacitance measurements have indicated that cAMP amplification of insulin secretion involves both PKA-dependent and PKA-independent mechanisms (25–28). Although many proteins involved in the stimulus-secretion coupling and exocytosis of

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2The abbreviations used are: KATP channel, ATP-sensitive K+ channel; [Ca2+]i, cytoplasmic Ca2+ concentration; [Ca2+]pm, cytoplasmic Ca2+ concentration beneath the plasma membrane; [cAMP]pm, cAMP concentration beneath the plasma membrane; CFP, cyan fluorescent protein; DDA, 2′,5′-dideoxyadenosine; Epac, exchange protein directly activated by cAMP.
insulin granules have been identified as targets for PKA phosphorylation (25), inhibitors of this kinase have surprisingly small effects on glucose-induced insulin secretion (29, 30). Such modest effects may be expected if PKA only amplifies first phase secretion as indicated by recent imaging of exocytosis with fluorescent tracers (31). The PKA-independent amplification of insulin secretion by cAMP is probably mediated by Epac2, which has been proposed to act via the small GTPase Rap-1 (24), the regulatory SUR1 subunit of the KATP channel (27) as well as via SNAP-25 (32), Rim2, and Piccolo (33), which are proteins involved in the exocytosis machinery.

The aim of the present study was to clarify mechanisms by which cAMP oscillations contribute to glucose-induced pulsatile insulin secretion in individual β-cells. The data indicate that cAMP amplifies both the first and the subsequent pulses of secretion via Epac2. PKA seems important for establishing pulsatile insulin release by promoting concomitant initial elevation of the subplasma membrane Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{pm}$) and [cAMP]$_{pm}$, but the kinase is not required for maintaining already manifested pulsatile insulin secretion from glucose-stimulated β-cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents of analytical grade and deionized water were used. Insulin, tolbutamide, 3-isobutyl-1-methylxanthine (IBMX), EGTA, forskolin, methoxyverapamil, 2',5'-dideoxyadenosine (DDA), SQ22536, H89, and KT5720 were from Sigma. The Epac agonist 8-(4-chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate (007) and its acetoxymethyl ester, and PKA antagonist 8-(4-chlorophenylthio)-adenosine 3',5'-cyclic monophosphorothioate, Rp-isomer, were from Biolog Life Science Institute (Bremen, Germany). The acetoxymethyl esters of the Ca$^{2+}$ indicators Fluor-4, Fluor-5F, Fura Red, and Indo-1 was performed with an evanescent wave microscopy setup built around an E600FN upright microscope (Nikon, Kanagawa, Japan) contained in an expanded by a rotating light shaping diffuser (Physical Optics Corp., Torrance, CA) before being refocused through a modified quartz dove prism (Axicon, Minsk, Belarus) with 70° angle to achieve total internal reflection. Laser lines were selected

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FIGURE 1. Glucose triggers oscillations of [Ca^{2+}]_{pm} and [cAMP]_{pm} that underlie pulsatile insulin release. A, evanescent wave microscopy recordings of PIP_{3} with GFP_{4}-Grp1 (black trace, reflecting insulin secretion kinetics), [cAMP]_{pm} with ∆RII-CFP-CaaX and Ca^{2+}-YFP (green trace), and [Ca^{2+}]_{pm} with Fluo-4 (red trace) in three separate MIN6 β-cells. Elevation of the glucose concentration from 3 to 11 mM triggers oscillations in all three messengers. Images of GFP_{4}-Grp1 fluorescence are from the time points indicated by the numbered arrowheads. B, simultaneous evanescent wave microscopy recordings of PIP_{3} with PH_{Akt}-CFP (black trace), [cAMP]_{pm} with Ca^{2+}-YFP and a nonfluorescent ∆RII-CaaX (green trace), and [Ca^{2+}]_{pm} with Indo-1 (red trace) during stimulation of MIN6 cells with 20 mM glucose, showing coordinated increases of [Ca^{2+}]_{pm} and [cAMP]_{pm} that precedes the increase of PIP_{3}. C, imposed elevations of cAMP by intermittent application of 50 μM IBMX trigger pulsatile insulin secretion (n = 13).
with interference filters (Semrock, Rochester, NY) in a motorized filter wheel equipped with a shutter (Sutter Instruments, Novato, CA) blocking the beam between image captures. The coverslips with attached cells were used as exchangeable bottoms of an open 200-μl superfusion chamber. The chamber was mounted on the custom-built stage of the microscope such that the coverslip was maintained in contact with the dove prism by a thin layer of immersion oil. Fluorescence from the cells was collected through a 40 × 0.8-NA water immersion objective (Nikon), selected with interference filters (Semrock) at 405/35 nm (center wavelength/half-bandwidth) and 483/32 nm for Indo-1; 485/25 nm for CFP; 530/50 nm for GFP, Fluo-5F, and Fluo-4; 542/27 nm for YFP; and a >645-nm glass filter for Fura Red, and detected with a back-illuminated EMCCD camera (DU-887; Andor Technology, Belfast, Northern Ireland) under MetaFluor (Molecular Devices, Downingtown, PA) software control. If not otherwise stated, images were acquired every 2–5 s using exposure times in the 100–200-ms range.

