REGULAR PAPER

Glucose-induced cAMP elevation in β-cells involves amplification of constitutive and glucagon-activated GLP-1 receptor signalling

Hongyan Shuai[1,2] | Yunjian Xu[2] | Parvin Ahooghalandari[2] | Anders Tengholm[2] 🔵

[1]School of Basic Medicine Sciences, Dali University, Yunnan, China

[2]Department of Medical Cell Biology, Biomedical Centre, Uppsala University, Uppsala, Sweden

Correspondence
Anders Tengholm, Department of Medical Cell Biology, Uppsala University, Biomedical Centre, Uppsala, Sweden.
Email: anders.tengholm@mcb.uu.se

Abstract

Aim: cAMP typically signals downstream of G_s-coupled receptors and regulates numerous cell functions. In β-cells, cAMP amplifies Ca^{2+}-triggered exocytosis of insulin granules. Glucose-induced insulin secretion is associated with Ca^{2+}- and metabolism-dependent increases of the sub-plasma-membrane cAMP concentration ($[cAMP]_{pm}$) in β-cells, but potential links to canonical receptor signalling are unclear. The aim of this study was to clarify the role of glucagon-like peptide-1 receptors (GLP1Rs) for glucose-induced cAMP signalling in β-cells.

Methods: Total internal reflection microscopy and fluorescent reporters were used to monitor changes in cAMP, Ca^{2+} and ATP concentrations as well as insulin secretion in MIN6 cells and mouse and human β-cells. Insulin release from mouse and human islets was also measured with ELISA.

Results: The GLP1R antagonist exendin-(9-39) (ex-9) prevented both GLP1- and glucagon-induced elevations of $[cAMP]_{pm}$, consistent with GLP1Rs being involved in the action of glucagon. This conclusion was supported by lack of unspecific effects of the antagonist in a reporter cell-line. Ex-9 also suppressed IBMX- and glucose-induced $[cAMP]_{pm}$ elevations. Depolarization with K^+ triggered Ca^{2+}-dependent $[cAMP]_{pm}$ elevation, an effect that was amplified by high glucose. Ex-9 inhibited both the Ca^{2+} and glucose-metabolism-dependent actions on $[cAMP]_{pm}$. The drug remained effective after minimizing paracrine signalling by dispersing the islets and it reduced basal $[cAMP]_{pm}$ in a cell-line heterologously expressing GLP1Rs, indicating that there is constitutive GLP1R signalling. The ex-9-induced reduction of $[cAMP]_{pm}$ in glucose-stimulated β-cells was paralleled by suppression of insulin secretion.

Conclusion: Agonist-independent and glucagon-stimulated GLP1R signalling in β-cells contributes to basal and glucose-induced cAMP production and insulin secretion.

KEYWORDS
adenylyl cyclase, exendin-(9-39), glucagon, glucagon-like peptide-1, insulin secretion, pancreatic islets

1 | INTRODUCTION

Appropriate insulin secretion from pancreatic β-cells is critical for glucose homeostasis and defective β-cell function may consequently lead to impaired glucose tolerance and overt diabetes mellitus.[1] Glucose is the major physiological regulator of insulin release but secretion is also modulated by neural factors and hormones. Glucagon from pancreatic α-cells, and the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide from enteroendocrine L- and K-cells respectively amplify glucose-stimulated insulin release.[2-4] Glucose stimulus-secretion coupling involves uptake and metabolism of the sugar in the β-cell, an increased intracellular ATP/ADP ratio, closure of ATP-sensitive K^+ (K_{ATP}) channels, membrane depolarisation and Ca^{2+} influx through voltage-dependent channels. The resulting increase of the cytoplasmic Ca^{2+} concentration triggers exocytosis of insulin secretory granules.[4,5] Glucose metabolism also generates factors that amplify insulin secretion by enhancing exocytosis without influencing the triggering Ca^{2+} signal.[3,5] One such amplifying factor is cAMP. It is produced by adenylyl cyclases and degraded via phosphodiesterases and amplifies insulin secretion by activating protein kinase A and Epac2.[6] Adenylyl cyclase activity is typically stimulated by activation of $G_s\alpha$-coupled receptors, like glucagon and GLP-1 receptors,[6,7] but also glucose stimulation elevates cAMP in β-cells.[8-13] Recordings of cAMP and protein kinase A activity in single β-cells have demonstrated that the glucose-induced cAMP increase often is characterized by oscillations,[10,12,13] which along with similar oscillations of the cytoplasmic Ca^{2+} concentration contribute to the generation of pulsatile insulin release.[6,10]

The mechanisms underlying glucose-induced cAMP formation in β-cells remain unclear. It is well established that cAMP production is stimulated by Ca^{2+},[14,15] which is explained by the expression of Ca^{2+}-activated adenylyl cyclase isoforms.[13,16,17] Ca^{2+}-inhibited adenylyl cyclase isoforms[18,19] and Ca^{2+}-activated phosphodiesterases[20-22] appears less important but may contribute to the generation of oscillations.[22,23] In addition to Ca^{2+}, there is a stimulatory effect of cell metabolism on adenylyl cyclases,[10] which might be due to increase of ATP,[10] the substrate for cAMP production, and lowering of AMP,[24] an inhibitor of adenylyl cyclase activity.[25] It has been suggested that paracrine glucagon release from α-cells is crucial for the β-cell cAMP response.[26,27] Glucagon may activate not only glucagon receptors but also GLP-1 receptors.[27-30] Studies in islets from GLP-1 receptor-deficient mice have shown lower glucose-induced cAMP production,[31] and GLP-1 receptor inhibition with exendin-(9-39) (ex-9) reduces glucose-stimulated insulin secretion in mouse and human islets[27,30,31] and perfused mouse pancreas.[29]

In this study, we used live-cell imaging techniques and ELISA hormone detection to clarify the involvement of $G_s\alpha$-coupled receptors for glucose-induced changes of the sub-plasma membrane cAMP concentration ($[cAMP]_{pm}$) in MIN6 cells and primary mouse and human β-cells. We demonstrate that constitutive agonist-independent, and glucagon-activated GLP-1 receptor signalling is involved in basal and glucose-stimulated cAMP production and insulin secretion.

2 | RESULTS

2.1 | GLP-1 receptor antagonism inhibits both GLP-1- and glucagon-stimulated cAMP production in β-cells

Recordings of $[cAMP]_{pm}$ with a ratiometric translocation biosensor in single MIN6-cells and primary β-cells within intact mouse and human pancreatic islets exposed to 3 mmol/L glucose showed that all cell preparations responded as expected to both GLP-1 (Figure 1A-C) and glucagon (Figure 1D-F) at 10 nmol/L with a prompt increase of $[cAMP]_{pm}$. In most cells, the $[cAMP]_{pm}$ elevation was sustained throughout the stimulation period, whereas in a few cases the response was transient (not shown). The GLP-1 receptor antagonist ex-9 suppressed not only the $[cAMP]_{pm}$ increase induced by GLP-1 (Figure 1A-C,H), but also that of glucagon (Figure 1D-F,I), and there was a slight effect also on basal $[cAMP]_{pm}$ (Figure 1G,H). The effect of ex-9 was reversible and $[cAMP]_{pm}$ often reached near maximally stimulated levels within 10-15 minutes after washout of the inhibitor.

2.2 | GLP-1 and ex-9 do not affect glucagon receptor signalling whereas glucagon promotes cAMP elevation via GLP-1 receptors

