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ARTICLE

Cyclic AMP triggers glucagon-like peptide-1 secretion from the GLUTag enteroendocrine cell line

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Abstract
Aims/hypothesis To investigate the pathways by which cyclic AMP (cAMP) stimulates glucagon-like peptide-1 (GLP-1) secretion, using the GLUTag enteroendocrine cell line.
Materials and methods GLP-1 release from GLUTag cells was measured in response to agents that increase cAMP, and single cells were studied by fluorescence calcium imaging and electrophysiology to evaluate the underlying pathways.
Results Pituitary adenylate cyclase-activating polypeptide increased cAMP levels and stimulated GLP-1 release from GLUTag cells. Agents that increase cAMP levels, including forskolin plus 3-isobutyl-1-methylxanthine (fsk/IBMX), triggered a rise in the intracellular calcium concentration and enhanced the response to glucose by increasing both the number of cells responding to glucose and the magnitude of calcium responses in individual cells. Importantly, fsk/IBMX also stimulated GLP-1 release and intracellular calcium elevation even in the absence of nutrients. fsk/IBMX triggered membrane depolarisation and the firing of action potentials, associated with a +14 mV shift in the voltage-dependence of activation of hyperpolarisation-activated currents and the closure of a background potassium conductance.
Conclusions/interpretation We show here that cAMP elevation directly triggers GLP-1 release and enhances the secretory response to other stimuli like glucose, by modulating hyperpolarisation-activated currents and the background potassium current. cAMP-elevating pathways and the cAMP-modulated conductances in L cells present important targets for the development of therapeutic GLP-1 secretagogues.

Keywords Cyclic AMP · Gastro-entero pancreatic factors · Glucagon-like peptide-1 · GLUTag cells

Abbreviations

$[Ca^{2+}]_i$	intracellular calcium concentration
cAMP	cyclic AMP
CGRP	calcitonin gene-related peptide
EPAC	cAMP–guanine nucleotide exchange factor
fsk/IBMX	forskolin plus IBMX
GIP	glucose-dependent insulinotrophic peptide
GLP-1	glucagon-like peptide-1
IBMX	3-isobutyl-1-methylxanthine
I_h	hyperpolarisation-activated current
K_{ATP}	ATP-sensitive potassium channels
PACAP	pituitary adenylate cyclase-activating polypeptide
PKA	protein kinase A

Introduction

Glucagon-like peptide-1 (GLP-1) is an incretin hormone, secreted in response to food ingestion, which stimulates insulin release from the endocrine pancreas. Together with glucose-dependent insulinotrophic peptide (GIP), it is responsible for up to 50% of normal insulin release following a meal [1]. As incretin hormones enhance glucose-stimulated insulin release, but do not on their own initiate insulin secretion, their action in vivo is glucose-dependent [2]. The new GLP-1-based glucose-lowering

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therapies, comprising GLP-1 analogues and agents that inhibit GLP-1 degradation, take advantage of this property to offer safer therapies for type 2 diabetes [3]. An alternative therapeutic strategy that is receiving increasing interest is to stimulate secretion of endogenous GLP-1 from intestinal L cells. As GLP-1 is co-secreted with the anorexigenic peptide, peptide YY, and the intestinally trophic hormone, GLP-2, enhancing L cell secretion is also of potential interest for appetite control [4] and to enhance intestinal epithelial proliferation in certain gastroenterological disorders [5].

A critical step towards the identification of GLP-1 secretagogues is to understand the different pathways involved in L cell stimulation, their relative importance in vivo and their potential synergistic or antagonistic interactions. L cells are scattered over the villi and crypts of the intestine, from the level of the jejunum down to the rectum [6]. Although secretion of GLP-1 is known to be triggered by food ingestion, it remains unclear exactly which cell populations respond at different stages after a meal, and how important luminal and neural/hormonal signals are for secretion from the different cell populations [7]. A range of stimuli enhancing GLP-1 secretion have been identified in whole-animal studies [8], as well as in model systems such as perfused intestine [9], cultured fetal rat intestinal cells [10], partially purified adult canine L cells [11] and enteroendocrine cell lines such as GLUTag [12], STC-1 [13] and NCI-H716 [14]. These include nutrients (e.g. sugars, amino acids, peptones, fats), hormones (e.g. GIP, calcitonin gene-related peptide [CGRP], gastrin-releasing peptide), and neurotransmitters (e.g. acetylcholine, γ-amino butyric acid) [2, 5, 7].

Single-cell measurements of electrical activity and calcium concentrations have shown that secretion from L cells, as in other endocrine cells, is dependent on elevation of the intracellular calcium concentration ($[Ca^{2+}]_i$), triggered by either membrane depolarisation and Ca^{2+} entry through voltage-gated Ca^{2+} channels or Ca^{2+} release from intracellular stores [14–17]. Our studies on GLUTag enteroendocrine cells have shown that glucose and amino acids (e.g. glutamine, alanine, glycine) trigger membrane depolarisation and the generation of action potentials in GLUTag cells, resulting in Ca^{2+} entry and the initiation of GLP-1 secretion [18–20].

