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ARTICLE

Calcium influx activates adenylyl cyclase 8 for sustained insulin secretion in rat pancreatic beta cells

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Abstract

Aims/hypothesis Insulin is a key metabolic regulator in health and diabetes. In pancreatic beta cells, insulin release is regulated by the major second messengers Ca^{2+} and cAMP: exocytosis is triggered by Ca^{2+} and mediated by the cAMP/protein kinase A (PKA) signalling pathway. However, the causal link between these two processes in primary beta cells remains undefined.

Methods Time-resolved confocal imaging of fluorescence resonance energy transfer signals was performed to visualise PKA activity, and combined membrane capacitance recordings were used to monitor insulin secretion from patch-clamped rat beta cells.

Results Membrane depolarisation-induced Ca^{2+} influx caused an increase in cytosolic PKA activity via activating a Ca^{2+}-sensitive adenylyl cyclase 8 (ADCY8) subpool. Glucose

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stimulation triggered coupled Ca^{2+} oscillations and PKA activation. ADCY8 knockdown significantly reduced the level of depolarisation-evoked PKA activation and impaired replenishment of the readily releasable vesicle pool. Pharmacological inhibition of PKA by two inhibitors reduced depolarisation-induced PKA activation to a similar extent and reduced the capacity for sustained vesicle exocytosis and insulin release.

Conclusions/interpretation Our findings suggest that depolarisation-induced Ca^{2+} influx plays dual roles in regulating exocytosis in rat pancreatic beta cells by triggering vesicle fusion and replenishing the vesicle pool to support sustained insulin release. Therefore, Ca^{2+} influx may be important for glucose-stimulated insulin secretion.

Keywords Adenylyl cyclase 8 · Ca^{2+} · Pancreatic beta cell · Protein kinase A · Vesicle pool

Abbreviations

ΔR	Normalised FRET ratio
ADCY	Adenyl cyclase
$[Ca^{2+}]_i$	Intracellular free Ca^{2+} concentration
cAMP	Cyclic AMP
$[cAMP]_i$	Intracellular free cyclic AMP concentration
CFP	Cyan fluorescent protein
C_m	Membrane capacitance
DDA	2′,5′-dideoxyadenosine
EPAC2	Exchange protein directly activated by cAMP 2
FRET	Fluorescence resonance energy transfer
GLP-1	Glucagon-like peptide1
IBMX	3-Isobutyl-1-methylxanthine
IRP	Immediately releasable pool
KD	Knockdown
PDE	Phosphodiesterase
PKA	Protein kinase A
RRP	Readily releasable pool
sh-ADCY8	ADCY8 short hairpin RNA
sh-Ctr	Scrambled control short hairpin RNA
TEA	Tetraethylammonium chloride
YFP	Yellow fluorescent protein
VGCC	Voltage-gated Ca^{2+} channel

Introduction

Insulin regulates blood glucose metabolism and helps maintain energy homeostasis. Impaired insulin secretion leads to glucose intolerance and diabetes. In response to increased blood glucose, the cytosolic ATP/ADP ratio in pancreatic beta cells increases [1]. This change leads to the closure of K_{ATP} channels, which depolarises the membrane, causing Ca^{2+} influx and subsequent exocytosis of insulin granules [2, 3]. Thus, elevated intracellular Ca^{2+} ($[Ca^{2+}]_i$) in pancreatic beta cells is known to be the major trigger for glucose-stimulated insulin secretion.

Cyclic AMP (cAMP) is another important second messenger that enhances vesicle fusion via both protein kinase A (PKA)-dependent and PKA-independent pathways, which are dependent on exchange protein directly activated by cAMP 2 (EPAC2) [4–6]. Endogenous incretin hormones, such as glucose-dependent insulinotropic polypeptide [7] and glucagon-like peptide1 (GLP-1) [8], act on membrane receptors to generate cytoplasmic cAMP and regulate insulin secretion. Glucose metabolism also triggers cytoplasmic cAMP elevation and oscillations in pancreatic beta cells, although the underlying mechanisms and the physiological significance of this process remain unclear [9–12].

Crosstalk between these key regulators of insulin secretion, the Ca^{2+} and cAMP signalling pathways, occurs within individual beta cells. Both Ca^{2+}-activated and Ca^{2+}-inhibited adenyl cyclase (ADCY) isoforms are expressed in insulin-secreting cells [13]. Ca^{2+}-activated phosphodiesterase (PDE) has been proposed to mediate cAMP oscillations in clonal MIN6 cells [14, 15]. Similarly, cAMP accumulation activates PKA, leading to phosphorylation of voltage-gated Ca^{2+} channels (VGCCs) on the plasma membrane [16, 17] and inositol 1,4,5-trisphosphate and ryanodine receptors on the endoplasmic membrane [18, 19]. Owing to the complexity of these interactions, the temporal and causal relationships between the Ca^{2+}- and cAMP/PKA-mediated pathways in beta cells remain undefined [11, 13–15, 20, 21]. It is unclear whether and how glucose or depolarisation triggers activation of the cAMP/PKA pathway, and vice versa.

In the present study, we used a genetically encoded AKAR3 reporter [22] to monitor the real-time activity of PKA in primary beta cells using fluorescence resonance energy transfer (FRET). By combining membrane capacitance (C_m) measurements with FRET imaging in individual beta cells, we showed that depolarisation-induced Ca^{2+} influx activates PKA via an adenylyl cyclase 8 (ADCY8) subpool and thus helps to replenish the readily releasable pool (RRP) of insulin granules during glucose stimulation.

Methods

Detailed materials and methods are available in the electronic supplementary materials (ESM Methods).