There is a high degree of homology between the GLP-1 and glucagon receptors and both hormones may activate both receptors.[28] To clarify to what extent GLP-1 and ex-9 act on glucagon receptors we took advantage of a reporter cell line that expresses glucagon receptors and $G_{15}\alpha$, which signals via phospholipase C, thus allowing receptor activation to be monitored as increases in $[Ca^{2+}]_{pm}$. Some cells exhibited fast $[Ca^{2+}]_{pm}$ oscillations prior to stimulation (not shown) and only cells with low and stable $[Ca^{2+}]_{pm}$ under basal conditions were selected for analyses. While 10 nmol/L glucagon induced prompt increase of $[Ca^{2+}]_{pm}$ with fast oscillations (Figure 2A,B,D), there was no effect of 100 nmol/L GLP-1 (Figure 2C). The glucagon effect was efficiently reversed by a glucagon receptor antagonist (GCGR-A; "compound 15"[32]; Figure 2B), whereas 0.1-1 μmol/L ex-9 lacked effect (Figure 2D,E). These data show that GLP-1 and ex-9 do not affect glucagon receptors at the concentrations tested. Recordings of $[cAMP]_{pm}$ from HEK293 cells, which lack endogenous glucagon and GLP-1 receptors, demonstrated
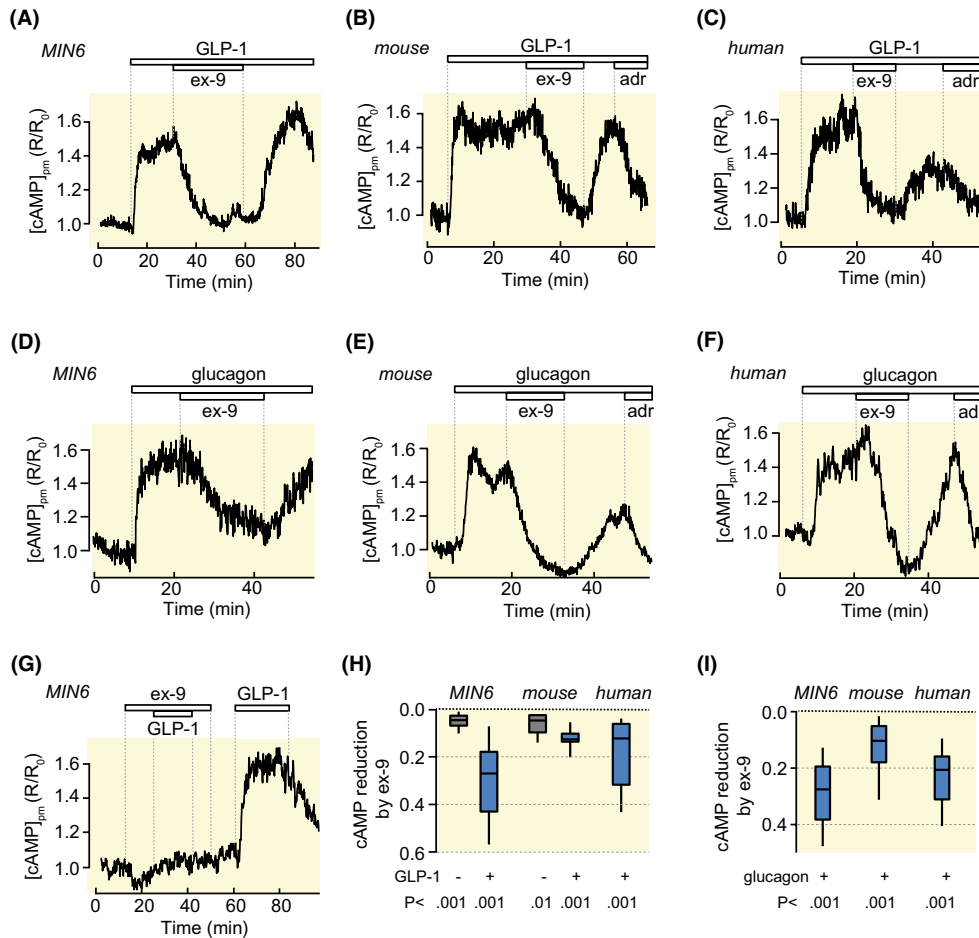
FIGURE 1 GLP-1 receptor antagonism inhibits both GLP-1- and glucagon-stimulated [cAMP]$_{pm}$ increases in β-cells. A-C, [cAMP]$_{pm}$ recordings from a single MIN6 β-cell (A, representative for 26 cells from four experiments), and single mouse (B, 36 cells from eight experiments) and human (C, representative for 20 cells from five experiments with islets from two donors) β-cells within intact islets, showing that ex-9 prevents the [cAMP]$_{pm}$ elevation induced by 10 nmol/L GLP-1. The [cAMP]$_{pm}$-lowering effect of 10 µmol/L adrenaline (adr) is used to verify the identity of the islet β-cells. D-F, Similar [cAMP]$_{pm}$ recordings from single MIN6 (D, representative for 25 cells from four experiments), mouse (E, representative for 20 cells from six experiments) and human (F, representative for 14 cells from 6 experiments with islets from three donors) β-cells showing that ex-9 prevents [cAMP]$_{pm}$ elevations induced by 10 nmol/L glucagon. G, Recording from a MIN6 β-cell showing that ex-9 reduces basal [cAMP]$_{pm}$ and prevents the GLP-1-induced elevation. Representative for 23 cells from four experiments. H, I, Box plots of the magnitudes of the [cAMP]$_{pm}$ reductions induced by ex-9 in cells stimulated with GLP-1 (H) or glucagon (I). P values are given for comparisons of mean values with Student's paired *t* test

that expression of a GFP-tagged GLP-1 receptor construct rendered the cells responsive to both glucagon and GLP-1, and that the hormone-induced [cAMP]$_{pm}$ increases were inhibited by ex-9 (Figure 2F-I). The observation that ex-9 prevents glucagon-induced [cAMP]$_{pm}$ elevations in β-cells therefore indicates that glucagon mainly acts via GLP-1 receptors in these cells.

2.3 | GLP-1 receptors contribute to basal cAMP production and to glucose-stimulated [cAMP]$_{pm}$ elevation in islets and single β-cells

There is high turnover of cAMP in β-cells and basal cAMP production is balanced by phosphodiesterase-mediated degradation. Inhibition of phosphodiesterases with IBMX unmasks the basal cAMP formation and increases [cAMP]$_{pm}$ (Figure 3A-E). Ex-9 attenuated the effect of IBMX in both mouse and human β-cells (Figure 3B,D,E) showing that GLP-1-receptor signalling contributes to the basal adenylyl cyclase activity.

As reported previously,[10,12,33] high glucose concentrations triggered increases of [cAMP]$_{pm}$ in MIN6 as well as in primary mouse and human β-cells (Figure 3F-I). The responses were inhibited by ex-9 with half-maximal effect at 32 nmol/L and maximal effect at ~1 µmol/L in MIN6-cells (Figure 3J). Although the translocation cAMP sensor reports cAMP in the sub-membrane space, glucose and ex-9 influenced cAMP globally in the cytoplasm as shown by recordings based on wide-field imaging and a cytoplasmic FRET cAMP reporter (Figure 3K). To
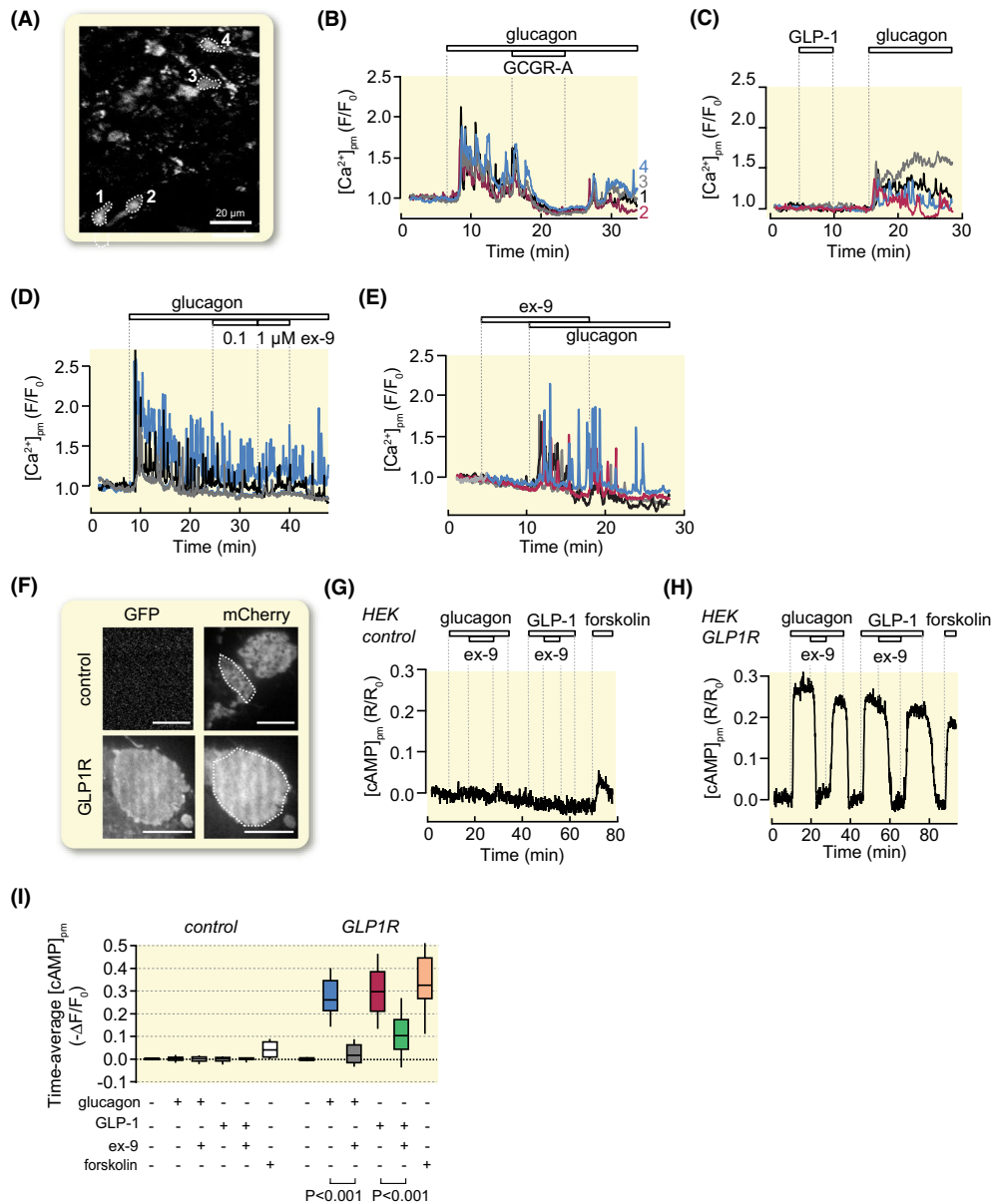
FIGURE 2 GLP-1 and ex-9 do not affect glucagon receptor signalling whereas glucagon promotes cAMP production via GLP-1 receptors. A-E, Recordings of $[Ca^{2+}]_{pm}$ from single glucagon receptor-expressing reporter cells loaded with the fluorescent Ca^{2+} indicator fluo-4. A, TIRF image (491/527 nm exc/em) of fluo-4-loaded reporter cells. B, Glucagon (10 nmol/L) induces $[Ca^{2+}]_{pm}$ signalling that is reversibly inhibited by 1 μmol/L of glucagon receptor antagonist (GCGR-A). Representative example traces from the cells highlighted in (A) out of 102 cells in seven experiments. C, GLP-1 (100 nmol/L) lacks effect while glucagon triggers $[Ca^{2+}]_{pm}$ signalling. Representative for 61 cells from six experiments. D, Ex-9 fails to reverse $[Ca^{2+}]_{pm}$ signalling evoked by glucagon. Representative for 135 cells from eight experiments. E, Pre-exposure to 1 μmol/L ex-9 does not prevent the $[Ca^{2+}]_{pm}$-elevating effect of 10 nmol/L glucagon. Representative for 61 cells from 4 experiments. F-I, Recordings of $[cAMP]_{pm}$ from HEK293 cells expressing GFP-tagged GLP-1 receptors. F, TIRF images of HEK293 cells transfected with an mCherry-based cAMP translocation biosensor together or not with a GFP-tagged GLP-1 receptor construct. Scale bar, 10 μm. G, Effects of 10 nmol/L glucagon, 1 μmol/L ex-9, 10 nmol/L GLP-1 and 10 μmol/L forskolin on $[cAMP]_{pm}$ in control cells lacking GLP-1 receptors. Representative for 24 cells from three experiments. H, Similar as in G but with cells expressing GLP-1 receptors. Representative for 43 cells from three experiments. I, Box plots of the time-average $[cAMP]_{pm}$ from experiments as shown in G and H. P values are given for the comparison of mean values with Student's paired t test