Whereas many of the identified stimuli of GLP-1 secretion exhibit variable efficacy depending on the experimental preparation, elevation of cyclic AMP (cAMP) has been repeatedly found to release GLP-1 in a range of different studies [11, 14, 15, 21, 22]. A number of hormones and neurotransmitters that can activate adenylate cyclase have been identified in the enteric nervous system [23] and the intestinal epithelium, e.g. GIP, CGRP, pituitary adenylate cyclase-activating polypeptide (PACAP) and noradrenaline (norepinephrine). Of these, GIP, CGRP and β-adrenergic agonists have been shown to stimulate GLP-1 release [9, 24–26], suggesting that hormonal, paracrine and/or neural circuits may play a role in regulating secretion from L cells. The first identified action of cAMP elevation on L cells was stimulation of proglucagon gene transcription [12]. This has been subsequently shown to involve both protein kinase A (PKA)-dependent and -independent pathways, the latter involving cAMP–guanine nucleotide exchange factors (EPACs) [27]. There is also evidence, however, that cAMP stimulates GLP-1 secretion as well as its synthesis [21]. In pancreatic beta cells, stimulation of insulin release by cAMP involves the glucose-dependent recruitment of previously unresponsive cells and amplification of the secretory pathway downstream of a calcium rise [28–30]. In GLUTag cells, however, we found that forskolin and 3-isobutyl-1-methylxanthine (fsk/IBMX) stimulated GLP-1 secretion in the absence as well as the presence of glucose [22]. The aim of this study was to investigate further the pathways underlying the stimulation of GLP-1 release by cAMP in GLUTag cells.

Materials and methods

Cell culture GLUTag cells were cultured in Dulbecco's modified Eagle's medium containing 5.5 mmol/l glucose, as described previously [22]. The GLUTag cell line was originally derived from a colonic tumour taken from a transgenic mouse expressing SV40 large T-antigen under the control of the proglucagon promoter [12], and has proved to be a reliable model of GLP-1 secretion as it exhibits sensitivity to a range of physiological stimuli.

GLP-1 secretion For secretion experiments, cells were plated in 24 well culture plates coated with Matrigel (BD Biosciences, Oxford, UK), and allowed to reach 60–80% confluence. On the day of the experiment, cells were washed twice with 500 µl nutrient-free standard bath solution supplemented with 0.1 mmol/l diprotin A (Ile–Pro–Ile) and 0.1% (wt/vol.) BSA. For experiments in 30 mmol/l KCl, the concentration of NaCl was reduced equivalently. Experiments were performed by incubating cells with test reagents in the same solution for 2 h at 37°C. The final DMSO concentration was adjusted to 0.1% for all conditions, including controls. After the incubation period, medium was collected and centrifuged to remove any floating cells. GLP-1 was assayed using an ELISA specific for GLP-1(7–36) amide and GLP-1(7–37) (Linco GLP-1 active ELISA-kit; Biogenesis, Poole, UK).

cAMP measurements Cells in 24 well plates were treated as described for secretion experiments. Test agents were

applied in 10 mmol/l glucose for 15 min at 37°C and cells were subsequently lysed for 10 min with 500 μl/well 0.25% dodecyltrimethylammonium bromide in 50 mmol/l sodium acetate (pH 5.8) containing 0.02% (w/v) BSA at room temperature. cAMP in the lysate was assayed by ELISA (GE Healthcare, Little Chalfont, UK) and cell lysis was confirmed using 0.1% Trypan Blue solution.

$[Ca^{2+}]_i$ measurements Cells were plated in Matrigel-coated glass bottom dishes (MatTek, Ashland, MA, USA) 1–3 days prior to use and loaded with fura-2 by incubation in 2 μmol/l of the acetoxymethylester (Molecular Probes, Leiden, the Netherlands) for 30 min in bath solution containing 1 mmol/l glucose at room temperature. Cells were then washed with bath solution, and the experiment started within 10 min. Dishes were mounted on an inverted fluorescence microscope (Olympus IX71; Southall, UK) with a ×40 oil-immersion objective. Excitation at 340 and 380 nm was achieved using a 75 W xenon arc lamp with a monochromator (Cairn Research, Faversham, UK) controlled by MetaFluor software (Universal Imaging; Cairn Research) and emission was recorded with a CCD camera (Orca ER, Hammamatsu; Cairn Research). Free cytoplasmic Ca^{2+} concentrations were estimated for individual cells from background-subtracted fluorescence using the equation of Grynkiewicz et al. [31] assuming a K_d of 224 nmol/l for fura-2. Minimal and maximal signals were recorded in the presence of 5 μmol/l ionomycin in 5 mmol/l EGTA/0 mmol/l Ca^{2+} and 5 mmol/l Ca^{2+}, respectively, at the end of the experiment. In subsequent analysis, the background level for each cell was obtained by averaging $[Ca^{2+}]_i$ in control solution, and the response to test agents was defined as the maximum concentration (averaged over 20 s) achieved during their application.

Electrophysiology Cells were plated into 35 mm dishes 1–3 days prior to use. Experiments were performed on single cells and well-defined cells in small clusters. Microelectrodes were pulled from borosilicate glass (GC150T; Harvard Apparatus, Edenbridge, UK) and the tips coated with refined yellow beeswax. Electrodes were fire-polished using a microforge (Narishige, London, UK) and had resistances of 2.5–3 MΩ when filled with pipette solution. Membrane potential and currents were recorded using an Axopatch 200B (Axon Instruments, Union City, CA, USA) linked through a Digidata 1320A interface and pCLAMP software (Axon Instruments). Electrophysiological recordings were made using the perforated-patch configuration of the patch-clamp setup, at 22–24°C.

Solutions and chemicals The perforated-patch pipette solution contained (mmol/l): 76 K_2SO_4, 10 KCl, 10 NaCl, 55 sucrose, 10 HEPES and 1 $MgCl_2$, (pH 7.2 with KOH), to which amphotericin B was added to a final concentration of 200 μg/ml. The standard bath solution contained (mmol/l): 5.6 KCl, 138 NaCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES (pH 7.4 with NaOH). Calcium currents were recorded in bath solution containing (mmol/l): 5.6 CsCl, 118 NaCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 20 tetraethylammonium chloride (TEACl; pH 7.4) with the addition of 0.3 μmol/l tetrodotoxin (TTX). The perforated-patch solution for recording calcium currents contained (mmol/l): 76 Cs_2SO_4, 10 KCl, 10 NaCl, 1 $MgCl_2$, 5 HEPES (pH 7.35 with CsOH), to which amphotericin B was added to a final concentration of 200 μg/ml.