Cell culture and transfection Pancreatic islet beta cells from adult Wistar rats (150–250 g) were isolated and cultured as previously described [23]. Briefly, rats were killed by cervical dislocation and islets were obtained from the pancreas by collagenase P digestion. After short-term tissue culture

(4–24 h) in RPMI 1640 medium, single beta cells were isolated by treating the islets with 0.025% trypsin (Invitrogen, Carlsbad, CA, USA). Transfection was conducted using the Neon 10 μl transfection system MPK10096 (Invitrogen). Experiments were performed 24–72 h after transfection, or >72 h for ADCY8 knockdown (KD).

Real-time FRET and Ca^{2+} imaging Live imaging of the AKAR3 fluorescent PKA reporter was performed using a Zeiss 710 inverted confocal microscope (Carl Zeiss, Oberkochen, Germany). AKAR3 was excited using a 405 nm laser, and a simultaneous two-channel mode was used for emission detection: one channel for cyan (466–489 nm) and the other for yellow (519–535 nm). The FRET ratio was calculated as $R=F_{YFP}/F_{CFP}$, and normalised as $\Delta R=\Delta R'/R$, where $\Delta R'$ is the absolute change in the FRET ratio, to take into account variation in the basal FRET ratio among different cells. For simultaneous imaging of $[Ca^{2+}]_i$ and FRET, cells were preloaded with 5 μmol/l Rhod-2 AM (Invitrogen) for <10 min, and the switched mode of frame-scan was used to alternately detect FRET and Ca^{2+} signals. Ca^{2+} signals were detected at 543 nm excitation and 560–620 nm emission. Images were acquired at 0.5 Hz, and the temperature was maintained at 28–32°C using a TempModule S (Carl Zeiss). Control solutions and drugs were applied locally to individual cells during recording using a multichannel microperfusion system [24].

Electrophysiology and membrane capacitance recording Whole-cell and perforated whole-cell configurations were used as previously described [3, 25]; the latter configuration was used during imaging to avoid loss of fluorescence intensity resulting from protein leakage into the pipette. For H-89 inhibition experiments, cells were dialysed with a solution containing 15 μmol/l H-89 for >7 min. Beta cells were characterised as healthy cells with a C_m of >4 pF and no Na^+ current [23]. The standard extracellular solution contained 118 mmol/l NaCl, 20 mmol/l tetraethylammonium chloride (TEA), 5.6 mmol/l KCl, 2.6 mmol/l $CaCl_2{\cdot}2H_2O$, 1.2 mmol/l $MgCl_2$, 5 mmol/l D-glucose and 5 mmol/l HEPES at pH 7.4. The intracellular solution contained 152 mmol/l $CsCH_3SO_3$, 10 mmol/l CsCl, 10 mmol/l KCl, 1 mmol/l $MgCl_2$ and 5 mmol/l HEPES, with pH adjusted to 7.35 using CsOH.

Detection of insulin release Insulin release from beta cells was measured by ELISA as previously described [25]. For ELISA, we used Krebs-Ringer buffer (KRB; 5 mmol/l KCl, 120 mmol/l NaCl, 15 mmol/l HEPES, pH 7.4, 24 mmol/l $NaHCO_3$, 1 mmol/l $MgCl_2$, 2 mmol/l $CaCl_2$ and 1 mg/ml BSA). Cells were treated with 105 mmol/l KCl for 1 min and the incubation solution was then collected for analysis. After incubation for 10 min with KRB (vehicle), PKA blocker (H-89) or ADCY8 blocker (2′,5′-dideoxyadenosine [DDA]), cells were treated with KCl for a further 1 min. All samples were then centrifuged at 16,000*g* for 5 min, and insulin levels in the supernatants were determined.

Statistical analysis All data were collected and analysed using Igor software (WaveMetrix, Lake Oswego, OR, USA). The means ± SEM were calculated, and the Student's *t* test was used to compare treatment effects. Statistical significance was set at $p<0.05$.

Results

Depolarisation induces PKA activation in pancreatic beta cells AKAR3, a genetically encoded fluorescent PKA reporter, was used to visualise PKA activity in beta cells. AKAR3 is a fusion peptide comprising cyan fluorescent protein (CFP), a phosphoamino acid-binding domain (14-3-3τ), a PKA-specific phosphorylatable peptide and yellow fluorescent protein (YFP). 14-3-3τ mediates signal transduction by binding to a PKA-phosphorylated substrate peptide. The resulting intramolecular conformational change alters the distance between CFP and YFP, thus leading to a reversible FRET signal [22]. As expected, when AKAR3 was transfected into primary beta cells, fluorescence was homogeneously distributed within the cytoplasm (Fig. 1e). The addition of 100 μmol/l 3-isobutyl-1-methylxanthine (IBMX; a PDE inhibitor) increased PKA activity, as shown by an increase in ΔR (Fig. 1a, d; $n=13$). Therefore, the AKAR3 probe can detect changes in PKA activity in live primary beta cells.

Stimulation of cells with 105 mmol/l KCl for 25 s ($n=14$) also evoked a robust increase in ΔR, comparable with that evoked by IBMX, followed by a slower (1–2 min) return to baseline (Fig. 1b, d). This ΔR value could not have been affected by intracellular pH because the pH did not alter during KCl treatment (ESM Fig. 1). A longer depolarisation period (100 s, $n=10$) did not lead to a further increase in ΔR (Fig. 1c, d), but was associated with a longer recovery time (time constant, tau) to the basal level (tau=171±45 s). In contrast, a longer application of IBMX ($n=10$) induced a much greater increase in ΔR (0.42±0.05), but with a similar recovery time (tau=54±9 s) as that following a 25 s IBMX treatment (Fig. 1c, d). Therefore, membrane depolarisation-induced PKA activity is saturable and reaches its peak value after 25 s KCl stimulation.