clarify whether the effect of ex-9 reflected GLP-1 receptor signalling activated by paracrine secretion of GLP-1 or glucagon in the islet, the inhibitor was applied to single, dispersed β-cells superfused with medium containing 20 mmol/L glucose. Like

for β-cells within intact islets, ex-9 caused a pronounced reduction of the glucose-induced $[cAMP]_{pm}$ elevation in single cells, sometimes reaching below the baseline (Figure 4A-C). A potential effect of endogenous GLP-1 production may be
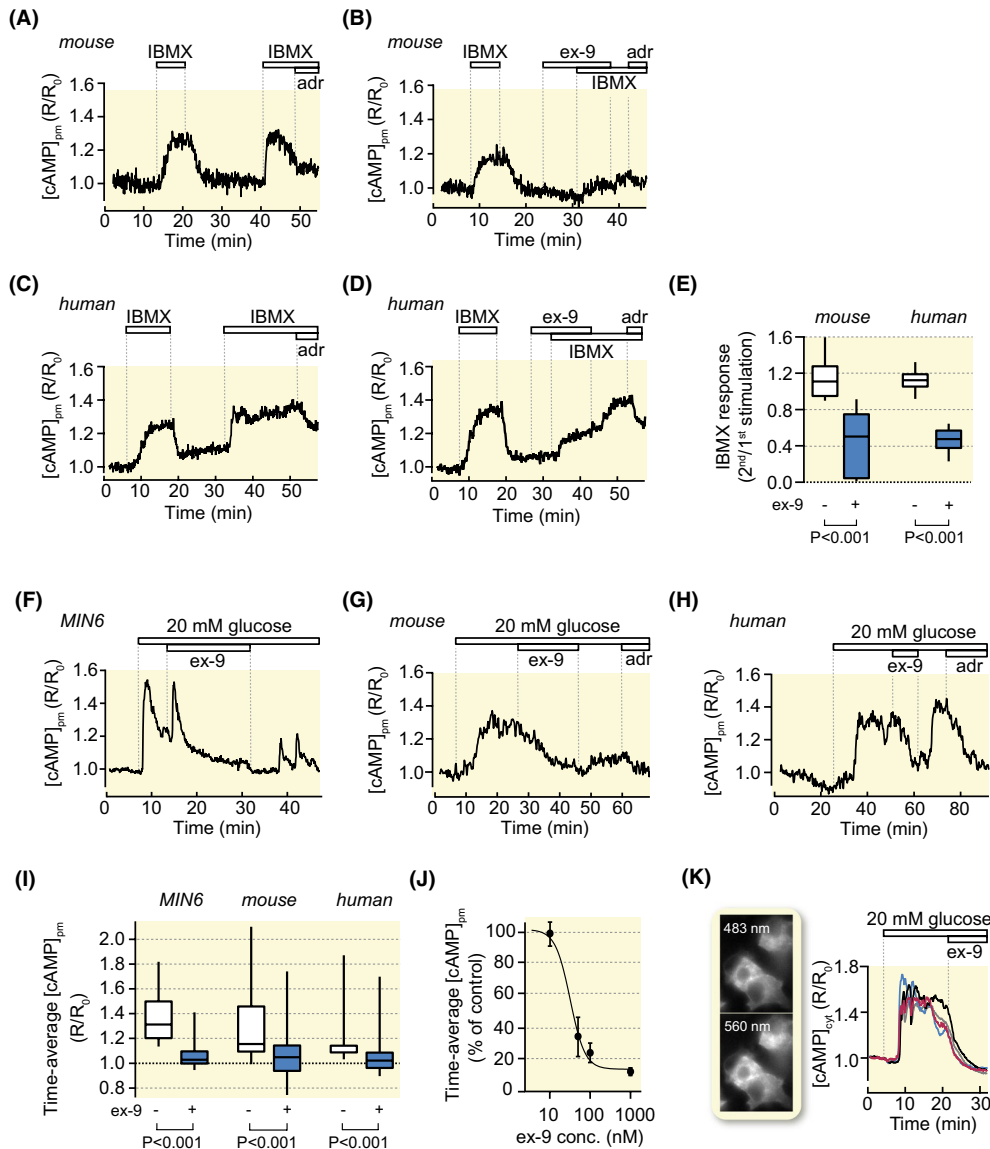
FIGURE 3 GLP-1 receptor antagonism suppresses basal cAMP production and glucose-induced $[cAMP]_{pm}$ elevations. A, $[cAMP]_{pm}$ recording from a single β-cell within an intact mouse islet during two consecutive exposures to IBMX. Representative for 27 cells from three experiments. B, As in A but with 1 µmol/L ex-9 introduced before the second IBMX exposure. Representative for 57 cells in nine experiments. C and D, As in A and B but with human β-cells. Representative for 28 cells for control and 22 cells with ex-9, both groups derived from four experiments with islets from a single donor. E, Box plots of the amplitudes of the second IBMX stimulation normalized to the first based on experiments as shown in A-D. F, $[cAMP]_{pm}$ recording from a single MIN6 β-cell exposed to an increase of the glucose concentration from 3 to 20 mmol/L. Ex-9 at 1 µmol/L reversibly suppresses the glucose-induced $[cAMP]_{pm}$ elevation. Representative for 84 cells from six experiments. G, H, $[cAMP]_{pm}$ recordings from β-cells within intact mouse (G, representative for 28 cells from 11 experiments) or human islets (H, 24 cells from five experiments with islets from four donors), showing that ex-9 suppresses the $[cAMP]_{pm}$ increase induced by elevation of glucose from 3 to 20 mmol/L. The lack of effect or lowering of $[cAMP]_{pm}$ in response to 10 µmol/L adrenaline (adr) is used as indication of β-cell identity. I, Box plots for $[cAMP]_{pm}$ after stimulation with 20 mmol/L glucose and exposure to ex-9. P values refer to statistical comparisons made with Wilcoxon signed-rank test. J, Means ± SEM for the effect of different concentrations of ex-9 on glucose-induced $[cAMP]_{pm}$ elevation in MIN6 β-cells. n = 7 to 24 cells for each data point. K, Wide-field fluorescence microscopy images and recordings of cytoplasmic cAMP from MIN6 cells stimulated by increase of glucose from 3 to 20 mmol/L and subsequently exposed to 100 nmol/L ex-9. Representative for 63 cells from three experiments

underestimated by the presence of dipeptidylpeptidase-4 (DPP-4) on islet cells.[34,35] However, 100 nmol/L of the DPP-4 inhibitor sitagliptin did not enhance $[cAMP]_{pm}$ elevation triggered by either glucose (Figure 4D,F) or GLP-1 (not shown) and lacked effect on basal $[cAMP]_{pm}$ (Figure 4E,F).

