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Aroma compound diacetyl suppresses glucagon-like peptide-1 production and secretion in STC-1 cells

Triona McCarthy[a,b], Christine Bruen[a], Fiona O'Halloran[a], Harriet Schellekens[b], Kieran Kilcawley[c], John F. Cryan[b] and Linda Giblin[a*]

[a] Food for Health Ireland, Teagasc Food Research Centre Moorepark, Fermoy, Cork, Ireland.

[b] Food for Health Ireland, Dept of Anatomy and Neuroscience, University College Cork, Ireland.

[c] Teagasc Food Research Centre Moorepark, Fermoy, Cork, Ireland.

* corresponding author Tel: +353-25-42614 Fax: +353-25-42340

Email Addresses: Triona Mc Carthy: triona.mccarthy@hotmail.com, Christine Bruen: bruen_christine@lilly.com, Fiona O'Halloran: Fiona.OHalloran@cit.ie, Harriet Schellekens: H.Schellekens@ucc.ie, Kieran Kilcawley: Kieran.Kilcawley@teagasc.ie, John Cryan: J.Cryan@ucc.ie, Linda Giblin: linda.giblin@teagasc.ie

Abbreviated running title: Diacetyl suppresses the satiety hormone GLP-1

Abstract

Diacetyl is a volatile flavour compound that has a characteristic buttery aroma and is widely used in the flavour industry. The aroma of a food plays an important role in food palatability and thus intake. This study investigates the effect of diacetyl on the satiety hormone, glucagon-like peptide (GLP-1), using the enteroendocrine cell line, STC-1. Diacetyl decreased *proglucagon* mRNA and total GLP-1 from glucose stimulated STC-1 cells. This dampening effect on GLP-1 appears to be mediated by increasing intracellular cAMP levels, increasing synthesis of the G protein coupled receptor, GPR120, and its recruitment to the cell surface. Voltage gated Ca_{2+} channels, K^{+}_{ATP} channels and the α–gustducin taste pathway do not appear to be involved. These findings demonstrate that components contributing to food palatability suppress GLP-1. This ability of diacetyl to reduce satiety signals may contribute to overconsumption of some palatable foods.

Keywords

Glucagon-like peptide 1

Diacetyl

Flavour compound

Satiety

GPR120

Chemical Compounds

Diacetyl (PubChem CID:650); Nicardipine (PubChem CID:41114); Nitrendipine (PubChem CID: 4507); Tolbutamide (PubChem CID 5505)

1. Introduction

The gut hormone glucagon-like peptide 1 (GLP-1) has attracted considerable interest in recent years due to its ability to enhance glucose-dependent insulin secretion, promote pancreatic β-cell proliferation and reduce food intake. It has also been reported that GLP-1 may reduce the rewarding and reinforcing properties of palatable foods (Dickson, Shirazi, Hansson, Bergquist, Nissbrandt, & Skibicka, 2012). Secretion of this nutrient-responsive hormone is impaired in obesity and type 2 diabetes (Toft-Nielsen, et al., 2001) and infusion of GLP-1 has been shown to improve glycemia and reduce food intake in obese patients (Nauck, et al., 1998).

GLP-1 is produced by L cells of the distal jejunum and ileum following tissue-specific proteolytic processing of the *proglucagon* gene (Baggio & Drucker, 2007). The arrival of carbohydrate, fat and protein in the gut lumen triggers GLP-1 release (Bruen, O'Halloran, Cashman, & Giblin, 2012). Inhibition of GLP-1 by food has not been extensively studied. Within the L cells, GLP-1 secretion occurs in response to an increase in intracellular levels of cyclic adenosine monophosphate (cAMP) and Ca^{2+} (Tolhurst, Reimann, & Gribble, 2009). Changes in these mediators are brought about by nutrient uptake pathways, metabolic closures of potassium channels and/or activation of nutrient-responsive G protein-coupled receptors (GPCRs) (Reimann, et al., 2012). These GPCRs play various roles in GLP-1 secretion. The taste GPCRs, T1r3/T1r2, and the G protein α-gustducin are involved in the secretion of GLP-1 in response to sugars (Jang, et al., 2007). On the other hand, free fatty acids (FFA) can induce GLP-1 secretion via the GPCRs, GPR40 and GPR120 (Hirasawa, et al., 2005; Reimann, et al., 2012). GPR120 is highly expressed in enteroendocrine L cells (Anbazhagan, et al., 2016; Hirasawa, et al., 2005) and is activated in response to unsaturated long-chain free fatty acids (FFAs) (Tanaka, Yano, Adachi, Koshimizu, Hirasawa, & Tsujimoto, 2008). Recent evidence also suggests that GPR120 plays an important role in the

orosensory detection and preference for fats (Cartoni, et al., 2010). GPR120 is classified as a $G_{q/11}$-coupled receptor, capable of increasing GLP-1 secretion via phospholipase Cβ and intercellular Ca^2 signalling (Blad, Tang, & Offermanns, 2012) although there is also some evidence of signalling promiscuity (Reimann, et al., 2012; Tsukahara, et al., 2015).

While the role of taste receptors and tastants in GLP-1 secretion has been widely investigated in recent years, there is less information available on whether aromatic compounds may also influence satiety signals. The aroma of a food plays an important role in food palatability and intake (Massolt, van Haard, Rehfeld, Posthuma, van der Veer, & Schweitzer, 2010; Ruijschop, Boelrijk, de Graaf, & Westerterp-Plantenga, 2009). While such effects may be mediated through neural pathways, it is also possible that aroma compounds may influence satiety signals. Indeed, it has been demonstrated that food-derived odorants present in the gut lumen may stimulate serotonin release via olfactory receptors present in human enterochromaffin cells (Braun, Voland, Kunz, Prinz, & Gratzl, 2007) and aroma intensity certainly influences perceived satiation (Ruijschop, et al., 2009).

Diacetyl (2,3-butanedione) is a volatile favour compound that occurs naturally in several foods, such as butter, milk, cheese, fruit and coffee and has a characteristic buttery aroma (Bartowsky & Henschke, 2004). It is primarily produced by citrate fermenting lactic acid bacteria during pyruvate metabolism. It is widely used in the flavouring industry. This pleasant buttery aroma is perceived as a positive attribute by consumers and has been shown to play a significant role in food preference and palatability (Liggett, Drake, & Delwiche, 2008).

The present study was undertaken to evaluate whether diacetyl alters GLP-1 production and secretion, using the murine secretin tumor cell line (STC-1). STC-1 is a popular and reliable enteroendocrine model to investigate gut hormone production and secretion. Similar to native L cells, STC-1 cells secrete GLP-1 in response to sugars, peptides, fatty acids, sweeteners,

bitter tastants, food bioactives, hormones and bile (McCarthy, Green, Calderwood, Gillespie, Cryan, & Giblin, 2015). However levels of response may differ to the native state (Kuhre, et al., 2016). STC-1 cells also express GPR120, taste receptors (T1r1, T1r2, T1r3) and α-gustducin (Hirasawa, et al., 2005; Wu, Rozengurt, Yang, Young, Sinnett-Smith, & Rozengurt, 2002) and are recognized as a good model for taste signalling (Saitoh, Hirano, & Nishimura, 2007).