Rather than generating persistent membrane depolarisation, as seen with KCl, glucose induces short trains of action potential spikes in beta cells [15, 26]. Therefore, we used a perforated whole-cell voltage clamp, which keeps most of the intracellular metabolites intact during recording [3], to measure PKA activity evoked by a single depolarisation event. A 500 ms single-step depolarisation from −70 to

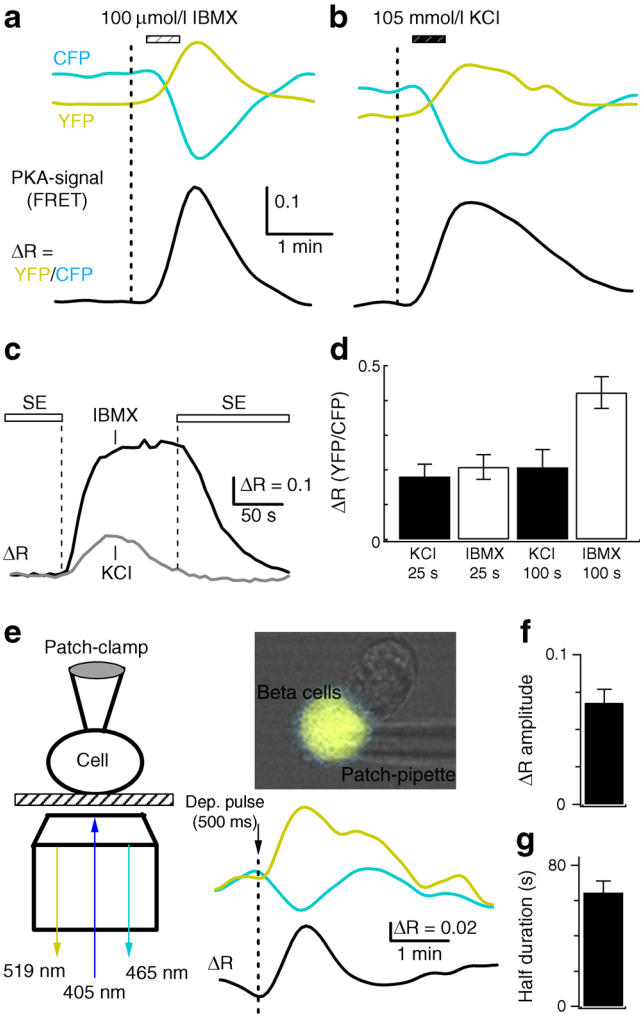

Fig. 1 FRET imaging of depolarisation-induced PKA activation in primary beta cells. (**a**, **b**) Changes in CFP (cyan) and YFP (yellow) signal intensity and ΔR (black) during the application of 100 µmol/l IBMX or 105 mmol/l KCl for 25 s to pancreatic beta cells. (**c**) ΔR changes during the application of 100 µmol/l IBMX or 105 mmol/l KCl for 100 s to the same cells (n=10). (**d**) Summary of ΔR changes, as measured in (**a**–**c**). (**e**) Experimental set-up and representative patch-clamp recording of AKAR3-expressing beta cells. Changes in CFP and YFP intensity and ΔR during a single 500 ms depolarisation event from −70 mV to 0 mV are also shown. (**f**, **g**) ΔR amplitude and half duration of FRET signals, as described in (**e**) (n=8 out of 32 responsive cells). Dep., depolarisation; SE, standard extracellular solution

0 mV, which is comparable with glucose-induced depolarisation, increased PKA activity in 25% of recorded beta cells (Fig. 1e, f; ΔR=0.08±0.01, n=8 out of 32 recorded cells). Although the ΔR increase was smaller, the recovery rate (tau=94±19 s) after depolarisation was similar to that induced by 25 s KCl stimulation (Fig. 1e, f). Thus, physiologically relevant depolarisation conditions can activate PKA in a proportion of beta cells.

Depolarisation-induced PKA activation is Ca^{2+} dependent To directly assess the temporal relationship between a depolarisation-evoked increase in $[Ca^{2+}]_i$ and PKA activation, we preloaded AKAR3-transfected beta cells with the Ca^{2+} indicator, Rhod-2 AM. The $[Ca^{2+}]_i$ increased rapidly following the application of 105 mmol/l KCl and then rapidly decreased to the basal level (Fig. 2c–e, rise time (RT)= 27±2 s; tau=63±15 s, n=11). These results contrast with the much slower changes in PKA activity (Fig. 2a, c; RT=59±5 s; tau=116±24 s, n=7). Latency between the maximum $[Ca^{2+}]_i$ and ΔR peaks was 28±5 s, indicating that PKA activation lags $[Ca^{2+}]_i$ elevation (Fig. 2f).

This temporal association suggests a causal relationship between increased $[Ca^{2+}]_i$ and PKA activation. To test this possibility, cells were sequentially stimulated with a KCl solution containing 2.6 mmol/l or 0 mmol/l Ca^{2+} for 25 s. When cells were depolarised in the Ca^{2+}-free solution, there was no PKA activation (Fig. 2a, b; n=7); however, the addition of 2.6 mmol/l Ca^{2+} partially restored PKA activation. Therefore, depolarisation-induced PKA activation is Ca^{2+} dependent.

When isolated single cells were challenged with 20 mmol/l glucose, oscillatory changes in PKA activity occurred at a frequency of approximately 0.3/min (Fig. 3a; n=8).