Drugs and chemicals were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated. CsCl and CsOH were from Alfa Aesar (Avocado Research Chemicals, Heysham, UK). 6-Benzoyl cAMP and 8-CPT 2-*O*-methyl cAMP were purchased from Biolog (Bremen, Germany). ZD7288 was from Tocris (Avonmouth, Bristol, UK). Forskolin and IBMX were prepared as 1,000× stocks in DMSO.

RT-PCR RT-PCR was performed as described previously [22] using the following specific primers, which were designed using sequence information from http://www.ensembl.org/mus_musculus/ (from 5′ to 3′): *Epac1* (also known as *Rapgef3*): 5′-GTGTGAGCTGGAGAAGAAAC-3′ and 5′-GGAACCTGTAGAATTGGGC-3′ (572 bp), *Epac2*: 5′-GCGATCTAGTGAAGATGTGG-3′ and 5′-CTTAT CATGTGGGGAGCTC-3′ (539 bp). The identity of positive bands in GLUTag cells was confirmed by direct sequencing.

Data analysis Data were analysed with Microcal Origin (Aston Scientific, Milton Keynes, UK), pClamp and Microsoft Excel software.

Calcium currents were fitted with a Boltzmann equation $y = (A_2 + [A_1 - A_2]/(1 + \exp([V_{0.5} - V_m]/k))) \times (V_m - V_{rev})$, where V_m is the voltage, A_1 is the conductance of the calcium current, A_2 is the background conductance, $V_{0.5}$ is the half-maximum voltage of activation, k is a constant, and V_{rev} is the reversal potential of the calcium current. Time constants of decay of the calcium current (τ) were fitted with a single exponential over the first 200 ms after the peak.

Hyperpolarisation-activated currents were fitted with a modified Boltzmann equation: $y = g_{max\,(I_h)} \times (V_m + 40)/\left(1 + e^{(V_m - V_{0.5})/k}\right) + g_b \times \left(V_m - V_{rev(b)}\right)$ where $g_{max\,(I_h)}$ is the maximum conductance of the I_h current, V_m is the membrane potential, $V_{0.5}$ is the half-maximum voltage of activation of I_h, k is a constant for I_h, g_b is the conductance of the background current, and $V_{rev(b)}$ is the reversal potential of the background current.

Results are presented as means±SE. Statistical significance was tested by Student's one-sample, two-sample or paired t test, as appropriate, using a threshold for significance of $p<0.05$.

Results

A variety of hormones have been shown to trigger secretion from GLP-1-secreting cells, including some which, in other cell types, are known to couple to adenylate cyclase activation and cAMP formation. In GLUTag cells, GIP and PACAP dose-dependently triggered GLP-1 release in the presence of 10 mmol/l glucose, with a maximum stimulation of 1.8- to 2-fold, and approximate EC_{50} values of 7 nmol/l for GIP and 1 nmol/l for PACAP (Fig. 1). Similar stimulation of GLP-1 release from GLUTag cells by GIP has been demonstrated previously [21]. These responses compare with a fourfold stimulation by fsk/IBMX (10 μmol/l of each) in parallel experiments. PACAP has previously been shown to stimulate cholecystokinin and secretin release from another enteroendocrine cell line, STC-1, associated with a rapid increase in intracellular cAMP that was only detectable in the presence of IBMX [32]. In GLUTag cells, 15 min incubations with PACAP or GIP (both at 10^{-7} mol/l, at which maximum secretion was observed) elevated cAMP concentrations ~1.5- to 2-fold in the presence of IBMX (Fig. 1b), but did not have detectable effects on cAMP levels in the absence of IBMX.

To investigate the actions of cAMP in further detail, we monitored calcium concentrations in GLUTag cells by measuring the 340:380 nm fluorescence ratio in cells loaded with fura-2. PACAP (10^{-7} mol/l) increased the fluorescence ratio and enhanced the response to glucose in many cells (Fig. 2a). In further experiments to dissect the underlying mechanism, we used fsk/IBMX rather than PACAP to elevate cAMP. This triggered qualitatively similar responses in calcium imaging studies. Glucose triggered an elevation of $[Ca^{2+}]_i$ in 26 out of 40 cells (Fig. 2b,c), with a mean increment of 204±50 nmol/l (n=40, p<0.001). In the presence of fsk/IBMX, however, 40 out of 40 cells responded to glucose (mean increment 759±160 nmol/l), and in 38 out of 40 cells glucose responsiveness was maintained 8–10 min after fsk/IBMX washout despite a return of the basal $[Ca^{2+}]_i$ to resting levels (Fig. 2b,c; mean glucose-triggered increment 731±150 nmol/l, p<0.001, vs response to first glucose application before fsk/IBMX). In the absence of glucose we also observed that fsk/IBMX itself triggered an increase in $[Ca^{2+}]_i$ in 51 out of 65 cells (Fig. 2d,e), with a mean increment of 288±37 nmol/l (n=65, p<0.001).