2. Materials and methods

2.1. Chemicals

Diacetyl (2,3 butanedione), KREBS ringers bicarbonate buffer, nicardipine, nitrendipine, tolbutamide, pertussis toxin, polyethylene glycol, DMSO, Hanks Balanced Salt Solution, DMEM media, glucose, L-glutamine, Foetal Bovine Serum, penicillin, streptomycin, 3-Isobutyl-1-methylxanthine (IBMX), forskolin, poly-l-lysine coated glass-slides, paraformaldehyde, HEPES, NaCl, EDTA, ethylene glycol tetraacetic acid, Nonident P40, dithiothreitol, Na_3VO_4, phenylmethysulphonyl fluoride, aprotinin, leupeptin, NaF, NaPPi, Tris-HCL, SDS, glycerol, bromophenol blue, Tween-20, Phosphate Buffered Saline (PBS), Tris buffered saline, Triton X-100, paraformaldehyde, Bovine Serum Albumin (BSA), donkey serum and β-actin horseradish peroxidase antibody were all sourced from Sigma Aldrich, Ireland. Alpha-linolenic acid was sourced from Cayman Europe, Estonia. Glyoxyl buffer was sourced from Ambion, USA. Rabbit anti-GPR120 antibody was purchased from Life Span Biosciences, USA. Donkey anti-rabbit Alexa 488 antibody was purchased from Santa Cruz Biotechnology, USA and horseradish peroxidase-conjugated anti-rabbit IgG was purchased from Jackson Immunoresearch, USA. Alamar Blue, NuPAGE 4-12 % Bis-Tris Gels and TOPO-TA cloning kit were purchased from Invitrogen, USA. QIAzol Lysis

Reagent Qiagen miRNeasy Mini Kit, QIAquick PCR Purification Kit and QuantiTect reverse transcription kit were sourced from Qiagen, UK. LightCycler 480 SYBR Green I Master kit was sourced from Roche Diagnostics, Germany. Halt Protease and Phosphatase Inhibitor were purchased from Thermo Fisher Scientific, USA. Total GLP-1 MSD® metabolic assay was purchased from Meso Scale Discovery, USA. The cAMP HTRF kit was purchased from Cisbio Bioassays, France. Pierce Biotechnology, USA supplied the BCA protein assay kit and the chemiluminescence Western blotting Substrate Kit. Polyvinylidene difluoride membranes were purchased from BIO-RAD, France. Non-fat dry milk and skim milk were purchased from Marvel, Premier Foods Limited, UK.

Stock solution of nicardipine (5 mM) was prepared in polyethylene glycol. Stock solution of nitrendipine (1 mM) was prepared in DMSO. Tolbutamide stock solution was prepared in DMSO at a concentration of 100 mM. Stock solution of Pertussis toxin (50 µg/ml) was prepared in distilled H_2O. All test compounds were prepared fresh, immediately before experiments in KREBS Ringers bicarbonate buffer (0.0468 g/l $MgCl_2$, 0.34 g/l KCl, 7 g/l NaCl, 0.1 g/l Na_2HPO_4, 0.18 g/l NaH_2PO_4, 1.8 g/l D-glucose, 1.26 g/l $NaHCO_3$) unless stated otherwise. Alpha-linolenic acid (100 µM) was prepared in pre-warmed KREBS and sonicated to aid solubility.

2.2. STC-1 exposure to diacetyl and blockers

STC-1 was received as a kind gift from by Prof. B. Wice (Washington University, St. Louis, USA) with permission from Prof. D. Hanahan (Swiss Institute for Experimental Cancer Research, Switzerland). STC-1 cells were cultured in DMEM containing 4.5 g/l of glucose and L-glutamine and supplemented with 17.5 % foetal bovine serum, 100 U/ml of penicillin and 100 mg/l of streptomycin. Cells were passaged upon reaching 80-90 % confluence. Experiments were performed with cells at passage numbers 15-30 (Gonzalez-Abuin,

Martinez-Micaelo, Blay, Green, Pinent, & Ardevol, 2014). STC-1 cells were seeded into 6-well plates at a density of 1.5 x 10^6 cells/well and incubated at 37 °C, 5 % CO_2, for 18 h prior to exposures. Cell monolayers in each well were washed with KREBS (1 ml) and then pre-incubated for 1 h with KREBS (500 µl). Following the pre-incubation period, KREBS was aspirated off and replaced with 1 ml of diacetyl, freshly prepared in KREBS, at various concentrations. Cells were exposed to diacetyl solution for 10 min, 30 min, 1 h, 2 h, 3 h and 4 h in sealed plates at 37 °C, 5 % CO_2. STC-1 cells were incubated with the blocker of interest for 15 min prior to exposure to 1000 ppb of diacetyl for a further 4 h. The final working concentrations of the blockers, prepared in KREBS buffer, were as follows: 5 µM nicardipine and nitrendipine, 100 µM tolbutamide and 10 µg/ml pertussis toxin, as previously described (Chen, Wu, Reeve, & Rozengurt, 2006; McLaughlin, Lomax, Hall, Dockray, Thompson, & Warhurst, 1998; Ristoiu, Pluteanu, Flonta, & Reid, 2002).

2.3. Cellular viability

Following the 1 h pre-incubation step in 10 µl of KREBS, 100 µl of various concentrations of diacetyl (500 ppb, 1000 ppb and 2000 ppb) were added to 1 x 10^5 STC-1 cells/well. Alamar Blue (10 µl) was added to each well and the plate was incubated for 4 h at 37 °C. Fluorescence was measured at 570 nm. STC-1 cell viability in the presence of each blocker was also tested. Data were expressed as % viability of STC-1 cells exposed to test compounds compared to STC-1 cells exposed to KREBS alone.

2.4. Real time-PCR

Following exposures, cell monolayers were washed with 1 ml of Hanks Balanced Salt Solution. Cells were then lysed, using QIAzol Lysis Reagent and total RNA was isolated from cell suspensions using the Qiagen miRNeasy Mini Kit, according to the manufacturer's

instructions. Total RNA was quantified spectrophotometrically, using the Nanodrop 1000 (Thermo Fisher Scientific, USA) and the integrity assessed by electrophoresis in a 1.5 % glyoxyl gel with 1 X glyoxyl buffer. Complementary DNA was prepared from 1 µg of RNA, using the Qiagen QuantiTect reverse transcription kit. Real time PCR was performed in a LightCycler 480 instrument (Roche Diagnostics, Germany) to quantify *proglucagon* (of which exon 4 codes for GLP-1), *GPR40* and *GPR120* mRNA levels. All primers were designed across intron/exon boundaries. Primers for murine *proglucagon* were designed, based on the GenBank sequence (accession Z46845): Forward primer 5' -AGGGACCTTTACCAGTGATGTGA- 3', Reverse primer 5'-ACGAGATGTTGTGAAGATGGTTGT -3'. The annealing temperature for amplification was 56 °C. Primers for murine *GPR40* were designed, based on the Genbank sequence (accession AB095745): Forward Primer 5' –TGCCCGTCTCAGTTTCTCCATTC- 3', Reverse primer 5' –TGTTCCCAAGTAGCCAGTGACCAG- 3'. Primers for murine *GPR120* were designed, based on the Genbank Sequence (accession AY288424): Forward primer 5' GGCCCAACCGCATAGGAGAAAT- 3', Reverse primer 5' –TGAAGGCCACCACCCAGAAGAAAA- 3'. The annealing temperature for amplification for *GPR40* and *GPR120* primers was 50 °C. Plasmid standards for *proglucagon* were created by cloning an amplified PCR product into the pCR4-TOPO vector, using the TOPO-TA cloning system, according to the manufacturer's instructions. Standards for *GPR40* and *GPR120* were created from amplified PCR products, which were purified using a QIAquick PCR Purification Kit, according to the manufacturer's instruction. The cloned amplicon and PCR products were confirmed by sequencing (Beckman Coulter Genomics, UK). For RT-PCR standards, linearized plasmid DNA or PCR products were quantified, using the Nanodrop 1000 (Thermo Fischer Scientific, USA), and a series of dilutions from 10^9 to 10^2copies were generated. For each 10 µl of Lightcycler reaction, 1 µl of test cDNA or

serially-diluted standard was used. The LightCycler 480 SYBR Green I Master kit was used for quantification, using 0.5 µM concentrations of both the forward and reverse primers.