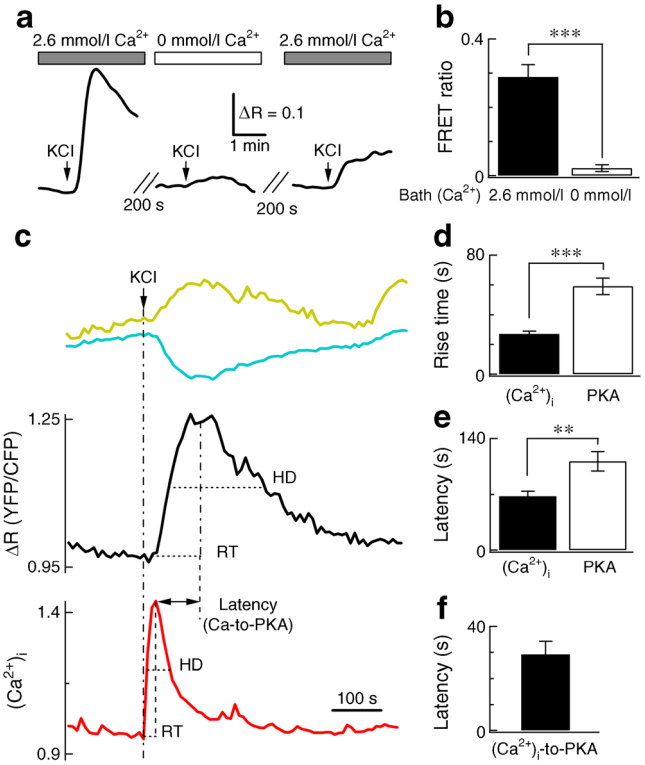

Fig. 2 Depolarisation-induced PKA activation is Ca^{2+}-dependent in primary beta cells. (**a**) Depolarisation-induced PKA changes in single beta cells suspended in 2.6 mmol/l Ca^{2+} or 0 mmol/l Ca^{2+} containing 2 mmol/l EGTA (n=7). (**b**) Graph showing PKA signals, as measured in (**a**). (**c**–**f**) Simultaneous recordings of FRET and $[Ca^{2+}]_i$ in single beta cells (n=11). $[Ca^{2+}]_i$ was measured using Rhod-2 AM. RT, rise time; HD, half-height duration; Latency (Ca-to-PKA), latency between $[Ca^{2+}]_i$ and PKA signal peaks; **p<0.01; ***p<0.001

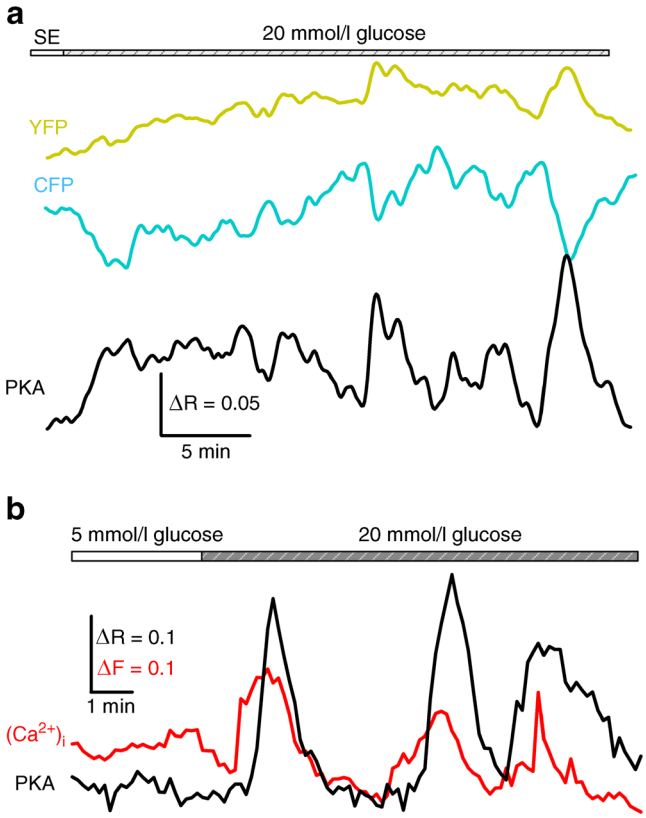

Fig. 3 Glucose-induced PKA oscillations are coupled to $[Ca^{2+}]_i$ oscillations in beta cells. (**a**) PKA oscillations induced by 20 mmol/l glucose (n=8). (**b**) Simultaneous recording of oscillatory $[Ca]_i$ (ΔF, red) and PKA (ΔR, black) signals induced by 20 mmol/l glucose in a representative cell bathed in standard extracellular solution (SE) (n=5)

Simultaneous monitoring of $[Ca^{2+}]_i$ and PKA activity showed a good temporal association between $[Ca^{2+}]_i$ spikes and ΔR spikes (Fig. 3b), implying a link between Ca^{2+} influx and PKA activation in glucose-mediated physiological processes, including glucose-stimulated insulin secretion.

ADCY8 is required for Ca^{2+}-dependent PKA activation Ca^{2+} modulates the intracellular free cAMP concentration ($[cAMP]_i$) and PKA activity via either Ca^{2+}-sensitive ADCY or Ca^{2+}-sensitive PDE [27]. Because depolarisation/Ca^{2+}-induced PKA activation showed different kinetics (slower decay rate) from those evoked by PDE inhibition (Fig. 1a, b), we speculated that ADCYs may play an important role. ADCY8 is activated by Ca^{2+}/calmodulin and participates in glucose- and GLP-1-mediated cAMP production in pancreatic beta cells [13, 28]. We therefore used RNA interference (RNAi)-based ADCY8 KD to determine the role of ADCY8 in depolarisation-evoked PKA activation.