Similar results in pancreatic beta cells have been attributed to contributions from separate cAMP-dependent pathways involving EPAC and PKA [33]. By RT-PCR, we detected both EPAC1 and EPAC2, suggesting that this pathway might similarly operate in GLUTag cells (Fig. 3a). To attempt to distinguish whether EPAC and PKA mediate calcium responses to fsk/IBMX, we tested the effect of two membrane-permeant cAMP analogues: 6-benzoyl cAMP, a relatively selective activator of PKA over EPAC, and 8-CPT 2-*O*-methyl cAMP, an activator of EPAC but not the PKA [34]. As shown in Fig. 3c, both 6-benzoyl cAMP and 8-CPT 2-*O*-methyl cAMP (both at 100 μmol/l) triggered increases in $[Ca^{2+}]_i$ and enhanced the response to glucose. Whilst these findings suggest a possible role for EPAC and PKA, the data do not exclude the possibility that both cAMP and the analogues act on other cAMP-binding proteins such as ion channels. In secretion studies, however, the PKA inhibitor H89 (10 μmol/l) inhibited fsk/IBMX-triggered GLP-1 release by ~25% (Fig. 3b), suggesting a role for PKA in cAMP-triggered GLP-1 release.

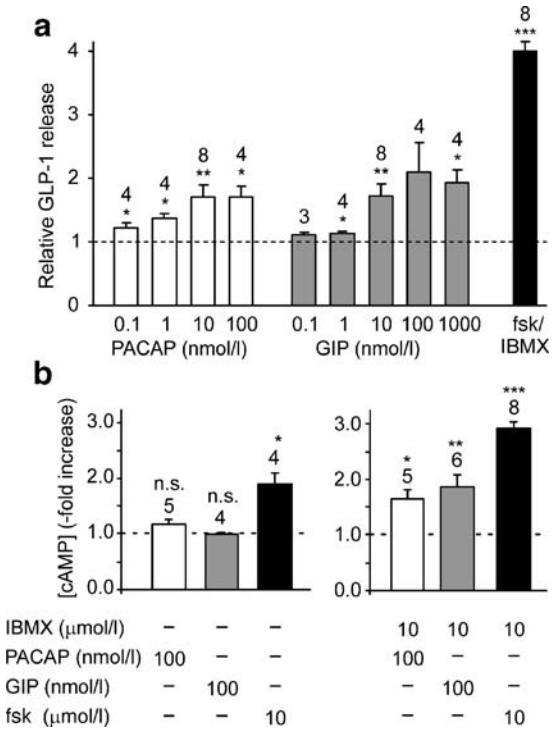

Fig. 1 Hormonal stimulation of GLP-1 release from GLUTag cells. **a** GLP-1 secretion from GLUTag cells incubated for 2 h with 10 mmol/l glucose plus the additions indicated: fsk/IBMX (10 μmol/l of each), PACAP or GIP at different concentrations. Secretion was normalised to baseline secretion in 10 mmol/l glucose alone, measured in parallel on the same day (indicated by the dashed line). The dose-dependence of GLP-1 secretion triggered by GIP and PACAP was fitted using a logistic equation, with EC_{50} values of 7 and 1 nmol/l, respectively. The number of wells is given above each bar. Statistical significance, comparing secretion in 10 mmol/l glucose with and without the test agent, was tested using Student's t test, *p<0.05, **p<0.01, ***p<0.001. **b** cAMP concentrations in GLUTag cells incubated for 15 min with 10 mmol/l glucose plus the additions indicated: IBMX (10 μmol/l), PACAP (100 nmol/l), GIP (100 nmol/l) or fsk (10 μmol/l). The measured cAMP concentration was normalised to that measured in control wells, containing either 10 mmol/l glucose alone (left-hand panel) or 10 mmol/l glucose plus IBMX (right-hand panel). IBMX did not significantly alter background cAMP levels in 10 mmol/l glucose. The number of wells is given above each bar. Statistical significance, comparing the fold-elevation of cAMP by test agents, was assessed by one-sample Student's t test, *p<0.05, **p<0.01, ***p<0.001, n.s., not significant