2.4 | The GLP-1 receptor shows agonist-independent activity

To elucidate if there is constitutive activity of the GLP-1 receptor, the GFP-tagged receptor was expressed in HEK293 cells,
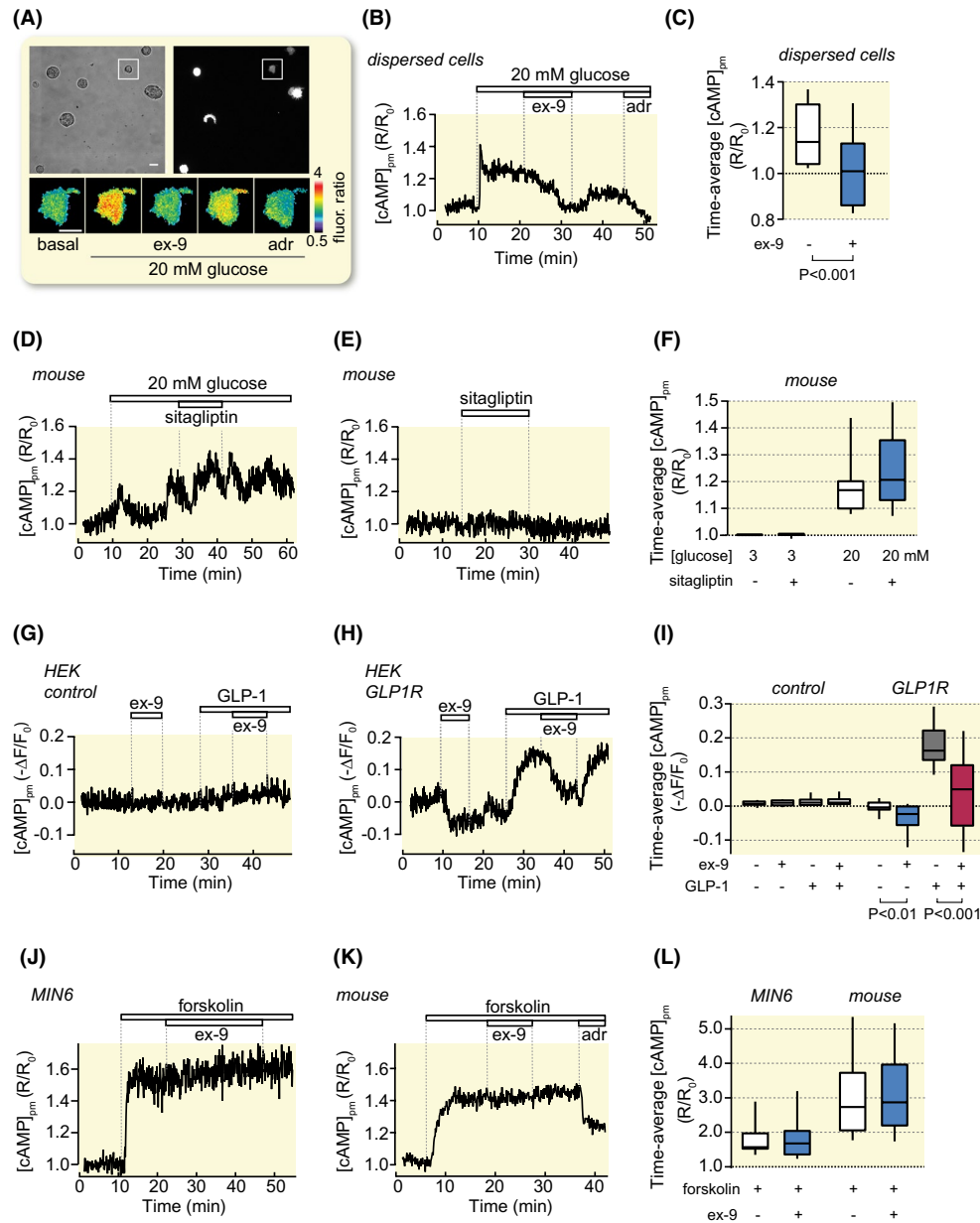
FIGURE 4 Effects of ex-9 and sitagliptin on glucose-, GLP-1- and forskolin-induced $[cAMP]_{pm}$ signals in dispersed β-cells, HEK293 cells and β-cell within intact islets. A, Transmitted light and TIRF images of dispersed mouse β-cells. The pseudo-coloured ratiometric $[cAMP]_{pm}$ image sequence shows the effect of an increase of glucose from 3 to 20 mmol/L, 1 μmol/L ex-9 and 10 μmol/L adrenaline (adr) in the single cell highlighted in the square. B, $[cAMP]_{pm}$ recording from the cell highlighted in (A). Representative for 13 cells from six experiments. C, Box plots of $[cAMP]_{pm}$ in cells exposed to 20 mmol/L glucose and ex-9 as in (B). The P value refers to comparison of the mean values with Student's t test. D, Effect of 100 nmol/L sitagliptin on $[cAMP]_{pm}$ in a β-cell within an intact mouse islet stimulated by an increase of the glucose concentration from 3 to 20 mmol/L. Representative for 25 cells from five experiments. E, Lack of effect of 100 nmol/L sitagliptin on $[cAMP]_{pm}$ in a mouse islet β-cell exposed to 3 mmol/L glucose. Representative for 42 cells from six experiments. F, Box plots of the effects of glucose and sitagliptin in experiments as shown in (D) and (E). G, H, Effects of ex-9 and GLP-1 on $[cAMP]_{pm}$ in single HEK293 cells lacking (G) or expressing (H) GLP-1 receptors. Representative for 21 and 29 cells from three experiments in each group. I, Box plots for the treatments shown in (G) and (H). P values refer to the comparison of mean values using Student's paired t test. J, K, $[cAMP]_{pm}$ recordings from a single MIN6 β-cell (J; Representative for 18 cells from three experiments) or a mouse islet β-cell (K; Representative for 47 cells from three experiments) showing that 1 μmol/L ex-9 lacks effect on $[cAMP]_{pm}$ elevations induced by 10 μmol/L forskolin. L, Box plots of the effects of forskolin and ex-9 on $[cAMP]_{pm}$ in experiments as in (J) and (K)