2.5. Secretion studies of Total GLP-1

Following the incubation period, 10 µl of 10 X Halt Protease and Phosphatase Inhibitor were added to each well to inactivate endogenous dipeptidyl peptidase-4 activity. The cellular supernatants were collected by aspiration and stored at -80 °C prior to analysis. Cellular supernatant levels of total GLP-1 were assayed, using an MSD® metabolic assay, according to the manufacturer's instructions. The MSD assay reports a minimum detection limit of 0.3 pM of total GLP-1. Total GLP-1 concentrations (pM) in the samples were quantified by interpolating the intensity of emitted light from standard curves generated in the same assays.

2.6. cAMP accumulation assay

Intracellular cAMP levels were measured, using a cAMP HTRF kit, according to manufacturer's instructions. Briefly STC-1 cells were seeded into a 96-well plate at a density of 6 x 10^5 cells/ml and incubated overnight at 37 °C, 5 % CO_2. Medium was aspirated off and the cell monolayers were washed with 100 µl of KREBS supplemented with 1 mM IBMX. The cells were incubated for 30 min in 25 µl of KREBS-IBMX solution. Cells were then exposed to 25 µl of diacetyl (1000 ppb) for a further 30 min. The positive control used was 1 µM forskolin, a known activator of the enzyme adenylyl cyclase, which causes an increase in intracellular cAMP. The negative control was KREBS-IBMX solution alone. After treatment, the reactions were stopped by addition of lysis buffer containing homogeneous time-resolved fluorescence reagents. Plates were then incubated for 60 min at room temperature, and time-resolved fluorescence resonance energy transfer signals were measured, using a FLUOstar Omega microplate reader (BMG Labtech, Germany).

220

221 *2.7. Immunocytochemistry*

222 For immunocytochemistry, STC-1 cells were seeded at a density of 8 x10^5 cells/ml in 24 well
223 plates containing poly-l-lysine-coated glass-slides and incubated overnight at 37 °C, 5 % CO_2
224 prior to exposure to diacetyl (1000 ppb) or α–linolenic acid (100 μM) for 30 min, 1 h and 4
225 h. As a control, cells were exposed to KREBS alone for the same time intervals. Following
226 exposure, cells were fixed with 4 % paraformaldehyde in PBS for 20min and permeabilized
227 in PBS supplemented with 0.1 % Triton X-100 for 5 min. Slides were than blocked with 10
228 % donkey serum diluted in PBS supplemented with 0.1 % Triton X-100. The slides were then
229 incubated overnight with rabbit anti GPR120 antibody (1:500 dilution), after which the slides
230 were washed and incubated for 2 h at room temperature with donkey anti-rabbit Alexa 488
231 antibody (1:1000). Primary and secondary antibodies were prepared in PBS supplemented
232 with 0.1 % Triton X-100 and 1 % donkey serum. Specificity of GPR120 antibody was
233 confirmed by the absence of fluorescent staining in HEK293T cells which do not express the
234 GPR120 receptor (Tanaka, Yano, Adachi, Koshimizu, Hirasawa, & Tsujimoto, 2008).
235 Specificity of the secondary antibody was confirmed by the absence of fluorescent staining
236 when the primary antibody was omitted (data not shown). Slides were analyzed, using an
237 inverted microscope set-up with a sensitive XM10 camera with an infrared cut filter, mercury
238 burner and fluorescence condenser (Olympus, Japan). Pictures were processed using Cell^F
239 Imaging Software (Olympus, Japan). For fluorescence data, intensity was determined to be
240 the mean pixel intensity for the entire cell using Image J software (National Institutes of
241 Health, USA). Staining was conducted in triplicate and, from an image of each staining; five
242 individual cells were selected for fluorescence intensity analysis.

243

244 *2.8. Western blotting*

STC-1 cells were seeded into 12-well plates at a density of 1.5 x 10^6 cells/well and incubated at 37 °C, 5 %CO_2, for 18 h prior to exposures. Following a 30 min, 1 h or 4 h exposure to KREBS alone, 1000 ppb of diacetyl or 100 mM α-linolenic acid, cells were lysed with lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 1 % Nonident P40, 0.5 mM dithiothreitol and 0.1 mM Na_3VO_4) containing protease inhibitors (0.1 mM phenylmethysulphonyl fluoride, 2 µg aprotinin/ml, 2 µg of leupeptin/ml, 0.02 mM NaF and 0.025 mM NaPPi) and centrifuged at 12,000 *g* for 10 min to remove insoluble debris. Protein content of the lysates was quantified using a BCA kit. The cell lysate was diluted in 4 X sample buffer (333 mM Tris-HCl, 3 % SDS, 26.7 % glycerol, 130 mM dithiothreitol and 0.2 % bromophenol blue) and heated for 10 min at 95 °C. Samples were loaded at 20 µg of protein per lane onto precast NuPAGE 4–12 % Bis-Tris Gels. The separated proteins were transferred onto polyvinylidene difluoride membranes by electroblotting. The membrane was blocked for 1.5 h with Tris buffered saline solution containing 0.1 % Tween-20 supplemented with 3 % non-fat dry milk and 2 % BSA. Membranes were incubated overnight at 4 °C with rabbit anti GPR120 antibody (1:1,000 dilution) in 0.1% Tween-20 supplemented with 1 % skim milk and 1 % BSA. After three washes in 0.1% Tween-20, the membranes were incubated for 1.5 h at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG (1:8,000). Visualisation was performed, using the enhanced chemiluminescence Western blotting Substrate Kit and GelDoc Image Reader (Las3000; Fujifilm, Japan). Sample loading of protein was corrected by staining with a 1:15,000 dilution of a β-actin horseradish peroxidase antibody.

2.9. Statistical analysis

STC-1 cell exposures to vehicle control (KREBS), diacetyl and blockers were performed in duplicate and on two separate occasions. For cellular viability assays, at least 2 wells on two

separate occasions were examined per test compound. RT-PCR assays were performed in triplicate. Total GLP-1 assays were performed in duplicate with both technical and experimental repeats.

Data were analysed by one-way analysis of variance (ANOVA), followed by, where appropriate, Fishers least significant difference (LSD) multiple comparison *posthoc* test. Means without a common letter differ significantly from each other ($P<0.05$).

3. Results

3.1. Diacetyl reduces proglucagon mRNA levels and total GLP-1 secretion in the presence of 10mM glucose

STC-1 cells were exposed to physiologically relevant concentrations of the volatile flavour compound, diacetyl. As in other studies (Zhou & Pestka, 2015), exposures were performed in the presence of the known stimulator, glucose. In our study, experiments were performed in KREBS Ringers bicarbonate buffer, which contains 10 mM glucose. Exposure of STC-1 cells to diacetyl resulted in a significant ($P < 0.05$) dose-dependent decrease in *proglucagon* mRNA levels compared to the vehicle control at all timepoints (Figure 1A). Increasing the exposure time from 10 min to 4 h led to an overall reduction in *proglucagon* mRNA levels (Figure 1A). This reduction in *proglucagon* mRNA coincided with a significant ($P < 0.05$) decrease in secreted levels of total GLP-1 compared with the control (Figure 1B). In STC-1 cells, the presence of diacetyl reduces *proglucagon* transcription and total GLP-1 secretion in response to glucose. In order to address potential cytotoxicity of diacetyl to STC-1 cells, the Alamar Blue assay was employed which is based on the ability of viable cells to reduce resazurin to resorufin. There was no significant difference in the ability of cells to reduce