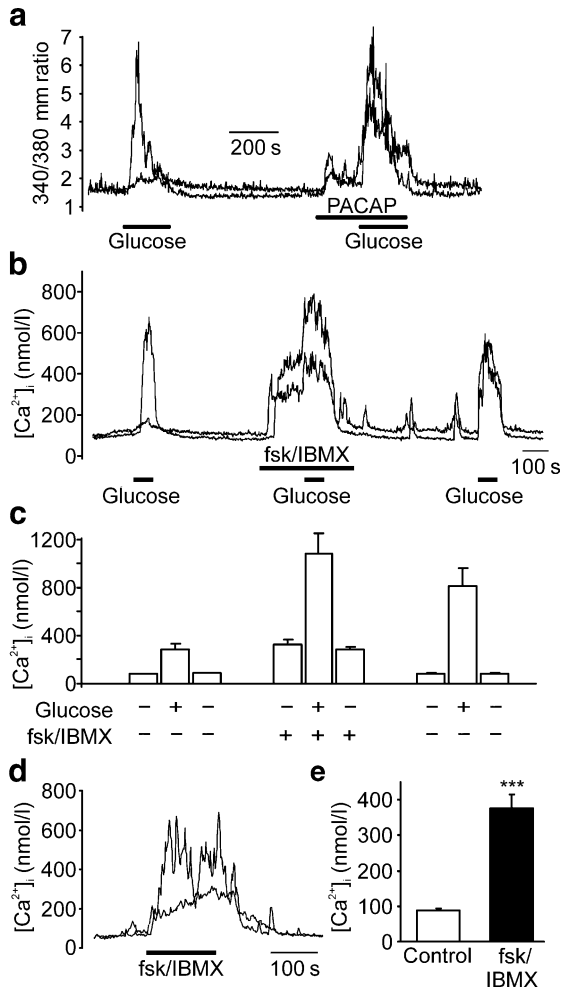

Fig. 2 Intracellular calcium responses to cAMP elevation. **a** $[Ca^{2+}]_i$, monitored as the ratio of fluorescence at 340 and 380 nm, in two individual GLUTag cells loaded with fura-2. PACAP (10^{-7} mol/l) and glucose (10 mmol/l) were added as indicated by the horizontal bars. **b** Calibrated $[Ca^{2+}]_i$ in two single cells in response to 10 mmol/l glucose and fsk/IBMX (10 μmol/l of each), added as indicated by the horizontal bars. **c** Mean $[Ca^{2+}]_i$ averaged over 20 s periods for experiments carried out as in **b** (n=36–40 cells from four separate experiments). Error bars indicate 1 SE. **d** $[Ca^{2+}]_i$ in two single cells in response to fsk/IBMX (10 μmol/l of each), added as indicated by the horizontal bar in the absence of nutrient. **e** Mean $[Ca^{2+}]_i$ averaged over 20 s periods for experiments carried out as in **d** (n=65 cells from six separate experiments). Error bars indicate 1 SE. ***p<0.001 by Student's t test

To evaluate the possible role of intracellular Ca^{2+} stores in fsk/IBMX-triggered GLP-1 release, we measured secretion following depletion of the endoplasmic reticulum Ca^{2+} stores using thapsigargin. Preincubation of GLUTag cells with 10 μmol/l thapsigargin did not impair responses to glucose or fsk/IBMX (data not shown), suggesting that the release of stored Ca^{2+} is not critical for cAMP-triggered GLP-1 release. By contrast, we showed previously that thapsigargin abolished GLP-1 secretion triggered by bombesin, which operates through inositol trisphosphate-mediated Ca^{2+} release [17].

Electrophysiological experiments were performed to investigate whether increasing the cAMP concentration affected the properties of the voltage-gated calcium current. In perforated-patch experiments, fsk/IBMX caused a modest, but non-significant, increase in the peak Ca^{2+} current of 12±5% (at +10 mV, n=4), without affecting the voltage-dependence of activation ($V_{0.5}$ +1±5 mV in control vs −2±3 mV in fsk/IBMX, n=4), or the time course of inactivation (τ at +10 mV 53±5 ms in control vs 56±2 ms in fsk/IBMX, n=4) (Fig. 4). In support of the idea that fsk/IBMX does not significantly affect calcium currents in GLUTag cells, we also found by fluorescence imaging that the calcium increment triggered by 17 mmol/l KCl was not significantly different in the absence and presence of fsk/IBMX (Fig. 4c, mean change in $[Ca^{2+}]_i$ 245±37 nmol/l vs 282±32 nmol/l, respectively).

An alternative explanation for the Ca^{2+} imaging results is that fsk/IBMX directly affects the membrane potential. To investigate this possibility, cells were monitored electrophysiologically in perforated-patch whole-cell recordings (Fig. 5) at low glucose concentrations (0.2–1 mmol/l).

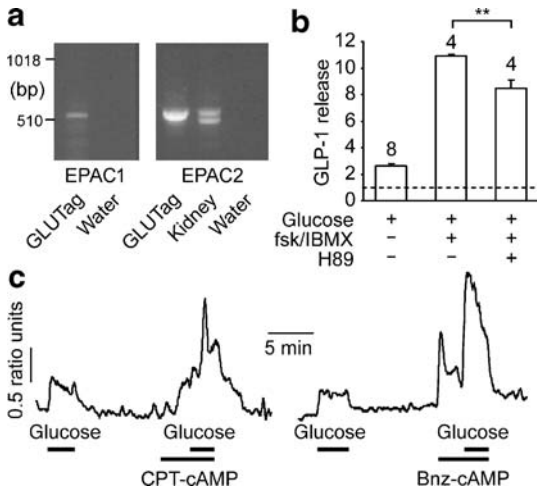

Fig. 3 EPAC- and PKA-dependent actions. **a** Expression of *Epac1* and *Epac2* in GLUTag cells, as determined by RT-PCR. The predicted band sizes are 572 and 539 bp, respectively. **b** GLP-1 secretion from GLUTag cells incubated for 2 h with 10 mmol/l glucose plus fsk/IBMX (10 μmol/l of each) and H89 (10 μmol/l) as indicated. Secretion was normalised to baseline secretion in the absence of glucose, measured in parallel on the same day (indicated by the dashed line). The number of wells is given above each bar. Statistical significance, comparing secretion in 10 mmol/l glucose plus fsk/IBMX with and without H89, was evaluated using Student's t test, **p<0.01. **c** $[Ca^{2+}]_i$, monitored as the ratio of fluorescence at 340 and 380 nm, in GLUTag cells loaded with fura-2. Glucose (10 mmol/l), 6-benzoyl cAMP (Bnz-cAMP, 100 μmol/l) and 8-CPT 2-*O*-methyl cAMP (CPT-cAMP, 100 μmol/l) were added as indicated by the horizontal bars. The vertical scale bar represents a change in the fluorescence ratio of 0.5. The mean of 15 cells from one experiment is shown for CPT-cAMP, and the mean of 19 cells is shown for Bnz-cAMP; these are representative of two and three experiments, respectively

Addition of fsk/IBMX resulted in a mean membrane depolarisation of +5.3±1.1 mV (n=6, p<0.01) and an increase in the mean action potential frequency from 0.07±0.05 to 0.4±0.1 Hz (n=6, p<0.05).