which completely lack paracrine signalling from glucagon or GLP-1. Recordings of $[cAMP]_{pm}$ showed that control cells did not respond to either ex-9 or GLP-1 (Figure 4G,I). However, in GLP-1-receptor-expressing cells, ex-9 reversibly lowered $[cAMP]_{pm}$ below the base-line (Figure 4H,I). This observation indicates that the GLP-1 receptor is constitutively active in the absence of ligand. The receptor-expressing cells readily responded to GLP-1 with $[cAMP]_{pm}$ elevation, an effect that
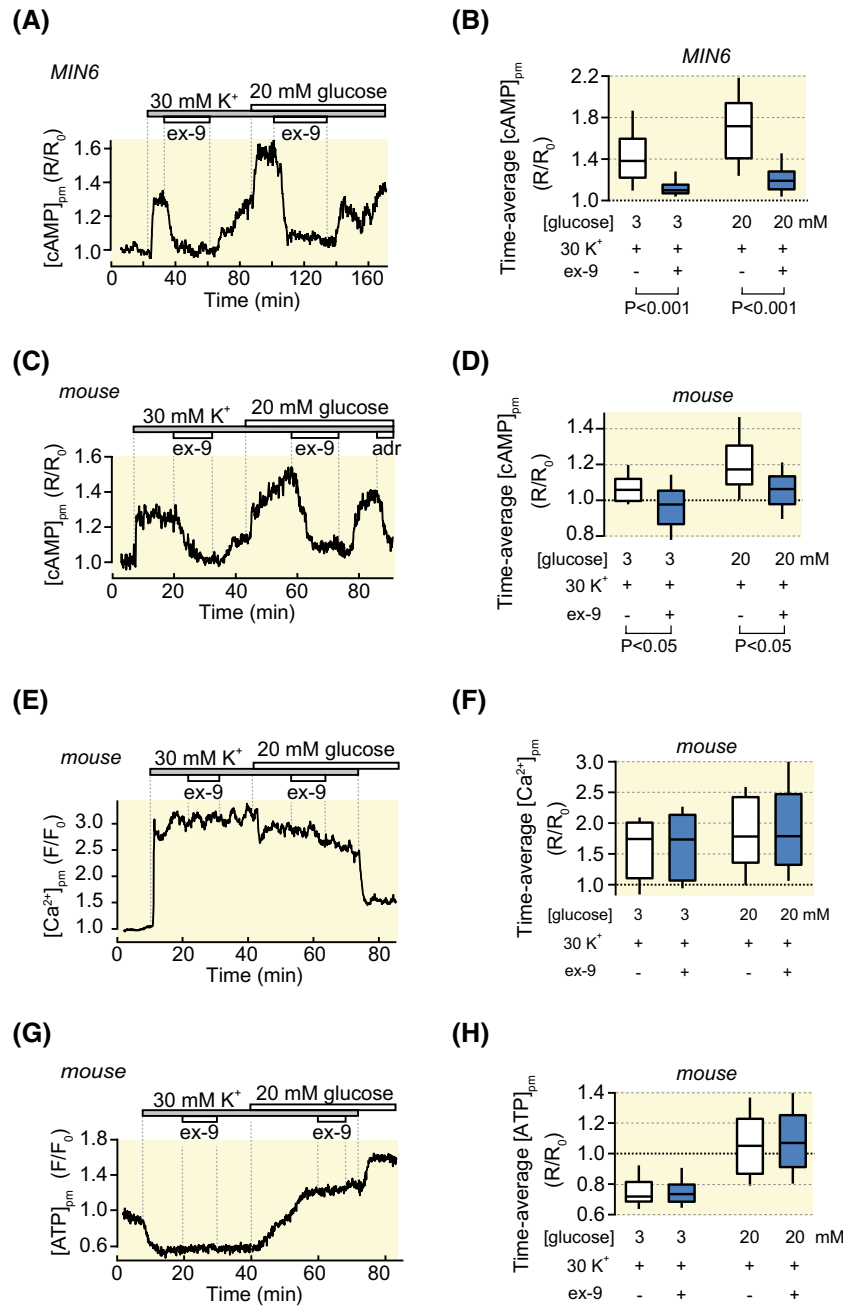
FIGURE 5 Ex-9 suppresses Ca^{2+}-stimulated $[cAMP]_{pm}$ increases. A, $[cAMP]_{pm}$ recording from a single MIN6 β-cell showing that 1 μmol/L ex-9 suppresses $[cAMP]_{pm}$ increases induced by depolarization and glucose stimulation in the continuous presence of 250 μmol/L diazoxide. Representative for 95 cells from five experiments. B, Box plots showing the effects of high K^+, glucose and ex-9 on $[cAMP]_{pm}$. P values refer to statistical comparisons made with Wilcoxon signed-rank test. C, Similar as in (A) but for mouse islet β-cells. The $[cAMP]_{pm}$-lowering effect of 10 μmol/L adrenaline (adr) is used to verify the identity of islet β-cells. Representative for 10 cells from five experiments. D, Box plots showing the effects of high K^+, glucose and ex-9 on $[cAMP]_{pm}$. P values are given for comparisons of mean values with Student's paired t test. E, $[Ca^{2+}]_{pm}$ recording from a single β-cell within an intact mouse islet showing that ex-9 lacks effect on depolarization-induced $[Ca^{2+}]_{pm}$ increases in the presence of diazoxide and 3 or 20 mmol/L glucose as in (C). Representative for 30 cells from eight experiments. F, Box plot for the effects of K^+, glucose and ex-9 on $[Ca^{2+}]_{pm}$. G, Recording of $[ATP]_{pm}$ under similar conditions as in (E). Representative for 42 cells from three experiments. H, Box plots showing the effects of K^+, glucose and ex-9 on $[ATP]_{pm}$

was counteracted by the antagonist. In contrast to the effect in hormone- and glucose-stimulated cells, ex-9 did not reduce $[cAMP]_{pm}$ in MIN6 cells stimulated with the adenylyl cyclase activator forskolin (Figure 4J-L) demonstrating that the drug does not exert an unspecific inhibition of cAMP formation.

2.5 | Ca^{2+}-stimulated cAMP formation involves GLP-1 receptor activity

Depolarization of MIN6 cells with 30 mmol/L K^+ in the presence of the K_{ATP}-channel opener diazoxide triggered

[cAMP]$_{pm}$ elevation, probably by activating Ca^{2+}-sensitive adenylyl cyclases,[13,16,17] and this effect was inhibited by ex-9 (Figure 5A,B). Increasing the glucose concentration from 3 to 20 mmol/L augmented the Ca^{2+}-triggered [cAMP]$_{pm}$ elevation, an effect that reflects stimulation of cAMP formation by cell metabolism,[10,24] and ex-9 reduced [cAMP]$_{pm}$ also under this condition (Figure 5A,B). Similar findings were made in primary mouse β-cells (Figure 5C,D). The effects of the GLP-1 receptor inhibitor on [cAMP]$_{pm}$ were not paralleled by alterations in [Ca^{2+}]$_{pm}$ (Figure 5E,F). It was also tested if ex-9 influenced cell metabolism. Recordings of [ATP]$_{pm}$ with the fluorescent reporter Perceval showed no effect of ex-9 but reduction of [ATP]$_{pm}$ in response to K^+ depolarization and increase with high glucose (Figure 5G,H), in line with previous reports.[36,37]

2.6 | The [cAMP]$_{pm}$-lowering effect of ex-9 does not involve activation of $G_i\alpha$

Since cAMP-lowering agonists often act on receptors coupled to the inhibitory G-protein $G_i\alpha$ we checked whether the effects of ex-9 might be explained by such a mechanism. To this end, MIN6-cells were treated with 200 ng mL^{-1} pertussis toxin for 18 hours prior to the [cAMP]$_{pm}$ imaging experiments. This treatment did not inhibit the effect of ex-9 (Figure 6A,C), while completely preventing $G_i\alpha$-mediated [cAMP]$_{pm}$ reduction by the α2-adrenergic agonist clonidine (Figure 6B,C).

2.7 | Ex-9-induced reduction of [cAMP]$_{pm}$ suppresses insulin secretion

Insulin secretion dynamics from single β-cells was monitored by recording the autocrine effect of insulin on the plasma membrane concentration of phosphatidylinositol 3,4,5-tris-phosphate ([PtdIns(3,4,5)P_3]$_{pm}$).[10,33] MIN6-cells responded to glucose stimulation with prompt [PtdIns(3,4,5)P_3]$_{pm}$ elevation followed by oscillations reflecting pulsatile insulin secretion (Figure 7A,B). This response was suppressed by ex-9 in a reversible manner. Addition of 100 nmol/L exogenous insulin caused stable [PtdIns(3,4,5)P_3]$_{pm}$ elevation, which was unaffected by ex-9, indicating that the GLP-1-receptor antagonist did not interfere with insulin receptor signalling (Figure 7B,D). Ex-9 also reversed glucose-elevated [PtdIns(3,4,5)P_3]$_{pm}$ in primary β-cells within intact mouse islets (Figure 7C,D). Similar results were obtained when insulin release was measured from perifused mouse islets using conventional ELISA. In islets exposed to 1 μmol/L ex-9, both first and second phase
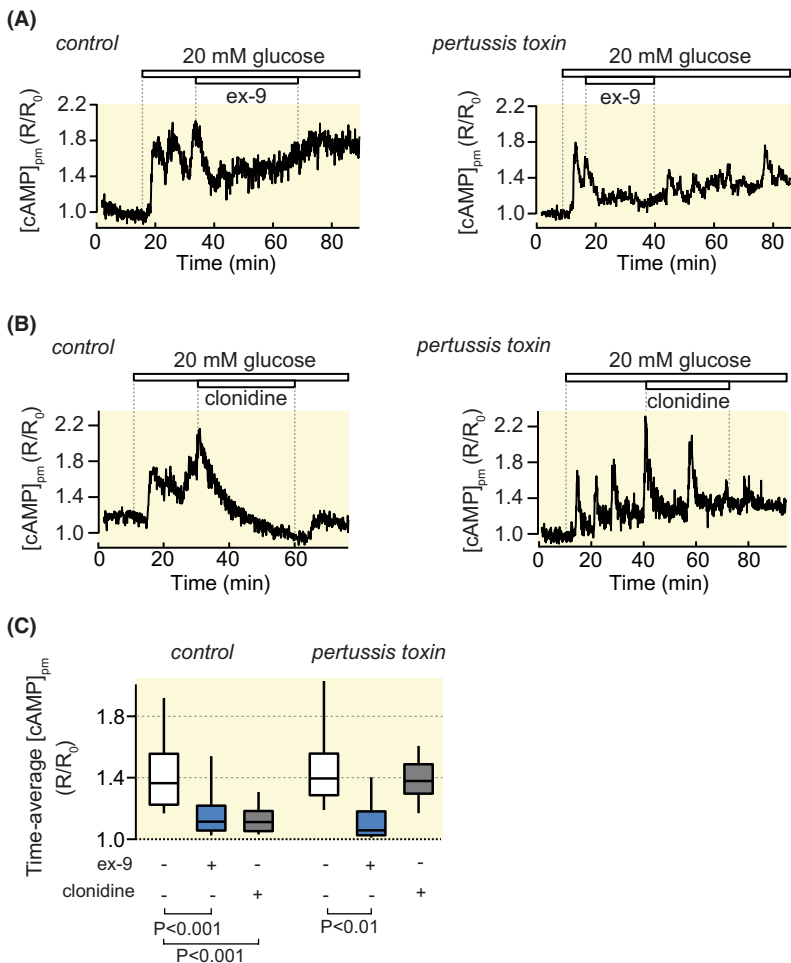