ADCY8 was efficiently silenced by ADCY8 short hairpin RNA (sh-ADCY8) transfection; a scrambled short hairpin RNA served as control (sh-Ctr). This effect was completely reversed by expression of a short hairpin RNA-resistant form of ADCY8 ('rescue') in INS-1 cells (Fig. 4a, b; n=3). Membrane depolarisation with 105 mmol/l KCl induced a robust ΔR increase in sh-Ctr-expressing cells (Fig. 4c, f; ΔR=0.08±0.02, n=20), but failed to activate PKA in most ADCY8-KD cells (Fig. 4d, f; ΔR=0.017±0.014, n=18). Treatment with 100 μmol/l IBMX (positive control) evoked similar ΔR changes in both ADCY8 KD and control cells (Fig. 4c, d). Depolarisation-evoked PKA activation was probably caused by reduced ADCY8 expression because it was completely rescued by ADCY8 overexpression (Fig. 4e, f; ΔR=0.17±0.04, n=5). Therefore, ADCY8 mediates depolarisation-evoked PKA activation in rat primary beta cells.

Depolarisation-induced PKA activation replenishes depleted vesicle pools To investigate the role of PKA in vesicle pool replenishment, we stimulated primary beta cells with

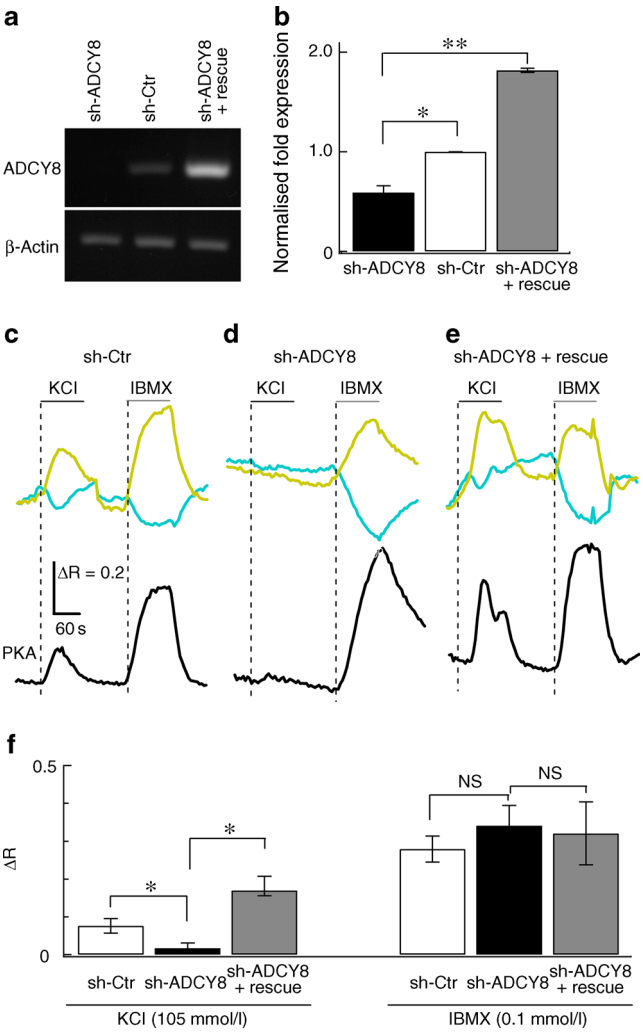

Fig. 4 ADCY8 is required for Ca^{2+}-dependent PKA activation in pancreatic beta cells. (**a**, **b**) Sh-ADCY8 KD and rescue efficiency in INS-1 cells, as assessed by RT-PCR and real-time PCR (n=3). (**c**–**e**) PKA changes induced by high KCl and IBMX in control (sh-Ctr; n=20), ADCY8-KD (sh-ADCY8; n=18) and rescued (sh-ADCY8 + rescue; n=5) beta cells. (**f**) Graphs summarising results from (**c**–**e**). p<0.05; ** p<0.01

depolarising pulse (or stimulus) trains from −70 to 0 mV at 100 ms intervals, and simultaneously measured ΔR (i.e. PKA activity) and C_m (i.e. exocytosis) in single beta cells (Fig. 5a). Each pulse train comprised five 50 ms depolarising pulses to deplete the immediately releasable pool (IRP1), followed by eight 500 ms depolarising pulses to induce exocytosis from the readily releasable pool (RRP1) [29]. A second identical stimulus pulse train was repeated 2 min after the first pulse train (IRP2 and RRP2, respectively). Recovery was estimated by determining the IRP2/IRP1 and RRP2/RRP1 ratios in the same cells.