To examine the nature of the underlying depolarising currents, we studied the cells in voltage clamp. As we have previously shown that GLUTag cells express hyperpolarisation activated channels [16], which are known to be modulated by cAMP in other tissues, we first used a voltage protocol suitable for I_h measurements. I_h currents were triggered by holding cells at −40 mV, and stepping for 2 s to sequentially more hyperpolarised potentials (Fig. 5b). Following addition of fsk/IBMX, I_h appeared at more depolarised potentials and showed a more rapid rate of activation (Fig. 5c,d). The voltage at which I_h currents were half-maximally activated was shifted from −85.7±1.9 mV to −72.1±1.7 mV (n=6, p<0.001), without any change in the maximum conductance.

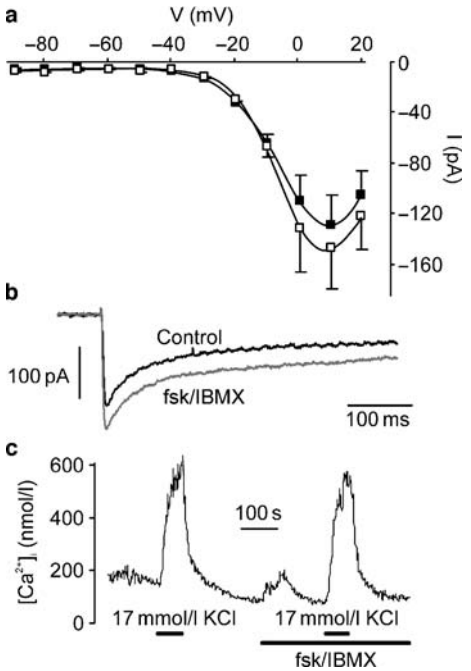

Fig. 4 Voltage-gated calcium currents. **a** Voltage-dependence of the peak calcium current in response to voltage steps to the different potentials shown from a holding potential of −70 mV in perforated-patch experiments. Current (I)–voltage (V) relationships are shown in the absence (closed squares) and presence (open squares) of fsk/IBMX (10 μmol/l of each). Error bars represent 1 SE (n=4). The line shows a fit of the data with a Boltzmann equation $y = (A_2 + [A_1 - A_2]/(1 + \exp([V_{0.5} - V_m]/k))) \times (V_m - V_{rev})$, with the following values in the absence and presence of fsk/IBMX, respectively: A_1 6.1, 4.9 nS; A_2 0.05, 0.06 nS; $V_{0.5}$ +2.1, −1.5 mV; k 8.7, 7.1; V_{rev} +40, +46 mV. **b** Representative traces showing the current response to a voltage step from −70 to +10 mV, in the absence and presence of fsk/IBMX. **c** Calibrated $[Ca^{2+}]_i$ in a GLUTag cell loaded with fura-2, and stimulated by 17 mmol/l KCl and/or fsk/IBMX (10 μmol/l of each), as indicated by the horizontal bars

To investigate whether the activation of I_h by cAMP underlies the responsiveness of GLUTag cells to fsk/IBMX, we tested the effect of ZD7288, which blocks ~70% of the I_h current in GLUTag cells at a concentration of 10 μmol/l [16]. In secretion studies, 100 μmol/l, but not 10 μmol/l, ZD7288 significantly inhibited fsk/IBMX-triggered GLP-1 release (Fig. 6a). In electrophysiological experiments, fsk/IBMX still triggered membrane depolarisation of +4.8±0.9 mV in the presence of 10 μmol/l ZD7288 (n=9; not significantly different from the depolarisation of 5.3 mV in the absence of ZD7288) and the firing of action potentials (Fig. 6b), consistent with the incomplete block of secretion by ZD7288. To investigate whether residual I_h currents could explain the depolarisation observed in ZD7288, we recorded the response to a series of voltage ramps from −90 to −40 mV before and after fsk/IBMX application in the continued presence of

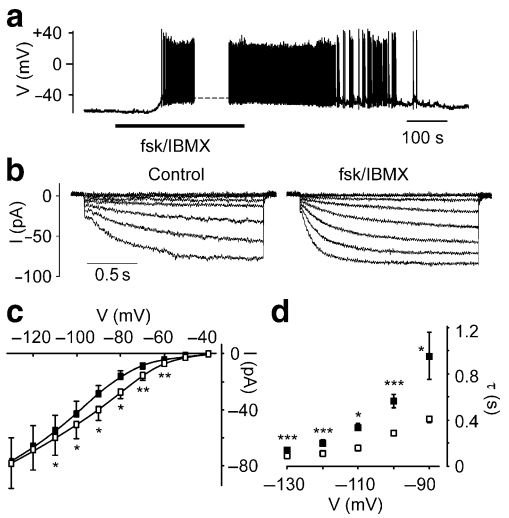

Fig. 5 Hyperpolarisation-activated channels. **a** Membrane potential of a single GLUTag cell monitored in a perforated-patch experiment. The cell was maintained in 0.2 mmol/l glucose, and fsk/IBMX (10 μmol/l of each) added as shown by the horizontal bar. The gap represents a period of ~2 min, during which the cell was switched to voltage clamp. **b** Current (I) responses to 2,000 ms voltage steps of −10 mV increments (from −40 mV to −110 mV) from a holding potential of −40 mV, in a perforated-patch voltage-clamp recording applied at ~0.25 Hz. The same cell was recorded in control solution containing 1 mmol/l glucose (left) and in the additional presence of fsk/IBMX (right). **c** Current–voltage relationship of the mean current at the end of voltage steps recorded as in **b**, in the absence (closed squares) and presence (open squares) of fsk/IBMX. Error bars represent 1 SE (n=5). The lines show fits of the data with a modified Boltzmann equation: $y = g_{max\,(I_h)} \times (V_m + 40)/\left(1 + e^{(V_m - V_{0.5})/k}\right) + g_b \times \left(V_m - V_{rev(b)}\right)$, with the following values, in the absence and presence of fsk/IBMX, respectively: $g_{max\,(I_h)}$ 0.69, 0.74 nS; $V_{0.5}$ −86, −69 mV; k 10.9, 9.9; g_b 0.17, 0.12 nS; $V_{rev(b)}$ −39, −34 mV. *p<0.05, **p<0.01 by paired t test. **d** Voltage-dependence of the time constant of activation of I_h. Current traces obtained as in **b** at different voltages were fitted with a single exponential of time constant τ. Control, filled squares; fsk/IBMX, open squares (n=5–6). Error bars represent 1 SE. Statistical significance, comparing τ in the absence and presence of fsk/IBMX at each voltage, was tested using Student's t test, *p<0.05, ***p<0.001