FIGURE 6 The [cAMP]$_{pm}$-lowering effect of ex-9 does not involve activation of $G_i\alpha$. [cAMP]$_{pm}$ recordings from single MIN6 β-cells pre-treated or not with pertussis toxin. A, Pertussis toxin does not prevent 1 μmol/L ex-9 from suppressing the [cAMP]$_{pm}$ increase induced by elevation of the glucose concentration from 3 to 20 mmol/L. Representative for 25 cells from four experiments in the control and 54 cells from eight experiments in the pertussis-toxin-treated group. B, Pertussis toxin prevents the suppression of glucose-induced [cAMP]$_{pm}$ elevation by 100 nmol/L clonidine. Representative for 23 cells from 3 experiments in the control and 20 cells from three experiments with pertussis toxin treatment. C, Box plots showing the effects of glucose, ex-9 and clonidine on [cAMP]$_{pm}$. P values refer to statistical comparisons made with Wilcoxon singed-rank test

glucose-stimulated insulin secretion was reduced by approximately 50% compared to control (Figure 7E,F; $P < .2$-$.05$; $n = 9$ and 7 for control and ex-9 respectively). In four experiments with human islets, glucose-stimulated secretion was reduced by 42%, but this effect did not reach statistical significance, probably because of the low number of observations (Figure 7G,H).

3 | DISCUSSION

The present study focused on the mechanisms underlying cAMP generation in pancreatic β-cells and reinforces the role of GLP-1 receptors for both glucose- and glucagon-induced

cAMP signalling. It is not surprising that the GLP-1 receptor antagonist ex-9, a truncated form of the GLP-1-receptor agonist exendin-4 from *Heloderma suspectum* venom,[38] completely reversed [cAMP]$_{pm}$ elevations induced by GLP-1, but the peptide turned out to be equally efficient in counteracting the effects of glucagon. It has been demonstrated that glucagon may activate β-cell GLP-1 receptors,[27-30] but patch-clamp studies in mouse β-cells have shown that ex-9 inhibits the effects of GLP-1 but not those of glucagon on exocytosis, suggesting that the two hormones act via different receptors.[39] Our data support that ex-9 fails to interfere with glucagon receptor signalling, but indicate that GLP-1 receptors in islets are more promiscuous.
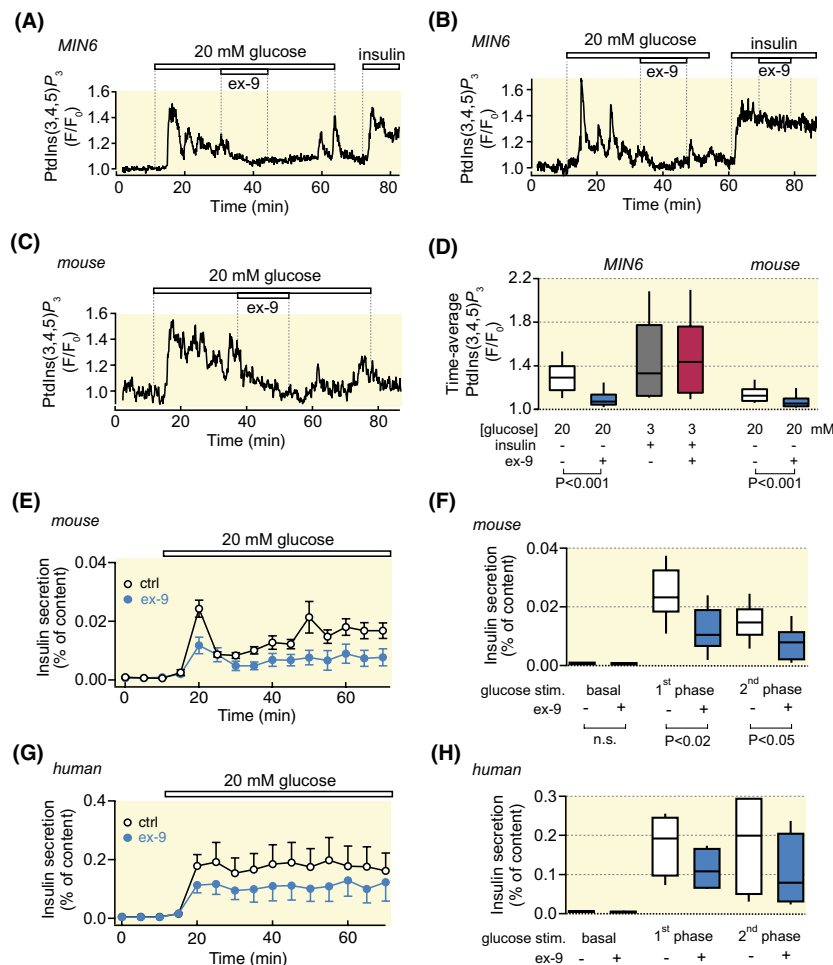