295 resazurin to resorufin in the presence of various concentrations of diacetyl compared to
296 vehicle control (Figure 2). These results indicated that diacetyl suppression of GLP-1
297 production and secretion is not due to cytotoxicity. GC/MS analysis of cellular supernatant
298 after 4 h exposure to diacetyl revealed that this volatile compound was still present in the
299 supernatant, albeit at a reduced level (data not shown).
300
301 *3.2. Is diacetyl suppression of total GLP-1 mediated via K^+, Ca^{2+} channels, taste pathways or*
302 *intracellular cAMP?*
303 To investigate whether the reduction of transcript levels of *proglucagon* mRNA by diacetyl
304 was mediated through Ca^{2+} channels, glucose stimulated STC-1 cells were preincubated with
305 L-type voltage-dependent Ca^{2+} channel blockers, nicardipine and nitrendipine, prior to
306 exposure to diacetyl. Both failed to restore *proglucagon* mRNA levels to those observed with
307 KREBS (Figure 3A). Indeed in both cases, there was a further small yet significant reduction
308 ($P < 0.05$) in *proglucagon* mRNA levels compared with exposure to diacetyl alone.
309 Preincubation with the K^+ channel blocker, tolbutamide, followed by exposure to diacetyl,
310 failed to restore *proglucagon* mRNA levels to vehicle control (Figure 3A). To examine if the
311 effects of diacetyl were mediated through taste signalling pathways, glucose stimulated STC-
312 1 cells were incubated in the presence of pertussis toxin, a known blocker of the α-subunit of
313 gustducin prior to exposure to diacetyl (Figure 3A). Blocking α-gustducin resulted in a 58-
314 fold decrease in *proglucagon* mRNA levels in glucose stimulated STC-1 cells compared with
315 the vehicle control and 17-fold decrease compared with levels observed with diacetyl (Figure
316 3A). These observed decreases were not as a result of decreases in cellular viability in the
317 presence of the various blockers (data not shown).
318 Secreted levels of total GLP-1 were measured in STC-1 cells stimulated with glucose in the
319 presence of the various blockers (data not shown). Nicardipine increased levels of secreted

total GLP-1 from glucose stimulated STC-1 cells by 2-fold ($P < 0.05$). GLP-1 levels remained unchanged with nitrendipine compared with vehicle control. STC-1 cells secreted significantly more GLP-1 in the presence of tolbutamide ($P < 0.05$) compared with glucose alone (vehicle control). Pertussis toxin had no effect on GLP-1 levels. To determine if the various blockers could block the inhibition of diacetyl on secreted GLP-1 levels, STC-1 cells were preincubated with each individual blocker prior to diacetyl exposure. None of the blockers were capable of altering levels of secreted GLP-1 from glucose-stimulated STC-1 cells exposed to diacetyl (Figure 3B).

The failure to respond to tolbutamide in a diacetyl background may indicate that diacetyl exposure causes cAMP levels to rise in STC-1 cells, closing K_{ATP}-channels (Reimann, 2010). Intracellular cAMP was therefore measured in a competitive immunoassay. Glucose-stimulated STC-1 cells exposed to 1000 ppb of diacetyl had a 5-fold increase in intracellular cAMP compared with cells incubated with glucose alone (Figure 4).

3.3. Diacetyl increases GPR120 mRNA levels and cell surface expression

As diacetyl is a methyl ketone and is structurally similar to methyl ketones produced during oxidation of free fatty acids, we investigated if GPR40 and GPR120 responded to diacetyl presence. Diacetyl had no significant effect on mRNA levels of the medium- to long-chain fatty acid receptor, *GPR40*, in glucose-stimulated STC-1 cells (Figure 5A). However, mRNA levels of the long chain fatty acid receptor, *GPR120*, were significantly ($P < 0.05$) increased in glucose-stimulated STC-1 cells exposed to diacetyl compared with KREBS (Figure 5B). To explore whether GPR120 proteins levels were also affected by diacetyl, time-course experiments was performed by immunofluorescent staining and Western blotting. Exposure of glucose-stimulated STC-1 cells, over a 4 h period, to diacetyl significantly ($P < 0.05$) and progressively increased GPR120 levels, both at protein and cell surface level, compared with

vehicle control (Figure 6). These increases with 1000 ppb of diacetyl (which equates to 11.7 μM) were also significantly higher than those observed with the GPR120 agonist, α-linolenic acid (100 μM), indicating that diacetyl is more potent.

4. Discussion

The widely used flavour ingredient diacetyl inhibits production and secretion of GLP-1 by intestinal endocrine cells *in vitro*. This damping effect on GLP-1 appears to be mediated by recruiting GPR120 to the cell surface, increasing intercellular cAMP levels and increasing GPR120 synthesis. The mechanism of GLP-1 reduction by diacetyl appears to be electroneutral, as evidenced by independence from K^+_{ATP} channels and voltage-gated Ca^{2+} channels. The α–gustducin taste pathway also does not appear to be involved in response to this flavour compound. Whether diacetyl enters the cell is unknown.

Diacetyl is structurally similar to methyl ketones produced during oxidation of free fatty acids. Ketones produced in this manner are mainly derived from fatty acids with 6 to 12 carbons. This may thus explain the ability of diacetyl to interact with GPR120. GPR120 is classified as a G_q protein-coupled free fatty acid (Blad, et al., 2012) that is internalized in the presence of long chain free fatty acids with concomitant increase in cytosolic Ca^{2+} but with no effect on cAMP levels (Hirasawa, et al., 2005). However, with the knowledge that GPR120 also plays roles in apoptosis (Katsuma, et al., 2005) and inflammatory response in intestinal L cells (Anbazhagan, et al., 2016; Tsukahara, et al., 2015), there is growing evidence that GPR120 can couple to alternative downstream pathways (Iakoubov, Izzo, Yeung, Whiteside, & Brubaker, 2007; Tsukahara, et al., 2015). Tsukahara et al. (2015) have recently demonstrated that activation of GPR120 can inhibit *proglucagon* production via G_s cAMP pathway in inflamed rat L cells and GLUTag cells (Tsukahara, et al., 2015). Treatment

of L-cells with 10 ng/ml of TNF-α, a pro-inflammatory cytokine, resulted in a 50 % decrease in *proglucagon* mRNA transcript levels. This effect was mediated by upregulating GPR120 and increasing cellular levels of cAMP. Similar to diacetyl, 30 ng/ml of TNF had no effect on the *GPR40* mRNA transcript whilst increasing *GPR120* mRNA by 250 % (Tsukahara, et al., 2015). Although the inflammatory status of STC-1 cells exposed to diacetyl, a GRAS food additive, was not monitored, cell viability was not altered.

In our study, STC-1 cells incubated with 1000 ppb of diacetyl resulted in 518 % increase in cAMP levels with a 70 % inhibition of *proglucagon* transcription and 93 % reduction in GLP-1 exocytosis compared with KREBS alone. Elevation of cAMP levels in STC-1 cells usually enhances transcription of *proglucagon* (Gevrey, et al., 2004; Islam, et al., 2009; Lotfi, et al., 2006). The *proglucagon* promoter contains a cAMP response element (CRE) at -291 bp to -298 bp. Increase in cAMP levels, leads to activation of the enzyme protein kinase A (PKA) which phosphorylates CRE-binding protein (CREB). Phosphorylated CREB then binds to other transcription factors (e.g. Pax-6, Isl-1) and usually recruits them to the promoter in order to bridge the pre-initiation complex (Gevrey, et al., 2004). In STC-1 cells, site-directed mutagenesis of the CRE site causes a 50 % reduction in the ability of forskolin to increase *proglucagon* promoter activity (Gevrey, et al., 2004). In GLUTag cells, cAMP rise by 10 µM; forskolin/IBMX stimulates GLP-1 exocytosis (Simpson, et al., 2007) but surprisingly does not increase GLP-1 exocytosis in STC-1 cells (Kuhre, et al., 2016). In GLUTag cells, this GLP-1 exocytosis is mediated through PKA phosphorylation of Kir6.2, the pore-forming subunit of K^+ channels (Reimann, 2010). This results in channel closure and an increase in intracellular Ca^{2+}, either *via* influx into the cell through voltage-gated Ca^{2+} channels or by mobilisation of intracellular Ca^{2+} stores (Reimann, 2010). In our study, neither the L-type voltage-dependent Ca^{2+} channel blockers, nicardipine and nitrendipine, nor the K^+ channel blocker, tolbutamide could influence the inhibitory effect of diacetyl on GLP-1

secretion, indicating differences in response to intracellular cAMP levels between GLUTag and STC-1 cells.