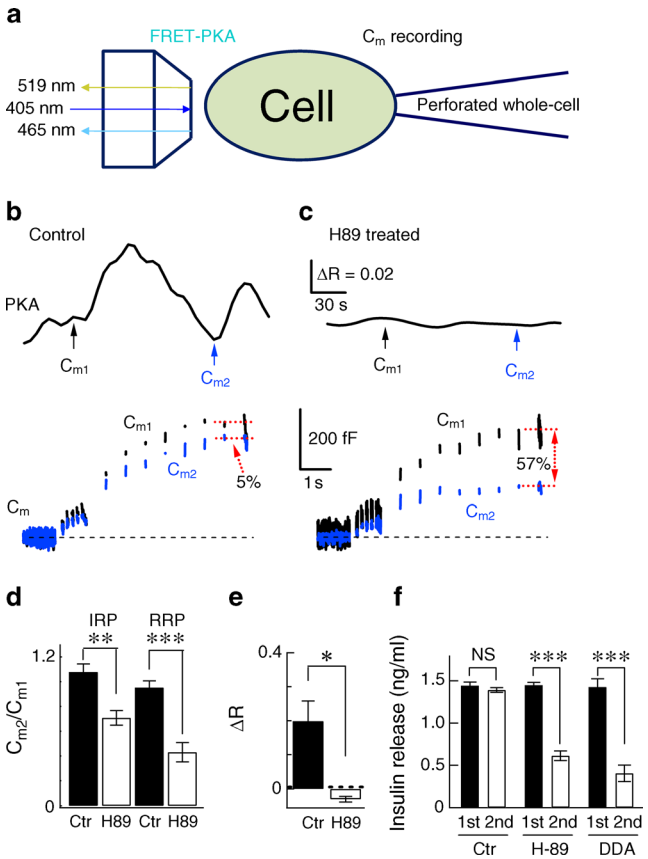

Fig. 5 Depolarisation-induced PKA activation is responsible for vesicle pool replenishment. (**a**) Representation showing simultaneous recording of PKA activity (ΔR) and C_m (exocytosis). (**b**) A train of depolarising pulses from −70 mV to 0 mV using voltage clamp-induced PKA (above) and exocytosis (below) signals in a single beta cell using a perforated whole-cell configuration. The stimulus train consisted of five 50 ms pulses to deplete vesicles from the IRP followed by eight 500 ms pulses to elicit exocytosis from the RRP, with 100 ms intervals between pulses. The stimulus trains were repeated twice, with a 2 min intervals between the first (C_{m1}, black) and second (C_{m2}, blue) stimulus train to allow vesicle pool replenishment. (**c**) Similar to (**b**) except that 15 μmol/l H-89 (PKA inhibitor) was present in the patch pipette. (**d**, **e**) Graphs showing C_{m2}/C_{m1} ratios for IRP and RRP (**d**) and PKA (**e**) evoked by the first pulse train in control (n=5) and H-89 treated (n=6) cells. (**f**) PKA activation is responsible for sustained insulin release from beta cells. Insulin release was stimulated by two pulses of KCl (105 mmol/l, 1 min; 10 min interval) and measured by ELISA. H-89 (15 μmol/l) or DDA (100 μmol/l) significantly inhibited insulin release following the second stimulus to 0.42 (p<0.01) and 0.25 (p<0.01) ng/ml, respectively (n=4). p<0.05; ** p<0.01; ***p<0.001

Typically, the first depolarising pulse train led to an increase in ΔR comparable to that evoked by a 100 s KCl stimulation (Fig. 5b, e; ΔR=0.20±0.06, n=5). The second pulse train was applied approximately 100 s later, when the cytoplasmic PKA activity had returned to the basal level (Fig. 5b). Under control conditions, C_m changes triggered by the second pulse train were similar to those triggered by the first (Fig. 5b, d; IRP2/IRP1 1.08±0.07, RRP2/RRP1 0.95±0.05), indicating complete replenishment of both pools (IRP and RRP). In contrast, 7–10 min after cell dialysis with a patch pipette containing H-89 (a membrane-permeable PKA inhibitor [30]), no increase in ΔR was induced by the first or second depolarising pulse train (Fig. 5c, e), indicating a complete blockade of PKA activation by H-89. The IRP (75±12 fF) and RRP (279±18 fF) induced by the first depolarising pulse train remained unchanged in the presence of H-89. However, the recovery of these pools before the second pulse train was severely impaired by PKA inhibition (Fig. 5c, d; IRP2/IRP1 0.71±0.06, RRP2/RRP1 0.43±0.08, n=6).

Next, we examined insulin release from dispersed islet beta cells using ELISA. Two sequential depolarisation events 10 min apart each triggered almost the same amount of insulin release in control cells (Fig. 5f). However, cells treated with either H-89 or the transmembrane ADCY inhibitor DDA during the interval between depolarising pulse trains exhibited significantly less insulin release in response to the second vs the first stimulus (Fig. 5f; n=4 for each experimental condition). Consistent with this, Rp-cAMPS, a more selective cAMP/PKA inhibitor, had similar inhibitory effects on both phases of glucose-induced insulin secretion (ESM Fig. 2). Taken together, these findings suggest that PKA activity stimulated by depolarisation and/or Ca^{2+} entry plays a central role in refilling insulin vesicle pools in rat primary beta cells.

ADCY8 activation facilitates sustained vesicle fusion Similar levels of PKA activation were induced by either a standard depolarising pulse train lasting for 5 s or a 100 s KCl stimulation (Figs 1d and 5e), suggesting that Ca^{2+} influx evoked by a 5 s pulse train maximally activates ADCY8 and PKA upon membrane depolarisation. In addition to Ca^{2+}, GLP-1 is known to activate ADCY8 via stimulation of the GLP-1 receptor [28]. In primary beta cells, 100 nmol/l GLP-1 evoked a much greater level of PKA activation compared with KCl (ESM Fig. 3; n=5). GLP-1-induced PKA activity was reduced by approximately 60% in ADCY8-KD cells (ESM Fig. 4), suggesting that ADCY8 also has a major role in hormone-induced cAMP/PKA activation. In addition, ADCY8 KD had a greater effect on GLP-1-triggered PKA activation (approximately 60% reduction; ESM Fig. 4) than on PKA activation evoked by membrane depolarisation (Fig. 4f), indicating that membrane depolarisation does not activate all of the

functional ADCY8 in beta cells. To confirm that the Ca^{2+}-responsive ADCY8 subpool is functionally important, we used a standard whole-cell patch-clamp configuration with an ATP- and cAMP-free intracellular solution to examine the role of ADCY8 in vesicle pool replenishment. In sh-Ctr-expressing cells, the increase in C_m induced by the second depolarising pulse train was somewhat lower than that of the first pulse train (by 33%) as a result of rundown (Fig. 6a, c; IRP2/IRP1 0.97±0.19, RRP2/RRP1 0.67±0.07; but see [5]). Thus, in control cells, the first pulse train evoked transient PKA activation that causes refilling of most of the IRP and RRP. In ADCY8-KD cells, the depolarisation-induced $[Ca^{2+}]_i$ rise was intact (ESM Fig. 5; n=11) and the first pulse train induced a C_m increase similar to that of control cells (Fig. 6b, c; IRP 15.0±2.8 fF, RRP 85.6±17.1 fF); however, secretion induced by the second pulse train was dramatically impaired (Fig. 6b, d; IRP2/IRP1 0.42±0.12, RRP2/RRP1 0.35±0.09). The recovery rate was markedly slower in ADCY8-KD cells than in control cells (Fig. 6a, d), indicating that replenishment of vesicle pools is impaired by ADCY8 KD. Taken together, these data suggest that activation of ADCY8 by depolarisation is responsible for replenishing insulin vesicle pools in rat beta cells.