FIGURE 7 Ex-9 perturbs insulin secretion dynamics. A, Recording of insulin secretion from a single MIN6 β-cell with a fluorescent PtdIns(3,4,5)P_3 biosensor. Ex-9 (1 µmol/L) reversibly suppresses the PtdIns(3,4,5)P_3 response induced by increase of the glucose concentration from 3 to 20 mmol/L. Insulin (100 nmol/L) is added at the end of the experiment as a positive control. Representative for 71 cells from 10 experiments. B, Similar experiment as in (A) showing that ex-9 does not affect insulin-triggered PtdIns(3,4,5)P_3 elevation. Representative for 31 cells from 6 experiments. C, PtdIns(3,4,5)P_3 recording as in (A), but from a β-cell within an intact mouse islet. Representative for 51 cells from 6 experiments. D, Box plots showing the effects of glucose, ex-9 and insulin on the time-average PtdIns(3,4,5)P_3 responses. P values refer to statistical comparisons made with Wilcoxon signed-rank test. E, Means ± SEM for insulin secretion from perifused mouse islets stimulated by an increase in glucose concentration from 3 to 20 mmol/L in the absence or presence of 1 µmol/L ex-9. F, Box plots showing the effect of ex-9 on insulin secretion at 3 mmol/L glucose (basal), at 5-10 min after stimulation with 20 mmol/L (1st phase) and at 20-60 min stimulation with 20 mmol/L glucose (2nd phase). N = 9 (control) and 7 (ex-9) independent experiments. P values refer to statistical comparisons made with Mann-Whitney U-test. G-H, As in E-F, but with human islets. N = 4 experiments with islets from 3 donors for both control and ex-9
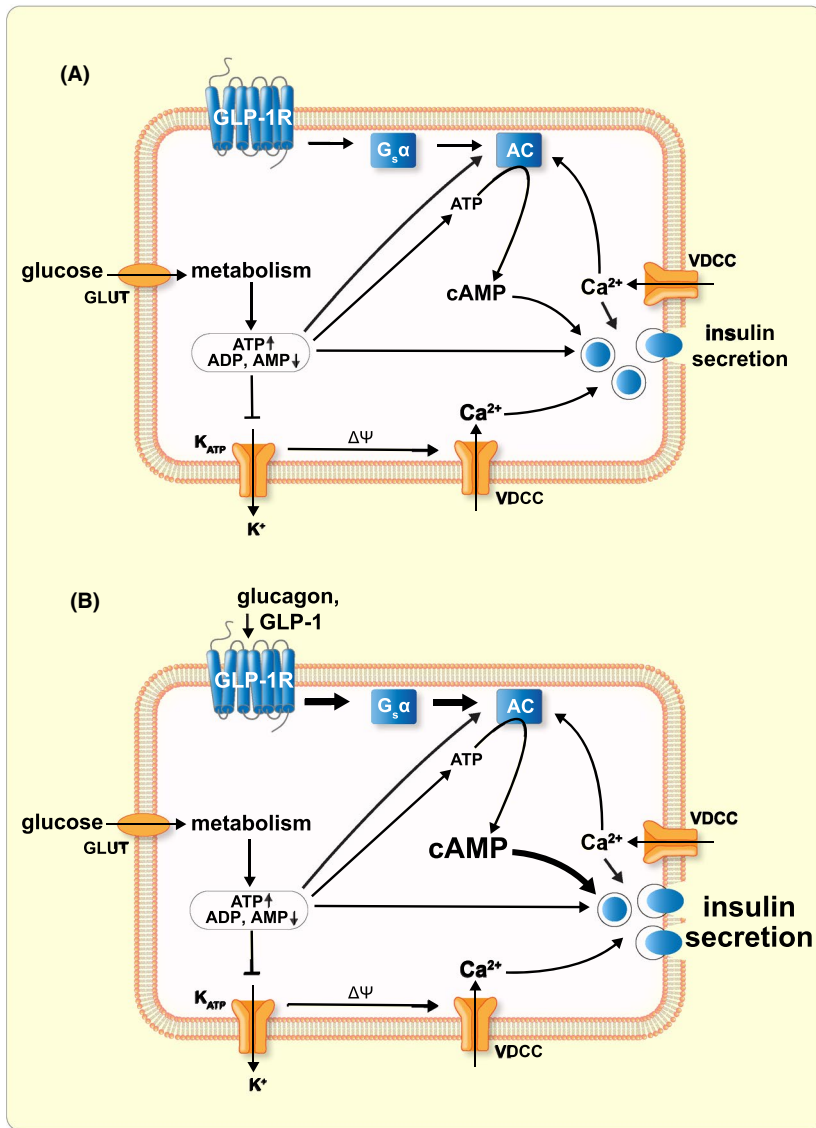
FIGURE 8 Proposed model for glucose- and hormone-induced cAMP formation in β-cells. A, Constitutive signalling from the GLP-1 receptor (in the absence of ligand) via $G_s\alpha$ and adenylyl cyclases (AC) contributes to glucose-induced cAMP formation and insulin secretion. Glucose metabolism results in increases of ATP and reductions of ADP and AMP. The change in nucleotide concentrations promotes AC activity by multiple mechanisms and cAMP enhances Ca^{2+}-triggered exocytosis. See text for details. Glucose metabolism triggers insulin secretion via K_{ATP} channel closure, membrane depolarization and influx of Ca^{2+} through voltage-dependent Ca^{2+} channels (VDCC). B, Activation of GLP-1 receptors by glucagon or GLP-1 stimulates $G_s\alpha$ and AC activity and the increased cAMP production leads to amplification of insulin secretion

The observation that ex-9 suppressed IBMX- and glucose-induced $[cAMP]_{pm}$ signalling in mouse and human islets in the absence of exogenously added GLP-1 or glucagon may reflect paracrine signalling from endogenous glucagon. This conclusion mirrors early observations that such activation of hormone receptors is critical for the glucose competence of β-cells,[26,40,41] a concept that has been reinforced in more recent studies.[27,29,30] Although GLP-1 has been reported to be produced in α-cells,[42,43] release of the bioactive hormone from these cells is very low[29] and it seems more likely that glucagon accounts for the GLP-1 receptor activation. In addition, there seems to be activity in the GLP-1 receptor also in the complete absence of agonist. There are divergent opinions about constitutive activity of the GLP-1 receptors. Agonist-independent activity have previously been reported,[44] but most studies have failed to observe such an effect.[45] In the present study, ex-9 lowered $[cAMP]_{pm}$ in single, dispersed islet cells, when paracrine influences would be minimal because of the dilution of any endogenous glucagon in the superfusion medium. Moreover, ex-9 lowered basal $[cAMP]_{pm}$ in HEK293 cells after heterologous expression of the GLP-1 receptor, an observation that cannot be explained by the presence of glucagon or glucagon-related peptides in the preparation. The conclusion that there is constitutive signalling in the GLP-1 receptor in the absence of ligand is in line with the suggestion that ex-9 acts as an inverse agonist.[46] The peptide has been found to reduce glucose-induced insulin secretion and cAMP production in normal mouse islets,[31] amino-acid-induced insulin secretion and cAMP content in SUR1−/− islets,[47] as well as basal cAMP production in rat islets.[28] The present data are consistent with these findings and extend the conclusions to human β-cells.

It is well-established that increases of $[Ca^{2+}]_{pm}$ in β-cells stimulate cAMP formation,[10,13-15,22,48] probably as a result of Ca^{2+}/calmodulin activation of the adenylyl cyclases AC1 and AC8.[49] It has also been demonstrated that cell metabolism may directly stimulate cAMP formation,[10] a concept that

has obtained support by mathematical modelling.[24] These two parallel mechanisms for cAMP formation were discriminated using a protocol which depolarizes the β-cell with a high K^+ concentration in the presence of diazoxide to evoke an increase of $[Ca^{2+}]_{pm}$, followed by increase of the glucose concentration to stimulate metabolism. The K_{ATP} channel activator prevents glucose from changing the membrane potential and increase $[Ca^{2+}]_{pm}$. GLP-1 receptor antagonism not only suppressed glucose-stimulated cAMP formation, but also that triggered by voltage-dependent Ca^{2+} entry was strongly impaired by pharmacological GLP-1 receptor antagonism. The effect on $[cAMP]_{pm}$ was not due to ex-9 influencing $[Ca^{2+}]_{pm}$ or $[ATP]_{pm}$, and it therefore seems that Ca^{2+}/calmodulin stimulation of adenylyl cyclase activity is facilitated by $G_s\alpha$ activity.

We also showed that ex-9 did not directly inhibit adenylyl cyclases, as it lacked effect on forskolin-stimulated cAMP generation. Moreover the possibility that the $[cAMP]_{pm}$-lowering effect was mediated by activation of the inhibitory G-protein $G_i\alpha$ could be excluded, since pertussis toxin failed to prevent the ex-9-induced $[cAMP]_{pm}$ lowering while completely abrogating the inhibitory effect of clonidine.

The present study leads to the conclusion that glucose-induced cAMP production in β-cells involves amplification of constitutive and glucagon-activated signalling through the GLP-1 receptor and that this mechanism is critical for glucose-stimulated insulin secretion (Figure 8). Glucose metabolism promotes cAMP formation by stimulating adenylyl cyclase activity directly by increasing availability of the substrate ATP and/or by decreasing the concentration of inhibitory AMP. Increases of $[Ca^{2+}]_{pm}$ also contribute to the elevation of $[cAMP]_{pm}$. Glucagon or GLP-1 agonism on the GLP-1 receptor leads to enhanced cAMP formation and insulin secretion and inhibition of receptor signalling accordingly leads to reduced cAMP formation and impaired insulin secretion without effect on the exocytosis-triggering $[Ca^{2+}]_{pm}$ signal. Incretin hormone effects are impaired in type 2 diabetes[50] and stimulation of GLP-1 receptor signalling is established as a successful therapeutic approach.[51,52] Reduced cAMP formation and cAMP-secretion coupling in β-cells have been reported in animal and in vitro models of the disease.[33,53-56] Future studies will elucidate to what extent lower inherent GLP-1 receptor signalling contributes to impaired β-cell function in diabetes.