The significant reduction by diacetyl of GLP-1 production in STC-1 cells in the presence of glucose appears to be specific, as neither the butterscotch fruity aroma, 2-butanone, nor the pineapple fruity odour, ethyl butyrate, altered *proglucagon* mRNA levels (unpublished data). However Jang et al. (2007) demonstrated that the food additive, lactisole, also significantly reduced GLP-1 secretion in sucrose-stimulated NCI-H716 cells (Jang, et al., 2007). Lactisole (2.5 mM) reduced GLP-1 secretion 1-fold in these human enteroendocrine cells incubated with 1-5mM sucrose compared to cells with sucrose alone (Jang, et al., 2007). Lactisole mediates its GLP-1 attenuation via the taste receptor, T1r3 (Jiang, et al., 2005) and is therefore added to food to suppress the perception of sweetness.

Gonzalez-Abuin et al. (2014) reported that 50 mg/l of grape seed procyanidin extract (GSPE) significantly reduced secreted levels of active GLP-1 from STC-1 cells stimulated with either 20 mM glucose or 5 mM proline plus 2.5 mM glucose (Gonzalez-Abuin, et al., 2014). Interestingly this flavonoid extract had no effect on GLP-1 secretion from STC-1 cells stimulated with 30 μM linoleic acid plus 10mm glucose (Gonzalez-Abuin, et al. 2014). GSPE appears to mediate its effect on STC-1 cells by the hyperpolarization of cellular and mitochondrial membranes, indicating that Na^+ chelation at the cation channel, TRPM5, maybe involved (Gonzalez-Abuin, et al. 2014). GSPE is likely to be perceived as bitter with a negative sensory attribute. The neuropeptide, galanin (100 nM) can also inhibit GLP-1 secretion by 75 % in primary duodenal cultures that are stimulated with 10 mM glucose and 100 μM IBMX (Psichas, Glass, Sharp, Reimann, & Gribble, 2016). This 29 amino acid peptide appears to mediate its inhibitory effect on stimulated enterendocrine cells by activating its GAL_1 receptor, employing G_i coupling pathways, reducing intercellular cAMP levels but not involving potassium channels (Psichas, et al., 2016). The nuclear receptor

Farnesoid X Receptor (FXR) is expressed in L-cells and activation of FXR in GLUTag cells resulted in a 50 % inhibition of glucose (5.6 mM)-induced proglucagon transcription (Trabelsi, et al., 2015). FXR physically interacts with the carbohydrate response element binding protein (ChREBP), implying that it interferes with ChREBP docking on the *proglucagon* promoter (Trabelsi, et al., 2015). Trabelsi et al. (2015) also observed that active FXR decreases glucose-induced GLP-1 secretion by inhibiting glycolysis and lowering intracellular ATP levels but not impeding membrane depolarization (Trabelsi, et al., 2015). Given the important role of GLP-1 in food intake and glucose homeostasis, it is surprising that the inhibition and fine-tuning of production and secretion of GLP-1 by foods has received little attention. To our knowledge, this is the first study which shows an inhibitory effect of a volatile aroma compound on satiety hormone production and secretion, albeit *in vitro*. Although there is extensive information available on (1) the mechanisms of neural responses to both orthonasal and retronasal aroma (Shirasu, et al., 2014), and (2) links between aroma, sensory exposure and satiation (Ruijschop, et al., 2009), there appears to be little information available on aroma directly modulating satiety signals. Massolt et al. (2010) did observe that subjects who smelled dark chocolate before eating it reported significantly higher levels of satiation, a result which correlated inversely with serum levels of the hunger hormone, ghrelin, but had no effect on serum GLP-1 (Massolt, et al., 2010). If the aroma of a food can dampen the gastrointestinal GLP-1 response to that food, the amount of that food consumed may increase. Overconsumption of palatable food is considered a major factor contributing to the global surge in obesity (Kenny, 2011).

In conclusion, the results of our study demonstrate that diacetyl reduces levels of *proglucagon* mRNA transcripts and total GLP-1 secretion, with an associated increase in cAMP levels and recruitment of GPR120 to the cell surface. It is feasible that inhibition of GLP-1 by palatable food components can contribute to overconsumption.

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583 **Figure 1. GLP-1 production and secretion in STC-1 cells exposed to diacetyl.** (A) STC-1
584 cells (1.5 x 10^6 cells/well) were exposed to various concentrations of diacetyl in KREBS,
585 which contains 10 mM glucose, from 10 min to 4 h. Different superscript letters (a-d)
586 indicate significant *proglucagon* mRNA differences within the same exposure time ($P<0.05$).
587 * indicates significant differences ($P < 0.05$) due to 500 ppb diacetyl after 10 min exposure
588 compared with 4 h exposure. ‡ indicates significant differences ($P < 0.05$) due to 1000 ppb
589 diacetyl after 10 min compared with 4 h. § indicates significant differences ($P < 0.05$) due to
590 2000 ppb diacetyl after 10 min compared with 4 h. **(B)** STC-1 cells (1.5 x 10^6 cells/well)
591 were exposed to 1000 ppb of diacetyl in KREBS for 4 h. Levels of total GLP-1 were
592 quantified in cell supernatant. Different superscript letters indicate significant differences in
593 total GLP-1 ($P < 0.05$). Both technical and experimental repeats were performed in duplicate
594 on two separate occasions.
595
596

597 **Figure 2. STC-1 cellular viability in the presence of various concentrations of diacetyl.**

598 Viability of STC-1 cells (1 x 10^5 cells/well) was determined, by Alamar Blue, after

599 incubation for 4 h with diacetyl. KREBS alone was the vehicle control and represented 100 %

600 cellular viability. Superscripts with a common letter are similar to each other ($P > 0.05$). Both

601 technical and experimental repeats were performed in duplicate on two separate occasions.

602

603 **Figure 3. GLP-1 production and secretion in STC-1 cells exposed to various blockers.**
604 (A) *proglucagon* mRNA levels: STC-1 cells (1.5 x 10^6 cells/well) were incubated with
605 various blockers (Ca^{2+} channel blockers (5 µM nicardipine or 5 µM nitrendipine), K^+channel
606 blocker (100 µM tolbutamide) or the α-gustducin inhibitor (10 µg/ml pertussis toxin)) for 15
607 min. Diacetyl (1000 ppb) was then added and cells incubated for a further 4 h. KREBS alone
608 is the vehicle control. Superscripts with a different letter indicate a significant difference ($P < 0.05$).
609 (B) Secreted levels of total GLP-1. Different superscripts indicate significant
610 differences ($P < 0.05$).
611

612

613 **Figure 4. Intracellular cAMP levels in STC-1 cells exposed to 1000 ppb of diacetyl for 4**

614 **h.** Each value represents the mean ± SEM. Vehicle control was KREBS alone. Means

615 without a common letter differ significantly from each other ($P < 0.05$).