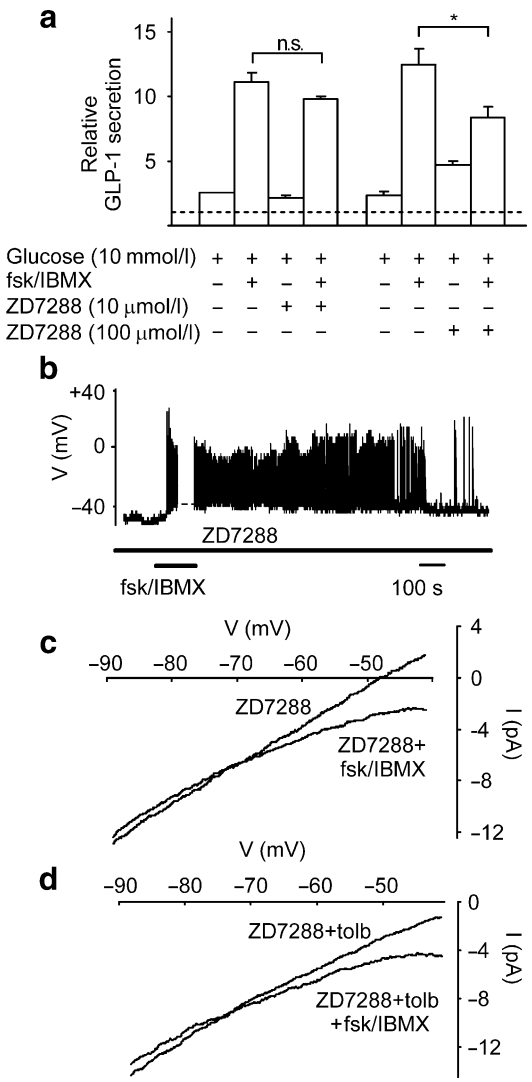

Fig. 6 Effect of fsk/IBMX following I_h inhibition. **a** GLP-1 secretion from GLUTag cells incubated for 2 h with 10 mmol/l glucose plus fsk/IBMX (10 μmol/l of each) and ZD7288 at the concentrations indicated. Secretion was normalised to baseline secretion (indicated by the dashed line) in the absence of stimuli, measured in parallel on the same day (error bars represent 1 SE; $n=3$ wells each). *$p<0.05$, n.s., non-significant by Student's t test. **b** Membrane potential of a single GLUTag cell monitored in a perforated-patch experiment. The cell was maintained in 0.2 mmol/l glucose plus 10 μmol/l ZD7288, and fsk/IBMX (10 μmol/l of each) added as shown by the horizontal bar. The gap represents a period of ~2 min, during which the cell was switched to voltage clamp. **c** Current (I)–voltage (V) relationships for the conductance remaining in the presence of ZD7288 (10 μmol/l) before and following addition of fsk/IBMX (10 μmol/l of each). Currents were triggered by repeatedly ramping the voltage over 2 s from −90 to −40 mV, from a holding potential of −70 mV. Twenty ramps were averaged in each condition. The data show the mean result from seven cells. **d** As in **c**, in the additional presence of 500 μmol/l tolbutamide (tolb). The data show the mean result from three cells

10 μmol/l ZD7288. Under these conditions, we observed that fsk/IBMX inhibited a current that reversed at about −70 mV (Fig. 6c), close to the reversal potential of K^+ ions.

As GLUTag cells contain functional ATP-sensitive potassium (K_{ATP}) channels, which are modulated by agents that increase cAMP in pancreatic beta cells [29], we examined the effect of fsk/IBMX in the presence of both ZD7288 and the K_{ATP} channel inhibitor, tolbutamide (500 μmol/l). Under these conditions we still observed the inhibition by fsk/IBMX of a conductance reversing at about −70 mV (Fig. 6d), suggesting that this is a K^+ current distinct from K_{ATP}. Overall the results suggest that elevation of cAMP triggers membrane depolarisation in GLUTag cells as a consequence of both activation of I_h currents and inhibition of a background K^+ conductance.

To investigate whether cAMP also enhances secretion downstream of membrane depolarisation, we tested the effect of fsk/IBMX in GLUTag cells depolarised with 30 mmol/l KCl plus 340 μmol/l diazoxide, which produces a robust elevation of $[Ca^{2+}]_i$. Under these conditions, in the absence of glucose, fsk/IBMX still stimulated secretion 2.3±0.2-fold ($n=4$). This is likely to reflect an action of cAMP downstream of Ca^{2+} entry, as calcium currents were not affected by fsk/IBMX (Fig. 4). In beta cells, a similar potentiating effect of cAMP has been reported but is glucose-dependent [30].