4 | MATERIALS AND METHODS

4.1 | Materials

Adrenaline, HEPES, 2-mercaptoethanol, poly-L-lysine, 3-isobutyl-1-methylxanthine (IBMX), GLP-1-(7-36)-amide and glucagon were purchased from Sigma-Aldrich (Stockholm, Sweden). DMEM, Lipofectamine 2000, trypsin, penicillin, streptomycin, glutamine, fluo-4-AM and fetal calf serum were from Invitrogen/Thermo Fisher Scientific (Carlsbad, CA, USA). Exendin-(9-39) was purchased from Bachem (Bubendorf, Switzerland). Pertussis toxin was from Tocris (Bristol, UK). The glucagon receptor inhibitor "compound 15"[32] was a kind gift from Novo-Nordisk. A plasmid encoding the GFP-tagged GLP-1 receptor was obtained from Prof Sebastian Barg, Uppsala, University. Plasmid or adenoviral vectors containing the two moieties of a cAMP translocation biosensor were generated as previously described.[10,48] The biosensor encodes a truncated and membrane-anchored PKA regulatory RIIβ subunit tagged with CFP and a PKA catalytic Cα subunit tagged with YFP. An alternative version of the sensor lacking fluorescence tag on the RIIβ subunit and with an mCherry tag on the Cα subunit was used for the experiments in Figure 2F-I and Figure 4G-I. A plasmid encoding GRP1 (general receptor for phophoinositides-1) fused to 4 tandem copies of (GFP_4-GRP1) was used to detect plasma membrane $PtdIns(3,4,5)P_3$ levels, which reflect insulin secretion with concomitant autocrine activation of insulin receptors and PI3-kinase.[10] An adenovirus-expressing Perceval was used for ATP recordings.[36] Glucagon-receptor-expressing Chem-1 reporter cells (Ready-to-Assay, HTS112RTA) were obtained from Merck Millipore (Solna, Stockholm, Sweden).

4.2 | MIN6-cell culture and transfection

Insulin-secreting MIN6-cells (passages 17-30)[57] were cultured in DMEM with 25 mmol/L glucose and supplemented with 15% fetal calf serum, 2 mmol/L L-glutamine, 50 μmol/L 2-mercaptoethanol, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were cultured at 37°C in a 5% CO_2 humidified air atmosphere. For imaging experiments, cells were seeded on poly-L-lysine-coated 25-mm coverslips. For each coverslip, 0.2 million cells were suspended in Optimen I medium (Invitrogen) containing 0.5 μL Lipofectamine 2000 (Invitrogen) and 0.2 to 0.4 μg of the cAMP or Ptdlns(3,4,5)P_3 biosensor plasmids and placed onto the centre of the coverslip. After 4 hours, when the cells were firmly attached, the transfection was interrupted by adding 3 mL complete cell culture medium. Cells were maintained in this medium for 24 to 48 hours.

4.3 | Islet isolation and virus transduction

Islets of Langerhans were collagenase-isolated from 6- to 9-month-old, normal-weight C57B16J female mice. The mice were housed in ventilated cages (up to 5 animals/cage) with a 12 hours dark/light cycle and with free access to water and

a standard mouse chow. All procedures for animal handling and islet isolation were approved by Uppsala animal ethics committee. After isolation, the islets were cultured for 1 to 2 days in RPMI 1640 medium containing 5.5 mmol/L glucose, 10% fetal calf serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at 37°C in a 5% CO_2 humidified air atmosphere. Human islets from eleven normoglycemic cadaveric organ donors (Table 1) were obtained via the Nordic Network for Clinical Islet Transplantation in Uppsala. All experiments with human islets were approved by the Uppsala human ethics committee. The isolated islets were cultured up to 7 days at 37°C in an atmosphere of 5% CO_2 in CMRL 1066 culture medium containing 5.5 mmol/L glucose, 100 U/mL penicillin and 100 μg/ml streptomycin, 2 mmol/L glutamine and 10% FBS. The islets or cells were infected 1 to 2 hours with cAMP or Ptdlns(3,4,5)P_3 biosensor adenoviruses at a concentration of 10^5 fluorescence forming units per islet, and this was followed by washing and further culture for 16-20 hours before use.

4.4 | Recordings of cAMP, Ca^{2+}, ATP and Ptdlns(3,4,5)P$_3$

Changes of the cAMP, Ca^{2+} and ATP concentrations in the sub-plasma membrane space ([cAMP]$_{pm}$, [Ca^{2+}]$_{pm}$ and [ATP]$_{pm}$) and of the plasma membrane concentration of the phospholipid phosphatidylinositol-3,4,5-trisphophate ([Ptdlns(3,4,5)P$_3$]$_{pm}$) were recorded with total internal reflection fluorescence (TIRF) microscopy. Before imaging, the cells or islets were pre-incubated for 30 minutes in experimental buffer containing in mM: NaCl 125, KCl 4.8, CaCl$_2$ 1.3, MgCl$_2$ 1.2 and HEPES 25 with pH adjusted to 7.40 with NaOH. For [Ca^{2+}]$_{pm}$ measurements, the cells were loaded with 1.3 μmol/L of the acetoxymethyl ester of the Ca^{2+} indicator Fluo-4 by 30 minutes of incubation at 37°C in experimental buffer. After the incubation, the islets were attached to poly-lysine-coated 25-mm coverslips. β-cells were identified on the basis of their large size and negative [cAMP]$_{pm}$ response to adrenaline, in contrast to alpha cells with small footprints and adrenaline-induced [cAMP]$_{pm}$ elevation.[12]

TIRF imaging was performed using a custom-built prism-based setup or an objective-based system as previously described.[23] Diode-pumped solid-state lasers (Cobolt, Solna, Sweden) provided excitation light for Fluo-4 (491 nm) and the fluorescent protein biosensors (445, 491 and 514 nm for CFP, GFP/Perceval and YFP, respectively). Emission wavelengths were selected with filters [485 nm/25 nm half-bandwidth for CFP, 527/27 nm for GFP/Perceval and 560/40 nm for YFP (Semrock Rochester, NY)] mounted in a filter wheel (Sutter Instruments). Fluorescence was detected with back-illuminated EMCCD cameras (DU-897, Andor Technology) under MetaFluor (Molecular Devices Corp, Downington, PA) software control. For time-lapse recordings, images or image pairs were acquired every 5 seconds. For the experiment in Figure 3K, the cells were transfected with the fluorescence resonance energy transfer (FRET)-based cAMP reporter EpacS^{H188}; Ref.58 The reporter was excited at 445 nm in the objective-based TIRF system with the laser angle set for wide-field illumination. Donor emission was recorded at 483 and sensitized emission from the acceptor at 560 nm. The data are presented as the 483/560 nm fluorescence emission ratio ("FRET ratio") normalized to the prestimulatory level.

4.5 | Measurements of insulin secretion

Groups of 15-16 islets were placed in a 10-μl teflon tube chamber and perifused at a rate of 60 μL/min using a pressurized

TABLE 1 Human islet donor characteristics

Islet preparation	Sex	Age y	BMI kg/m^2	HbA1c mmol/mol (%)	Related experiments
H1848	Male	58	21.4	unknown	Figure 3F
H1922	Male	55	25.8	36.6 (5.5)	Figure 3F
H2088	Male	68	25.6	39.9 (5.8)	Figures 1C and 3F
H2089	Male	30	26	34.4 (5.3)	Figures 1C and 3F
H2154	Male	66	24.3	38.8 (5.7)	Figure 1F
H2164	Male	67	24.6	34.4 (5.3)	Figure 1F
H2176	Female	64	25.7	37.7 (5.6)	Figure 1F
H2325	Female	71	30.8	Unknown	Figure 3C and 3D
H2512	Female	56	24.6	40.0 (5.8)	Figure 7G,H
H2516	Male	55	22.4	36.0 (5.4)	Figure 7G,H
H2517	Female	51	28.1	35.0 (5.4)	Figure 7G,H

air system (AutoMate Scientific, Berkeley, CA, USA) and equilibrated in experimental buffer containing 3 mmol/L glucose with or without 1 μmol/L ex-9 during 45 minutes. The perifusate was subsequently collected in 5-min fractions into ice-chilled 96-well plates with a non-binding-surface (Corning Inc Kennebunk, ME) while changing the glucose concentration to 20 mmol/L in the absence or continued presence of ex-9. The islets were retrieved and briefly sonicated in acid ethanol to determine insulin content. Insulin concentrations in the samples were determined with ELISA according to the manufacturer's instructions (Mercodia AB, Uppsala, Sweden).

4.6 | Data analysis

Image analysis was performed with MetaFluor. The cAMP concentration was expressed as the ratio of CFP over YFP fluorescence or as the relative changes of mCherry fluorescence (experiments in Figures 2G-I and 4G-I) after subtraction of the background and normalization to the prestimulatory level. For other fluorescent reporters, changes in fluorescence intensity were normalized to the initial fluorescence intensity after subtraction of background. Curve fitting was carried out using IGOR Pro (Wavemetrics, Lake Oswego, OR, USA) software. If not otherwise stated, all experiments were performed with at least three independent islet isolations or preparations of cells. Data are presented as box plots and statistical analyses were made with Student's t test, Wilcoxon signed-rank test or Mann-Whitney U test as appropriate.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
HS designed and performed all TIRF imaging experiments and analysed data. YX contributed widefield imaging experiments and PA immunoassay experiments. AT conceived the study, designed experiments, analysed data and wrote the paper. All authors critically revised the manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
Anders Tengholm https://orcid.org/0000-0003-4508-0836

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