616

617

618 **Figure 5. (A) *GPR40* mRNA levels and (B) *GPR120* mRNA levels in STC-1 cells** STC-1

619 cells (1.5 x 10^6 cells/well) were exposed to 1000 ppb of diacetyl for 4 h. Means without a

620 common letter differ significantly from each other ($P < 0.05$).

621

622

623

Figure 6. (A) GPR120 surface expression and (B) GPR120 protein levels in STC-1 cells. (A) For immunocytochemistry, seeded STC-1 cells (8 x 10^5 cells/ml) were exposed, for 30 min, 1 h or 4 h, to either KREBS alone or 1000 ppb of diacetyl or 100 μM α- linolenic acid. Rabbit anti GPR120 (1:500 dilution) antibody was applied to fixed cells. Staining was conducted in triplicate and from an image of each staining, five individual cells were selected for fluorescence intensity analysis. Data at each time point were analysed, using a one-way ANOVA with a Fisher's LSD comparison. Means without a common roman numeral, within the same time interval, differ significantly from each other ($P < 0.05$). (B) STC-1 cells were seeded into 12-well plates at a density of 1.5 x 10^6 cells/well and exposed for 30 min, 1 h or 4h to KREBS alone, 1000 ppb of diacetyl or 100 mM α-linolenic acid. Separated proteins were incubated with rabbit anti GPR120 antibody (1:1,000 dilution). Sample loading of protein was corrected by staining with a 1:15,000 dilution β-actin horseradish peroxidase antibody. Both technical and experimental repeats were performed in duplicate on two separate occasions.

Figure 1

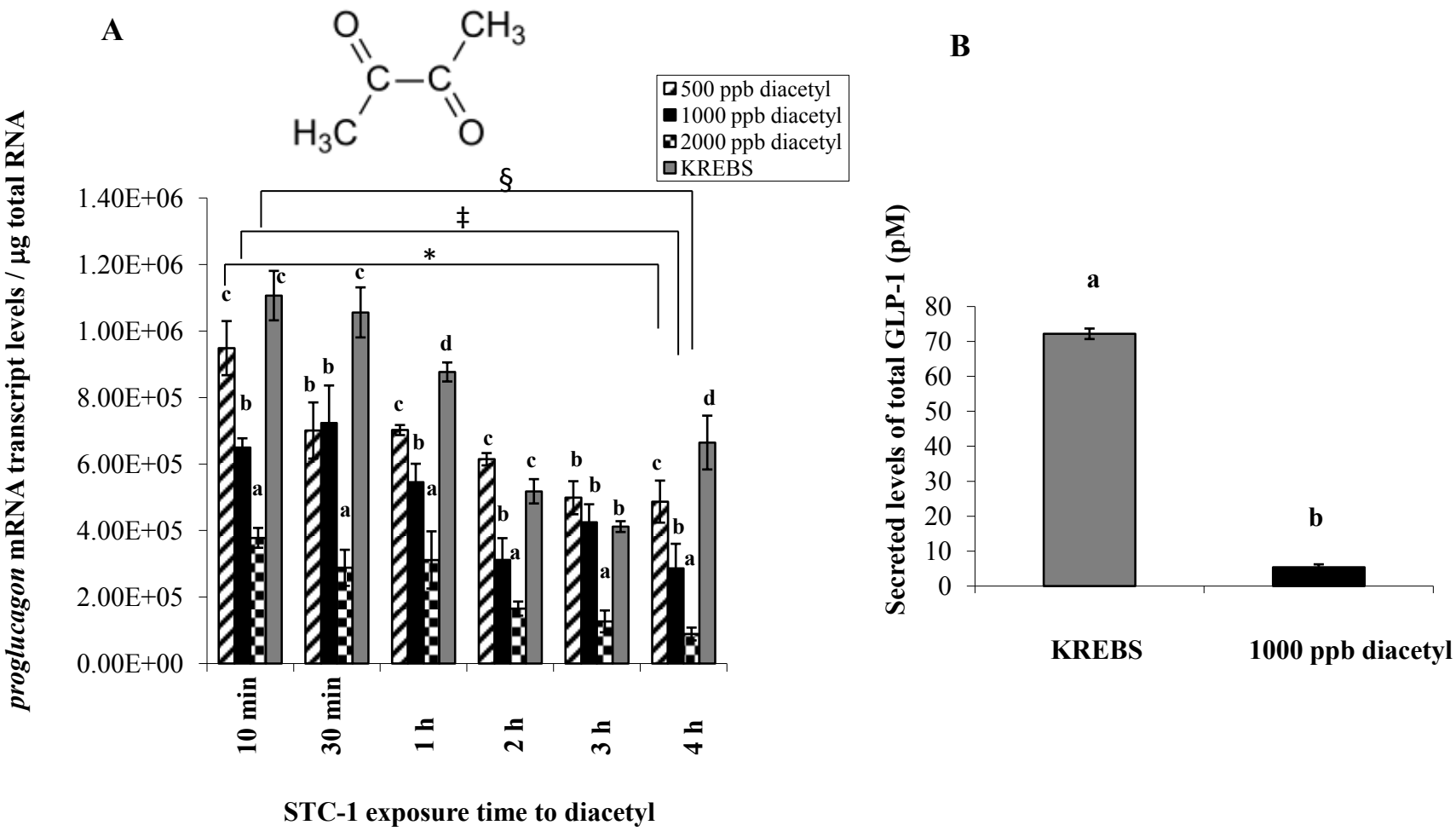

A
B
500 ppb diacetyl
1000 ppb diacetyl
2000 ppb diacetyl
KREBS
proglucagon mRNA transcript levels / µg total RNA
1.40E+06
1.20E+06
1.00E+06
8.00E+05
6.00E+05
4.00E+05
2.00E+05
0.00E+00
10 min
30 min
1 h
2 h
3 h
4 h
STC-1 exposure time to diacetyl
Secreted levels of total GLP-1 (pM)
80
70
60
50
40
30
20
10
0
KREBS
1000 ppb diacetyl

Figure 2

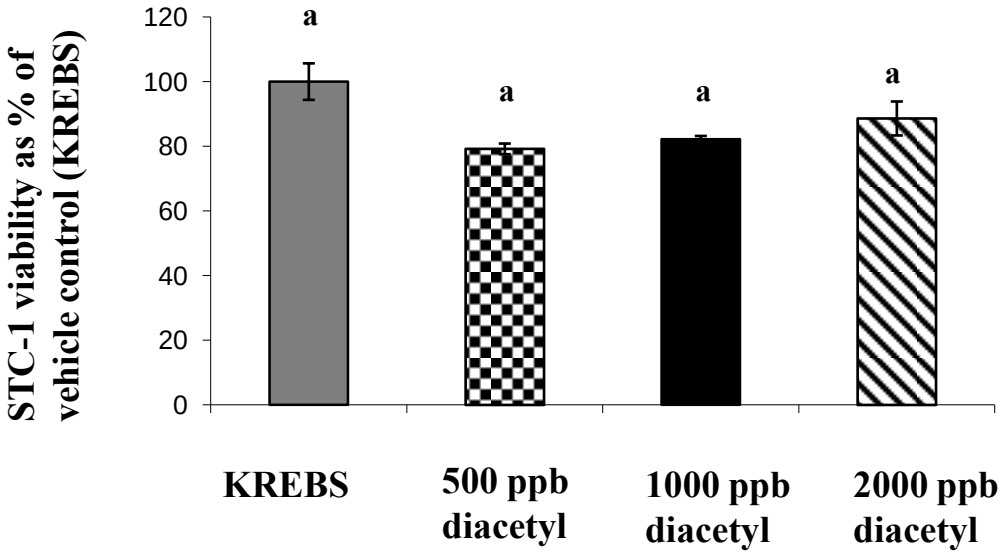

STC-1 viability as % of vehicle control (KREBS)
120
100
80
60
40
20
0
a
a
a
a
KREBS
500 ppb diacetyl
1000 ppb diacetyl
2000 ppb diacetyl