Electrophysiology—Membrane potential was recorded in the perforated patch whole cell configuration using an EPC-9 patch clamp amplifier and Pulse software (Heka Elektronik, Lamprecht/Pfalz, Germany). Patch electrodes were pulled from borosilicate glass capillaries, coated with dental wax, and fire-polished. The extracellular solution consisted of 138 mM NaCl, 5.6 mM KCl, 2.6 mM CaCl₂, 1.2 mM MgCl₂, 3 mM glucose, and 5 mM HEPES (pH 7.4 using NaOH). The pipette solution consisted of 76 mM K₂SO₄, 10 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 5 mM HEPES (pH 7.15 with KOH) supplemented with 0.24 mg/ml amphotericin B. Drugs were dissolved in extracellular solution and applied locally through a 100-μm capillary.

siRNA Treatment, Real-time PCR, and Western Blotting—Where indicated, MIN6 cells were transfected with 100 nM siRNA (Sigma-Aldrich) against Epac2 (5'-gaguacacggugugucucatt-3') or luciferase-GL3 as control (5'-cuaacgacgagucucgatt-3'), alone or together with GFP-Grp1, 24–72 h prior to experiments. Efficiency of knockdown was verified with real-time PCR and immunoblotting. Total mRNA were isolated from MIN6 cells
using RNeasy Plus mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions and reverse-transcribed with SuperScript™ III First-strand synthesis system for reverse-transcription-PCR (Invitrogen) using random primers. The real-time PCR was performed using SYBR® Green JumpStart Taq ReadyMix™ (Sigma-Aldrich) and the following primers: Epac2 sense, 5′-ggtgctagctagcatcacc-3′ and antisense, 5′-cctctcttagaacaattca-3′, β-actin sense, 5′-gtcagagagctcctacc-3′ and antisense 5′-gggaccagaagctcctcatc-3′. PCR products were normalized to the housekeeping gene β-actin, and expression levels are given relative to control according to the formula: fold change = 2^ΔΔCt, where ΔΔCt = [Ct(Epac2 siRNA) − Ct(β-actin)] − [Ct(Epac2 control) − Ct(β-actin control)].

The level of Epac2 protein was determined by Western blotting. Samples were prepared by washing the MIN6 cells twice with phosphate-buffered saline followed by lysis in a buffer containing 150 mM NaCl, 20 mM Tris (pH 7.4), 0.1% SDS, 1% Triton X-100, 0.25% sodium deoxycholate, 2 mM EGTA, and a protease inhibitor mixture (Sigma-Aldrich) for 15 min. After lysis, the preparations were collected and centrifuged at 12,000 × g for 10 min at 4 °C, and the supernatant was collected and mixed with SDS-PAGE sample buffer containing 25 mM Tris-HCl (pH 6.8), 0.1% SDS, glycerol, and 2-mercaptoethanol, and boiled for 5 min. 20 μl of each sample was then subjected to SDS-PAGE. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane, and immunoblot analysis was performed with a mouse polyclonal antibody against Epac2 (generous gift from Johannes Bos, Utrecht University) using an ECL Plus Western blot detection system (GE Healthcare). Immunoreactive bands were imaged on a Kodak image station 4000MM.