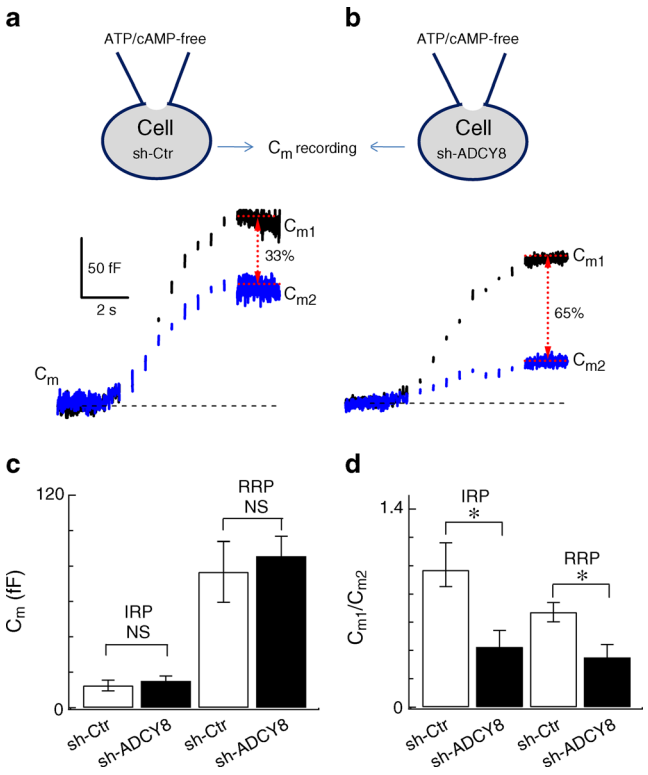

Fig. 6 ADCY8 is responsible for vesicle pool replenishment. (**a**, **b**) C_m recordings under whole-cell dialysis with an ATP- and cAMP-free solution in ADCY8 KD (n=11) and control (n=6) cells. (**c**, **d**) Graphs summarising exocytosis and C_{m2}/C_{m1} ratios for both IRP and RRP induced by the first pulse train, from (**a**, **b**). $p<0.05$

Discussion

A major finding of the present study is that depolarisation-induced Ca^{2+} influx has dual effects on insulin release in beta cells, by directly triggering vesicle fusion (for instant release) and indirectly promoting vesicle replenishment (for sustained release). Regarding the relationship between Ca^{2+} and vesicle pools, it has been established that intracellular Ca^{2+} triggers vesicle release via stimulating Ca^{2+}-dependent vesicle fusion to the plasma membrane in neurons and endocrine cells [24, 31–33], including beta cells [25, 34–36]. However, multiple mechanisms have been proposed to promote vesicle pool replenishment in excitable cells. In chromaffin cells and neurons, it is known that basal $[Ca^{2+}]_i$ level can modulate the vesicle pool [37, 38]. Previous pharmacological experiments established that cAMP also facilitates replenishment of the vesicle pool in beta cells via both PKA-dependent and EPAC2-dependent pathways [2, 4–6, 34]. Here, by combining PKA imaging with C_m recording, we found that ADCY8 links the Ca^{2+}- and cAMP/PKA-dependent pathways (described above) in rat primary beta cells. When using a physiologically relevant stimulation pattern (Fig. 3), the first depolarising pulse train caused not only the first phase of secretion and PKA activation but also facilitated exocytosis/insulin release induced by the second pulse train through vesicle pool replenishment supported by PKA activation (Figs 5 and 6). These findings suggest that Ca^{2+}- and ADCY8-dependent PKA pathways may also play critical roles in promoting sustained insulin release upon glucose challenge under physiological conditions.

The second important finding is the causal link between depolarisation-induced Ca^{2+} entry and enhanced PKA activity in rat primary beta cells. Previous studies using genetic indicators of cAMP levels in clonal MIN6 cells showed coordinated glucose-stimulated oscillations in Ca^{2+} and cAMP [14, 20], as well as Ca^{2+} influx and an ADCY8-induced cAMP signal [39]. A recent study reported that Ca^{2+} and cAMP oscillate in synchrony and that PKA activity lags behind cAMP changes such that an increase in PKA activity precedes the next round of Ca^{2+} signalling after depolarisation in MIN6 cells [15]. Differences between stimuli (KCl/glucose vs TEA) may account for the observed differences in lag times between different signals. In the present study, we demonstrated that a physiologically relevant depolarisation-triggered transient increase in $[Ca^{2+}]_i$ precedes the transient PKA activation by 28 s in primary beta cells (Fig. 2c). Moreover, the depolarisation-evoked PKA activation was abolished by removing extracellular Ca^{2+} (Fig. 2a, b). Glucose-stimulated PKA activity was also coupled to $[Ca^{2+}]_i$, although the lag time and the waveform linking them were variable (Fig. 3b). This variation may be caused by glucose promotion of cAMP production via metabolic coupling factors unrelated to Ca^{2+} [11, 20, 40]. These results established the important contribution of

elevated $[Ca^{2+}]_i$ to activation of the glucose-triggered cAMP/PKA pathway in native beta cells, as distinct from previous studies using cell lines.