Discussion

Elevation of cytoplasmic cAMP is a well-recognised stimulus of GLP-1 secretion in a variety of model systems, including perfused intestine, cultured fetal and adult intestinal cells and cell lines such as GLUTag and STC-1 [11, 14, 15, 21]. Although previously shown to increase transcription of the proglucagon gene through PKA-dependent and EPAC-dependent pathways [12, 27], there is also evidence that cAMP elevation directly enhances secretion [22]. We show here that cAMP acts as both a trigger and a potentiator of GLP-1 release from the GLUTag cell line, and that the triggering pathway involves the modulation of ion channel activity in the plasma membrane, leading to membrane depolarisation and action potential generation. The strong depolarising action of cAMP in the GLUTag cell may explain how fsk/IBMX triggers GLP-1 release from GLUTag cells even in the absence of added nutrient.

Two cAMP-sensitive conductances were identified electrophysiologically in GLUTag cells, which were distinct from those identified in studies on pancreatic beta cells [29]. We have previously shown that GLUTag cells express hyperpolarisation-activated channels, which are known to be modulated by cAMP in other cell types [35]. In resting GLUTag cells, with a membrane potential of −40 to −50 mV, the I_h current is negligible. However, fsk/IBMX

shifted the voltage-dependence of I_h activation by ~14 mV, which would induce a depolarising I_h current of ~0.5 pA at −50 mV. Although very small, this would be sufficient to trigger a depolarisation of a few mV in a GLUTag cell with a typical membrane resistance of 5 GΩ. In other cell types, I_h has been shown to modulate action potential frequency and pacemaker activity, as it is activated during the hyperpolarisation that follows an action potential [35]. Inhibiting I_h in GLUTag cells with ZD7288 did not prevent membrane depolarisation triggered by fsk/IBMX, and relatively high concentrations were needed to impair fsk/IBMX-triggered GLP-1 secretion. At such high concentrations, the specificity of ZD7288 must be questioned as it has been shown to inhibit exocytosis directly [36], although a generalised inhibition of exocytosis by 100 μmol/l ZD7288 in our experiment seems unlikely, as it increased rather than reduced the background secretion in 10 mmol/l glucose. The maintained depolarising effect of fsk/IBMX in the presence of ZD7288, however, suggests that modulation of I_h, whilst possibly contributing to the effect of fsk/IBMX in GLUTag cells, does not fully explain the results.

In the presence of ZD7288, we found that fsk/IBMX also inhibited a small current with a reversal potential of about −70 mV, probably therefore reflecting a K^+ flux. This was distinct from K_{ATP}, as it was not abolished by tolbutamide. Examination of the current–voltage relationships in Fig. 6c,d suggests that this conductance exhibits outward rectification, although the appearance of outward rectification could also be generated by an additional small ZD7288-resistant I_h current appearing at more negative potentials. The identity of this K^+ current remains unknown, but possible candidates are members of the two-pore K^+ channel family, such as TREK-1, which we have shown previously to be expressed in GLUTag cells by Affymetrix microarray analysis [16]. TREK-1 and TREK-2 (which was not represented on the original Affymetrix chip) are inhibited by cAMP via PKA and exhibit outward rectification in physiological solutions [37].

The finding that cAMP modulates the activity of ion channels in GLUTag cells makes it difficult to interpret fully the cAMP analogue data. Our data suggest that PKA plays a role in fsk/IBMX-triggered secretion as the calcium changes were mimicked by 6-benzoyl cAMP, and H89 reduced GLP-1 release in the presence of fsk/IBMX. It is less clear, however, whether EPAC contributes to cAMP-triggered secretion. Although 8-CPT 2-*O*-methyl cAMP triggered changes in $[Ca^{2+}]_i$, we cannot exclude the possibility that this analogue also targets cAMP-binding sites on ion channels.

In calcium imaging experiments, we observed that as well as triggering an increase in $[Ca^{2+}]_i$, fsk/IBMX and PACAP enhanced the response to glucose by increasing both the number of active cells and the magnitude of responses in individual cells. Activation of voltage-gated Ca^{2+} channels is unlikely to underlie these effects, as the calcium currents were not significantly affected by fsk/IBMX. The effects on $[Ca^{2+}]_i$ could, however, be explained by the depolarising action of cAMP. The small I_h current and reduced background K^+ conductance would tend to make the cells more excitable and enhance responses to other depolarising stimuli, such as those induced by nutrients.

Previous studies in GLUTag cells have shown that elevation of $[Ca^{2+}]_i$ is essential for exocytosis, indicating that agents that only enhance the secretory response downstream of the calcium signal or stimulate proglucagon gene transcription would be ineffective without an additional trigger that stimulates either Ca^{2+} entry or Ca^{2+} release from stores. We showed previously that a calcium signal can be initiated in GLUTag cells by nutrients such as glucose and glutamine, or by activation of ionotrophic γ-amino butyric acid or glycine receptors [18–20, 22]. We show here that hormones elevating cAMP may also initiate an increase in $[Ca^{2+}]_i$.

The nutrient-independent ability of cAMP to trigger electrical activity and potentiate secretion from GLUTag cells contrasts with the glucose-dependence of its actions on the pancreatic beta cell [28, 30]. Physiologically, the glucose-dependence of beta cell function is essential to ensure that insulin release is not stimulated when glucose concentrations are low, with the consequent risk of hypoglycaemia. In L cells, by contrast, an ability of adenylate cyclase-coupled hormones and neurotransmitters to trigger GLP-1 release in the absence of nutrient could provide a pathway for the indirect stimulation of L cells lower in the gut, where levels of luminal nutrients are low. Further evaluation of the effects of cAMP elevation in vivo will require careful experiments, as the majority of anaesthetic agents affect ion channels and receptors that are produced in either the enteric nervous system or L cells. However, our results, taken together with the previous literature, suggest that targeting receptors on L cells coupled to adenylate cyclase could provide a therapeutic strategy to stimulate GLP-1 release in vivo.

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