Data and Statistical Analysis—Image analysis was performed using MetaFluor, MetaMorph (Universal Imaging), and ImageJ (W. S. Rasband, National Institutes of Health) software. Fluorescence intensities are expressed as changes in relation to initial fluorescence after subtraction of background (ΔF/F0). Igor Pro (Wavemetrics, Lake Oswego, OR) and Illustrator (Adobe Systems, San José, CA) software were used for curve fitting and illustrations. All data are presented as mean values ± S.E. Differences were statistically evaluated by two-tailed Student’s t test.

RESULTS

Glucose Induces Concomitant Elevations of [Ca^{2+}]_{pm} and [cAMP]_{pm} which Trigger Pulsatile Insulin Release—MIN6 β-cells responded after a 2–3-min delay to elevation of the glucose concentration from 3 to 11 mM with slow oscillations of [Ca^{2+}]_{pm} and [cAMP]_{pm} reported with evanescent wave microscopy and the Ca^{2+} indicator Fluo-4 and a PKA-based translocation biosensor (36), respectively (Fig. 1A). Cells expressing the PIP2-binding translocation biosensor GFPα-Grp1 showed an analogous response. After an initial increase of the fluorescence (124 ± 13%, n = 36) there were pronounced oscillations from a slightly elevated level (frequency 0.21 ± 0.01 min⁻¹, n = 36; Fig. 1A). We have previously demonstrated that this PIP3 response reflects autocrine activation of insulin receptors and phosphoinositide 3-kinase, which in turn parallels insulin secretion (11, 21).

To clarify the temporal relationship between the messengers, we simultaneously recorded PIP2, [cAMP]_{pm}, and [Ca^{2+}]_{pm} measured in the same cell using modified versions of the biosensors to avoid spectral overlap between the signals. Fig. 1B shows that elevation of the glucose concentration caused concomitant elevations of [Ca^{2+}]_{pm} and [cAMP]_{pm} that were followed by a rise of PIP3. Although [Ca^{2+}]_{pm} increased before [cAMP]_{pm} in some cells and after in others, the PIP3 response was typically delayed further. In simultaneous recordings of [cAMP]_{pm} and PIP3, the glucose-induced increase in [cAMP]_{pm} preceded that of PIP3 by 14 ± 3 s (n = 30). Most of this delay is explained by the time required for insulin receptor signal transduction, PIP3 formation, biosensor diffusion, and association with the membrane. Accordingly, exposure of the cells to 100 nM exogenous insulin caused membrane translocation of GFPα-Grp1 after 10 ± 1 s (n = 13; data not shown).

To test whether oscillations in [cAMP]_{pm} are sufficient to drive pulsatile insulin secretion from glucose-stimulated β-cells, MIN6 cells exposed to 11 mM glucose were subjected to brief (2–5 min) applications of the phosphodiesterase inhibitor IBMX, which results in elevations of [cAMP]_{pm} (36). Each application of IBMX triggered a rise of PIP3 that returned to base line when IBMX was removed (n = 13). This effect is particularly evident in Fig. 1C, which shows a cell with an initial peak response to glucose followed by stable, suprabasal second phase secretion. Imposed oscillations of [cAMP]_{pm} can thus generate pulsatile insulin secretion from glucose-stimulated β-cells.

Proper Glucose Stimulation of Insulin Secretion Requires PKA Activity—Suppression of cAMP production by inhibition of adenyl cyclases with 50 μM DDA markedly reduced the PIP3 response to glucose stimulation (Fig. 2, A, B, and E). In cells pretreated with DDA, the initial PIP3 response amplitude was reduced to 64 ± 8% of control (p < 0.01, n = 17). Although the amplitudes were decreased, glucose still triggered PIP3 oscillations in most cells exposed to DDA (Fig. 2B). Similar results
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A

11 mM glucose

B

11 mM glucose
100 μM Rp-cAMPS

C

Fluo-5F response amplitude (ΔF/ΔF₀)