Our third finding is that an ADCY8 subpool in beta cell contributes to depolarisation-induced PKA activation. ADCY8 is an important mediator of cAMP accumulation evoked by incretin stimulation [28]. Previous studies also revealed that A kinase-anchoring protein 79/150 (AKAP79/150) specifically interacts with ADCY8 and thus has the potential to produce localised cAMP signalling evoked by local Ca^{2+} entry [41]. Here, we showed that the level of PKA activation following depolarisation triggered by 105 mmol/l KCl perfusion for 25–100 s was similar to that triggered by patch-clamp depolarisation for ≤5 s (Figs 1 and 5). This was not a consequence of probe saturation because 100 s IBMX evoked an even greater response in the same cells (Fig. 1c, d). Instead, our interpretation of the data is that an ADCY8 subpool is activated by membrane depolarisation. Although ADCY8 is almost completely absent from human beta cells [12, 42], this finding may help to reveal the roles of other ADCY isoforms, such as ADCY5, which is involved in human islet function [12].

In a physiological context, each wave within the glucose-induced membrane potential oscillations may maximally and repeatedly activate a proportion of the cAMP/PKA pathway. As shown in Fig. 3, PKA peaks triggered by 20 mmol/l glucose had a similar amplitude and dynamics to those evoked by a single train of depolarisation. Such an arrangement ensures sustained insulin vesicle trafficking and secretion in single primary beta cells. Here, consistent with previous reports [13, 40, 43], GLP-1 evoked a much greater level of PKA activation than that evoked by depolarisation alone (ESM Fig. 3), suggesting that incretins activate an additional ADCY8 subpool [43]. ADCY8 proteins activated at later time points may be more distant from VGCCs and thus incapable of activation by the depolarisation-induced Ca^{2+} influx alone (Fig. 7). Figure 7 summarises the results of present study: glucose stimulation generates a transient microdomain of high $[Ca^{2+}]_i$ that promotes insulin secretion through dual pathways, i.e. by directly triggering vesicle fusion and indirectly activating ADCY8/PKA to enhance vesicle pool replenishment. A VGCC-coupled ADCY8 subpool plays a central role in Ca^{2+}-evoked PKA activation, which is partially responsible for glucose- and GLP-1-stimulated cAMP production. This study

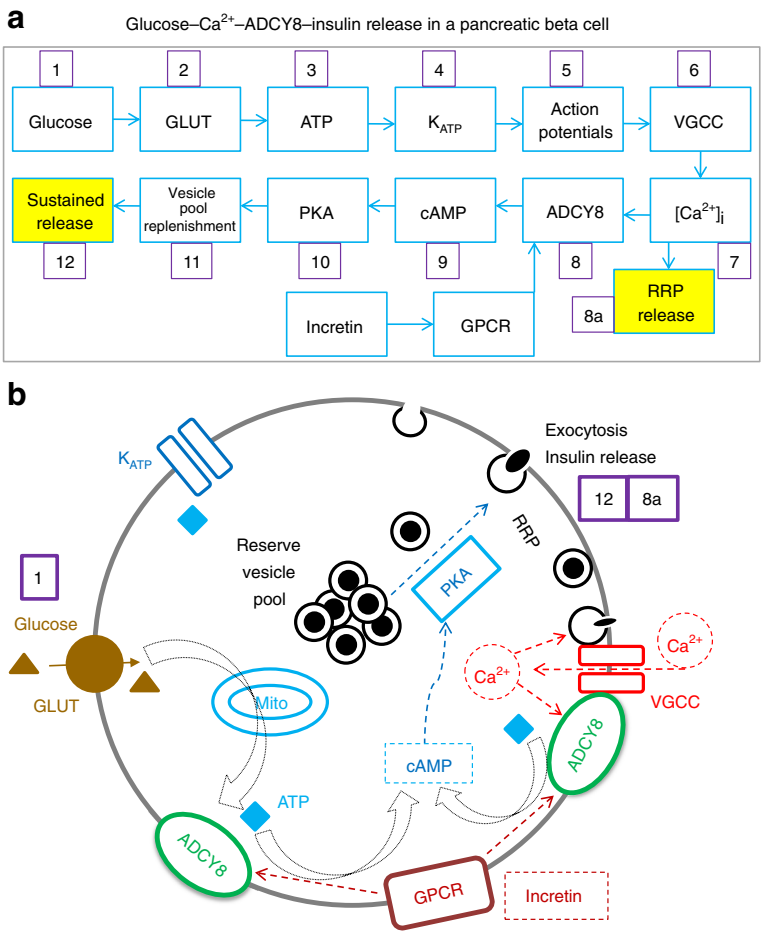

Fig. 7 Proposed model for the control of insulin release by the glucose/Ca^{2+}/ADCY8 pathway. (**a**) Following an increase in blood glucose (1), the GLUT delivers glucose into beta cells (2), followed by a mitochondria-mediated increase in the cytosolic ATP/ADP ratio (3). This change leads to K_{ATP} channel closure (4) and action potential firing (5). During membrane depolarisation, VGCCs open (6), leading to increased $[Ca^{2+}]_i$ (7), which triggers insulin vesicle exocytosis (8a). Increased $[Ca^{2+}]_i$ also stimulates ADCY8 activation (8), leading to cAMP production (9) and PKA activation (10). PKA activation is responsible for vesicle pool replenishment (11) and maintaining insulin secretion (12). Incretins (such as GLP-1) activate the additional VGCC-insensitive ADCY8 subpool and can trigger higher levels of cAMP/PKA pathway activation compared with depolarisation. (**b**) PKA activation stimulates reserve pool vesicles into the RRP necessary for sustained insulin secretion. Mito, mitochondrion

has improved our understanding of glucose- and Ca^{2+}-regulated vesicle trafficking and pulsatile insulin secretion [2, 3, 44] under normal physiological and diabetic conditions.

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