Figure 3

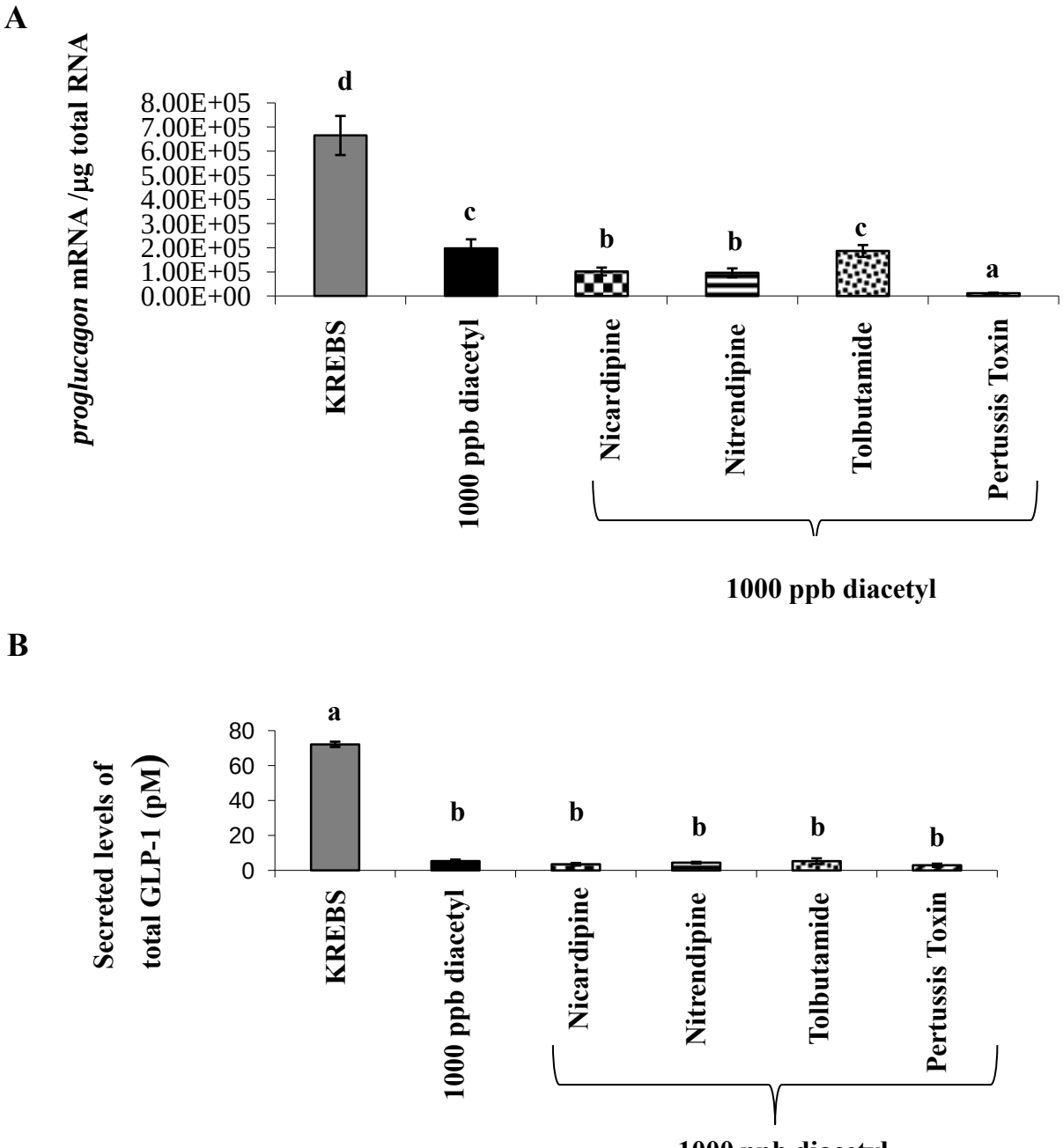

A
proglucagon mRNA /µg total RNA
8.00E+05
7.00E+05
6.00E+05
5.00E+05
4.00E+05
3.00E+05
2.00E+05
1.00E+05
0.00E+00
d
c
b
b
c
a
KREBS
1000 ppb diacetyl
Nicardipine
Nitrendipine
Tolbutamide
Pertussis Toxin
1000 ppb diacetyl
B
Secreted levels of total GLP-1 (pM)
80
60
40
20
0
a
b
b
b
b
b
KREBS
1000 ppb diacetyl
Nicardipine
Nitrendipine
Tolbutamide
Pertussis Toxin
1000 ppb diacetyl

Figure 4

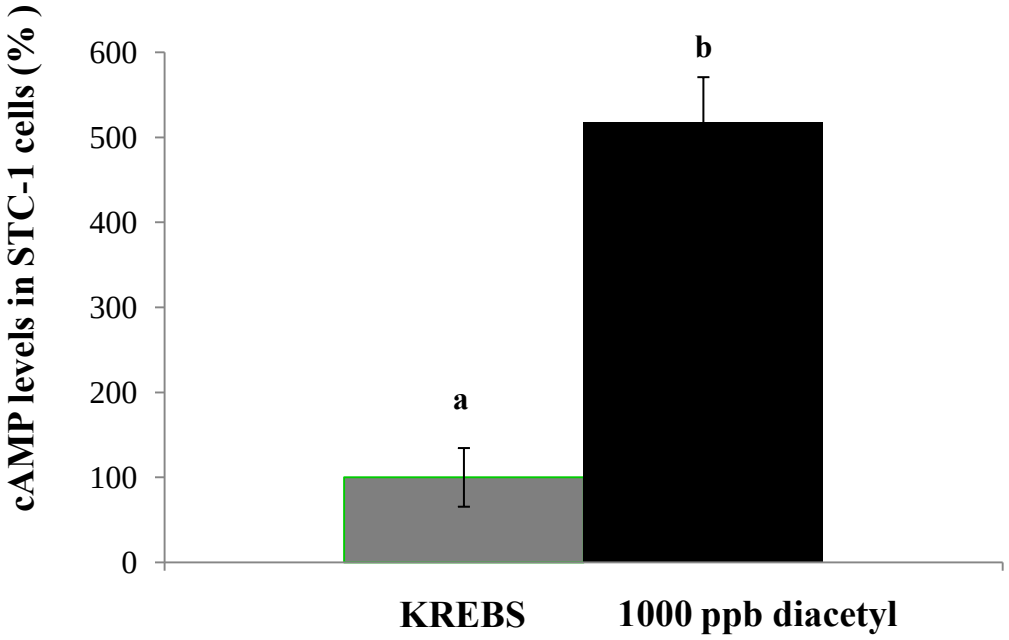
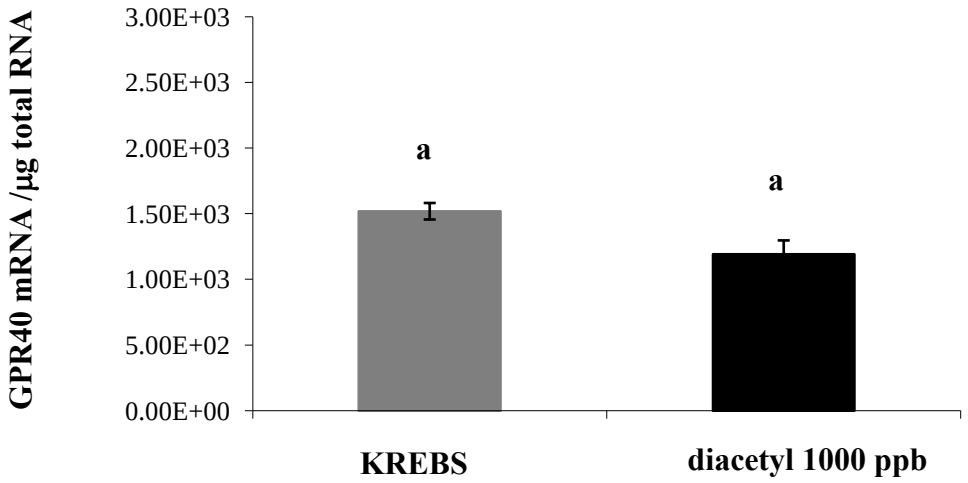

cAMP levels in STC-1 cells (%)
600
500
400
300
200
100
0
a
b
KREBS
1000 ppb diacetyl