Control Rp-cAMPS KT5720

D

Control

11 mM glucose

E

Rp-cAMPS

F

Control

11 mM glucose

G

KT5720

H

Control Rp-cAMPS

I

Control KT5720

J

cAMP behind cAMP ahead

Δtime (s)
were obtained with inhibitors of PKA. Accordingly, the amplitudes of the initial PIP₃ response to glucose were reduced to 66 ± 7% (p < 0.01, n = 35) and 41 ± 10% (p < 0.001, n = 19) of control by 100 μM Rp-8-CPT-cAMPS and 2 μM KT5720, respectively (Fig. 2, C–E), but the sugar still triggered PIP₃ oscillations. However, washout of the adenyl cyclase inhibitors or PKA inhibitors did not result in consistent normalization of the response amplitude within the time frame of the experiment. We also tested the importance of PKA for Ca²⁺ influx and insulin secretion elicited by depolarization with KCl, which preferentially triggers the fusion of primed readily releasable granules (39). When the cells were exposed to 30 mM KCl there was a rapid increase of [Ca²⁺]ᵢᵣm, followed after 16 ± 3 s (n = 14) by pronounced rise of GFPᵧ₋Grp1 fluorescence (92 ± 15% fluorescence increase, n = 14) and a decline to a sustained plateau (Fig. 2, F and G). The GFPᵧ₋Grp1 responses were not different in cells depolarized in the presence of Rp-8-CPT-cAMPS or DDA (Fig. 2, F–H), suggesting that cAMP/PKA is not required for the KCl-evoked exocytosis of primed granules. It was ascertained that the effects of the adenyl cyclase and PKA inhibitors were not due to a direct effect on phosphoinositide 3-kinase. Accordingly, slight tendencies of DDA and Rp-8-CPT-cAMPS to reduce PIP₃ formation induced by 300 nM insulin did not reach statistical significance (−11 ± 6%, n = 12 for DDA and −11 ± 8% for Rp-8-CPT-cAMPS; n = 8; data not shown). Similarly, when added to unstimulated cells, neither of the inhibitors affected the insulin-induced PIP₃ response (Fig. 2I). Together, these results indicate that activation of PKA is important for the ability of glucose to initiate insulin secretion properly.

**Temporal Dissociation of the Glucose-induced Initial [Ca²⁺]ᵢᵣm and [cAMP]ᵢᵣm Responses Results in Reduced Insulin Secretion**—We next investigated whether inhibition of PKA interferes with the glucose-induced elevation of [Ca²⁺]. MIN6 cells loaded with Fura-2 responded to elevations of the glucose concentration with slow, large amplitude [Ca²⁺]ᵢᵣm oscillations (first peak, 306 ± 40 mM; frequency, 0.27 ± 0.01 min⁻¹; n = 30; Fig. 3A). Inhibition of PKA with 100 μM Rp-8-CPT-cAMPS was without detectable effect on the magnitude of the glucose-induced [Ca²⁺]ᵢᵣm response (first peak, 340 ± 60 mM) and the frequency of the oscillations (0.32 ± 0.02 min⁻¹; n = 15; Fig. 3B). Similar results were obtained when Ca²⁺ was instead measured in the submembrane space with the much lower affinity indicator Fluo-5F (Fig. 3C).

Simultaneous evanescent wave microscopy recordings of [Ca²⁺]ᵢᵣm with Fura Red and PIP₃ with CFP-PHₐkt demonstrated that [Ca²⁺]ᵢᵣm started to increase 164 ± 5 s after glucose stimulation and PIP₃ after an additional 14 ± 3 s (n = 20; Fig. 3D). Inhibition of PKA with Rp-8-CPT-cAMPS reduced the delay between glucose stimulation and elevation of [Ca²⁺]ᵢᵣm to 134 ± 7 s (n = 11, p < 0.01) without significantly affecting the subsequent delay of the PIP₃ elevation (19 ± 5 s; Fig. 3, D and E). Similar results were obtained with KT5720 (data not shown). Although the CFP-PHₐkt fluorescence response to glucose was suppressed to 67% in the presence of PKA inhibitor (p < 0.05), there was no difference in the magnitude of the Fura Red response (Fig. 3H).

Also, the amplitude of the glucose-induced rise in [cAMP]ᵢᵣm was unaffected by PKA inhibition with 2 μM KT5720 (Fig. 3, F, G, and I). Moreover, the timing of the response was unchanged with a 176 ± 6 s (n = 100) delay between glucose stimulation and elevation of [cAMP]ᵢᵣm in control cells compared with 176 ± 10 s (n = 35) in KT5720-treated cells. However, simultaneous recordings of cAMP and PIP₃ indicated a striking shift in the timing of cAMP and insulin release. In control cells stimulated with 11 mM glucose alone, the initial increase in [cAMP]ᵢᵣm preceded that of PIP₃ in 78% of the cells, and the average time difference for all cells was 20 ± 4 s (n = 100; Fig. 3F). In contrast, during treatment with KT5720, the rise of [cAMP]ᵢᵣm typically lagged behind that of PIP₃, with an average time difference of 17 ± 6 s (n = 35; Fig. 3G). The earlier PIP₃ response reflects the shortened delay between glucose stimulation and elevation of [Ca²⁺]ᵢᵣm (see above), and under these conditions the amplifying cAMP signal has not yet been generated. Because the magnitudes of the [Ca²⁺]ᵢᵣm and [cAMP]ᵢᵣm elevations were unaffected, it seems likely that the temporal dissociation of the two signals explains the suppression of the early secretory response after PKA inhibition. Indeed, glucose elicited a 75% greater increase of PHₐkt-CFP fluorescence in control cells with [cAMP]ᵢᵣm increasing >10 s before PIP₃ than in the less common control cells with [cAMP]ᵢᵣm increasing >10 s after PIP₃ (n = 22; Fig. 3F).

To test the hypothesis further that dissociation of the triggering Ca²⁺ and amplifying cAMP signals underlies the impaired secretory response after PKA inhibition, the cells were stimulated by elevation of the glucose concentration from 3 to 11 mM, followed after 1 min by depolarization with the Kₐₜₚ channel blocker tobutamide. This treatment triggered Ca²⁺ influx before the glucose-induced cAMP signal was manifested and was associated with a smaller PIP₃ response (77 ± 5% of that observed with 11 mM glucose alone; p < 0.001, n = 141 and 157 cells with and without tobutamide, respectively; Fig. 4, A and
C). If glucose-induced Ca\(^{2+}\) influx was instead prevented by exposure to the hyperpolarizing K\(_{\text{ATP}}\) channel opener diazoxide for a longer period than normally required for the cAMP rise, the presence of a PKA inhibitor did not affect the PIP\(_3\) response when \([Ca^{2+}]_i\) was subsequently allowed to increase after washout of diazoxide (Fig. 4, B and C).

Because PKA-mediated phosphorylation has been reported to increase the K\(_{\text{ATP}}\) channel activity (40, 41), we hypothesized that PKA inhibition may accelerate the glucose-induced depolarization by decreasing K\(_{\text{ATP}}\) channel activity. The membrane potential was therefore recorded in MIN6 cells using the patch clamp technique in the perf...
rated patch configuration. In cells exposed to 3 mM glucose, PKA inhibition by Rp-8-CPT-cAMPS indeed caused depolarization, often associated with the appearance of action potentials (Fig. 4, D and E).

We next tested the possible role of Epac as mediator of cAMP-amplified insulin secretion. Whereas 100 μM Epac agonist 007 had little effect on the PIP3 response induced by rise of glucose from 3 to 11 mM under control conditions, it partially restored the response in cells treated with 100 μM PKA inhibitor Rp-8-CPT-cAMPS (Fig. 4, F and G) without changing the timing of the PIP3 signal (not shown). These results indicate that Epac is an important mediator of the glucose-induced cAMP effect on insulin secretion.

cAMP Dependence of Manifest Glucose-induced Pulsatile Insulin Secretion Is Mediated by Epac2—In contrast to the early glucose response, the established pulsatile insulin secretion was not reduced by inhibitors of PKA. Thus, after treatment with Rp-8-CPT-cAMPS (100 μM), KT5720 (2 μM), or H89 (10 μM) the amplitudes of the PIP3 oscillations were unaltered or even slightly increased, reaching 97 ± 4% (n = 38), 125 ± 10% (n = 35; p < 0.05), and 114 ± 6% (n = 27; p < 0.05) of control, respectively (Fig. 5, A–C). However, inhibition of adenylyl cyclases with 50 μM DDA or 400 μM SQ22536 reversibly suppressed the glucose-induced PIP3 response to 56 ± 5% (n = 39; p < 0.001) and 71 ± 4% (n = 51; p < 0.001) of control, respectively (Fig. 5, D–F). The action of cAMP is probably mediated by Epac because the agonist 007-AM restored the PIP3 response in cells treated with DDA or SQ22536 (Fig. 5, D–F). Similar results were obtained from the rat-derived β-cell line INS-1(832/13) (not shown). In addition, 007-AM restored spontaneously fading PIP3 oscillations in some glucose-stimulated cells (Fig. 5G).