Figure 5

A


GPR40 mRNA /µg total RNA
3.00E+03
2.50E+03
2.00E+03
1.50E+03
1.00E+03
5.00E+02
0.00E+00
a
a
KREBS
diacetyl 1000 ppb

B

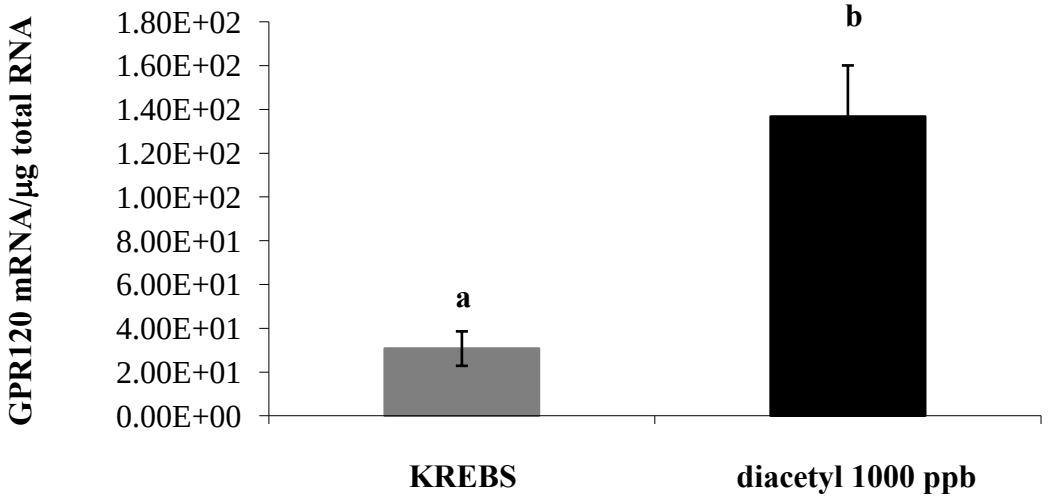
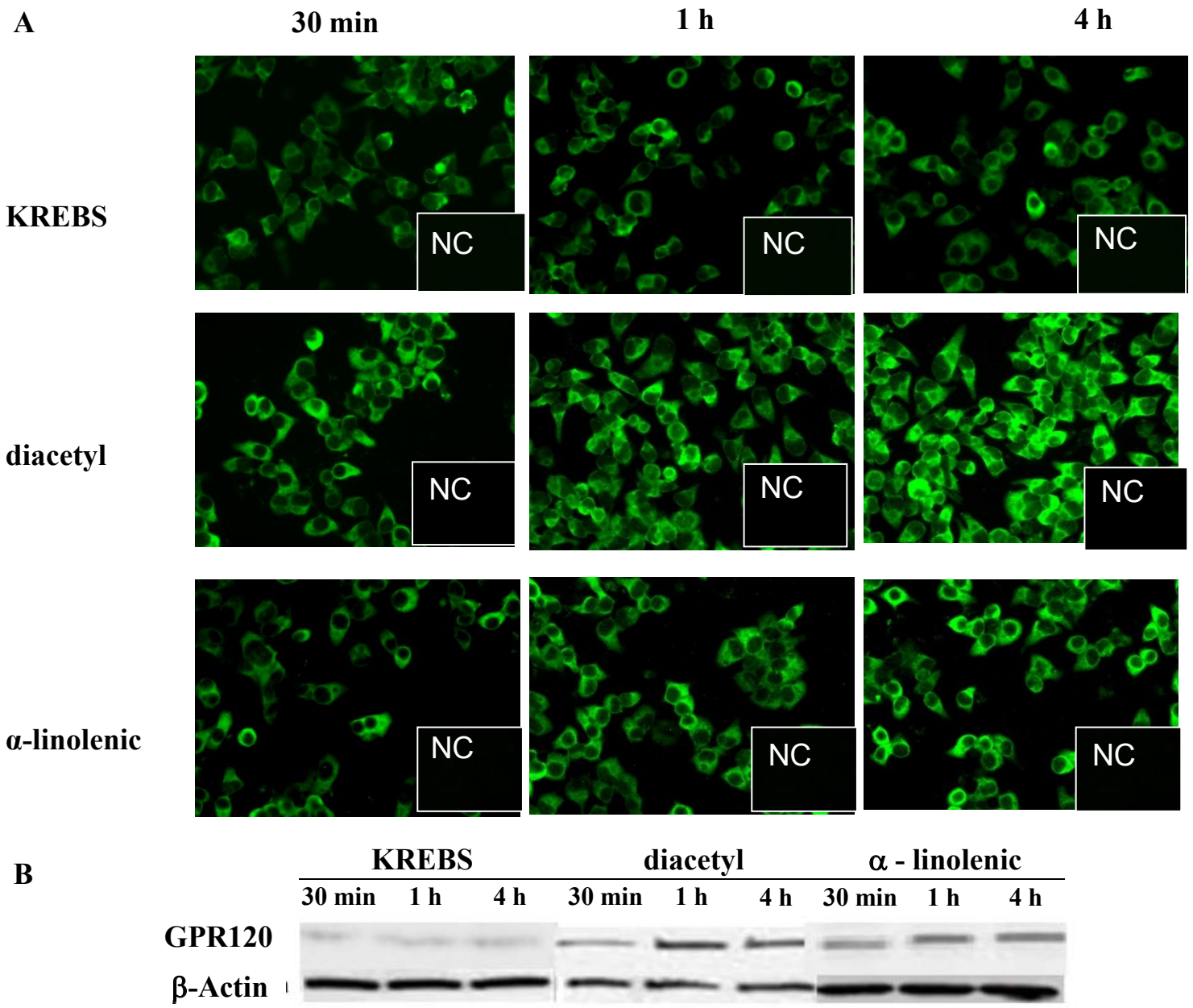

GPR120 mRNA/µg total RNA
1.80E+02
1.60E+02
1.40E+02
1.20E+02
1.00E+02
8.00E+01
6.00E+01
4.00E+01
2.00E+01
0.00E+00
a
b
KREBS
diacetyl 1000 ppb

Figure 6


A
30 min
1 h
4 h
KREBS
diacetyl
α-linolenic
NC
B
KREBS
diacetyl
α - linolenic
30 min
1 h
4 h
GPR120
β-Actin

Highlights:

- A volatile favour compound with a pleasant aroma supresses GLP-1, a satiety hormone
- Aromatic diacetyl recruits GPR120 to the cell surface and increases cAMP levels
- K^+_{ATP} channels, Ca^{2+} channels and the α-gustducin taste pathway are not involved