To pinpoint the role of Epac further, we next knocked down the
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**A**

![Graph](image)

**B**

![Graph](image)

**C**

![Graph](image)

**FIGURE 6.** Down-regulation of Epac2 expression suppresses glucose-induced pulsatile insulin secretion. A, Epac2 mRNA and protein expression in MIN6 cells detected with real-time PCR and Western blotting 24 h (mRNA) or 72 h (protein) after treatment with 100 nM siRNA targeted to Epac2 or luciferase as control. B, evanescent wave microscopy recording of membrane PIP3 concentration during elevation of the glucose concentration from 3 to 11 mM in single GFP4-Grp1-expressing MIN6 β-cells treated with 100 nM control (n = 82) or Epac2 siRNA (n = 72). C, means ± S.E. (error bars) of the amplitudes of the initial glucose-induced PIP3 peak and the average for the subsequent oscillations. 

Epac2 isoform. Treatment of the cells with 100 nM siRNA directed against Epac2 reduced the corresponding mRNA levels to 40% after 24 h compared with control siRNA (Fig. 6A). The knockdown of Epac2 protein was verified with Western blotting of whole cell extracts after 72 h (Fig. 6A). After Epac2 knockdown only 13% (n = 71) of cells responded to 007-AM with PIP3 elevation as compared with 66% (n = 59) in control siRNA treated cells (p < 0.001; data not shown). The glucose-induced PIP3 response was also markedly suppressed after Epac2 knockdown and the amplitude of both the initial and subsequent oscillations reached only 60% of control (n = 72–82 cells, p < 0.01; Fig. 6, B and C).

**DISCUSSION**

The present study shows that cAMP is important for both initiating and maintaining pulsatile insulin release from glucose-stimulated MIN6 β-cells. During initiation of secretion, PKA activity is required to coordinate Ca2+ and cAMP elevations temporally, thereby allowing Epac to amplify the Ca2+-triggered exocytosis. However, PKA is also important for establishing subsequent pulsatile secretion. In contrast, already established pulsatile insulin secretion is maintained independently of PKA activity, and the cAMP dependence is mediated primarily by Epac2.

Similar to two-photon excitation imaging studies of the exocytosis in intact pancreatic islets (31), the present data indicate that PKA is involved primarily during early glucose-induced insulin secretion. Although voltage-dependent Ca2+ channels are substrates for PKA (25), PKA inhibitors did not suppress secretion by inhibiting the glucose-induced [Ca2+]i response. Neither did PKA inhibition interfere with the glucose-induced elevation of [cAMP]pm. Several proteins involved in insulin granule exocytosis are targets for PKA (25, 42), and PKA has been found to amplify exocytosis downstream of granule priming in a process regulated by ATP (28). On the other hand, we did not observe any effect of PKA inhibition on KCl depolarization-induced insulin secretion, which supposedly reflects exocytosis of primed, readily releasable granules (39). This observation is in line with results from membrane capacitance measurements showing that PKA has little effect on the readily releasable pool of granules (26).

Basal PKA activity seems to be a prerequisite for concomitant elevation of [Ca2+]pm and [cAMP]pm. Thus, after inhibition of PKA, the glucose-induced elevation of [Ca2+]pm and insulin secretion preceded the rise of [cAMP]pm. It is noteworthy that both the channel-forming Kir6.2 and the regulatory SUR1 subunit of the KATP channel are PKA substrates and that phosphorylation has been reported to increase channel activity (40, 41). Decreased KATP channel phosphorylation therefore likely explains the accelerated depolarization and earlier secretory response to glucose stimulation after PKA inhibition. However, there was no change in the timing of the cAMP elevation. This observation underscores that Ca2+ and cAMP are independently regulated in β-cells. Coordination of the Ca2+ and cAMP signals seems to be required for an optimal exocytosis response. An interesting possibility is that the reduced insulin secretion after inhibition of PKA is caused by temporal dissociation of the messenger signals, such that Ca2+ triggers exocytosis before the amplifying cAMP signal is manifested (Fig. 7). In support of this idea, we found that the initial secretory response was reduced when the KATP channel antagonist tolbutamide was used to induce precocious depolarization in glucose-stimulated MIN6 cells. Moreover, PKA inhibitors were without effect after synchronization of Ca2+ and cAMP eleva-
glucose

Control

PKA inactive

Epac inactive

Ca\(^{2+}\) cAMP PIP\(_3\)

Initial response
Subsequent oscillations

FIGURE 7. Model for the effect of PKA and Epac on glucose-induced pulsatile insulin secretion. Under control conditions, elevation of the glucose concentration triggers after a delay concomitant rises of \([Ca^{2+}]_{pm}\), (red trace) and \([cAMP]_{pm}\) (black trace), which are followed by an increase of membrane PIP\(_3\) (green trace), reflecting the more pronounced initial peak of insulin secretion. During subsequent pulsatile secretion, \([Ca^{2+}]_{pm}\) and \([cAMP]_{pm}\) elevations slightly precede the rises of PIP\(_3\). When PKA is inhibited, the initial delay for increase of \([Ca^{2+}]_{pm}\) is shortened. Moreover, The \([Ca^{2+}]\)-triggered rise of PIP\(_3\) is less pronounced than under control conditions and occurs before the elevation of \([cAMP]_{pm}\). In contrast, inhibition of PKA during manifested pulsatile secretion does not alter the responses. Inactivation of Epac does not affect the glucose-induced \([Ca^{2+}]_{pm}\) and \([cAMP]_{pm}\) signals, but lowers the amplitude of both the first peak and the subsequent PIP\(_3\) responses.

In summary, we found that temporal coordination of \([Ca^{2+}]_{pm}\) and \([cAMP]_{pm}\) signals are important for optimal glucose-induced insulin secretion from individual \(\beta\)-cells. Although initiation of glucose-stimulated secretion involves both PKA- and Epac-dependent mechanisms, the cAMP dependence of established pulsatile insulin secretion is mediated primarily by Epac2. Because type 2 diabetes is characterized by loss of glucose-stimulated first-phase insulin secretion (46) and perturbation of pulsatile secretion (47), clarification of the underlying \(Ca^{2+}\) and cAMP signaling events is important for understanding and correcting the defective \(\beta\)-cell function in this disease.

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REFERENCES


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ult-granule fusion events (43). Although it has been proposed that Epac primarily regulates exocytosis of small synaptic-like vesicles rather than insulin-containing dense core vesicles (44), our results based on the autocrine effect of secreted insulin favor the latter alternative. We now observed that both the initial peak of glucose-stimulated insulin release and the subsequent pulsatile secretion rely on a PKA-independent cAMP effector. The involvement of Epac was verified by two independent sets of experiments. First, pulsatile secretion perturbed by adenylyl cyclase inhibitors was restored by an Epac activator. Second, down-regulation of Epac2 expression with siRNA suppressed both the initial PIP\(_3\) response and the subsequent oscillations. However, the mechanisms of Epac2 action are not entirely clear. In addition to activating the small GTPase Rap1, which recently was found to play an important role in cAMP-potentiated exocytosis in \(\beta\)-cells (24), it is possible that Epac acts via mechanism(s) involving the SUR1 subunit of the K\(_{ATP}\) channel and/or the exocytosis proteins Rim2, Piccolo (45), and SNAP-25 (32).

In summary, we found that temporal coordination of \([Ca^{2+}]_{pm}\) and \([cAMP]_{pm}\) signals are important for optimal glucose-induced insulin secretion from individual \(\beta\)-cells. Although initiation of glucose-stimulated secretion involves both PKA- and Epac-dependent mechanisms, the cAMP dependence of established pulsatile insulin secretion is mediated primarily by Epac2. Because type 2 diabetes is characterized by loss of glucose-stimulated first-phase insulin secretion (46) and perturbation of pulsatile secretion (47), clarification of the underlying \(Ca^{2+}\) and cAMP signaling events is important for understanding and correcting the defective \(\beta\)-cell function in this disease.
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