1 **The bioactive effects of casein proteins on enteroendocrine cell health,**
2 **proliferation and incretin hormone secretion.**

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5 Anna L. Gillespie¹ and Brian D. Green¹

6 ¹Institute for Global Food Security (IGFS), Queen's University Belfast, United Kingdom
7 Corresponding author at: agillespie14@qub.ac.uk. Institute for Global Food Security,
8 Queen's University Belfast, Stranmillis Road, Belfast, Northern Ireland BT9 6AG,
9 United Kingdom. Tel.: +44 (0)28 9097 6543.

10
11 **Abstract**

12 Previous studies suggest that **casein** exerts various anti-diabetic effects. However, it
13 is not known which **casein** proteins are bioactive, nor their effects on
14 enteroendocrine cells. This study evaluated the effects of **intact whole casein**, intact
15 individual proteins (**alpha, beta and kappa casein**) and hydrolysates on an
16 enteroendocrine cell line. High content analysis accurately monitored changes in cell
17 health and intracellular glucagon-like peptide-1 (GLP-1) content. Cheese ripening
18 duration and GLP-1 secretory responses were also considered. **Beta casein**
19 significantly stimulates **enteroendocrine** cell proliferation and all caseins were potent
20 GLP-1 secretagogues (except **kappa casein**). Interestingly the GLP-1 secretory activity
21 was almost always lost or significantly reduced upon hydrolysis with proteolytic
22 enzymes. Only pepsin-derived beta casein hydrolysates had significantly increased
23 potency compared with the intact protein, but this was diminished with prolonged
24 hydrolysis. In conclusion **casein** proteins are not detrimental to enteroendocrine
25 cells and **alpha and beta casein** are particularly beneficial stimulating proliferation
26 and GLP-1 secretion.

27
28 **Keywords:** Protein, casein, diabetes, incretin hormones, GLP-1, GIP, pGIP/Neo STC-1,
29 HCA

30
31 *1. Introduction*

32 Animal milk has been consumed by humans since ancient times (Caramia et al. 2012)
33 and it is an important source of nutrition for humans of all ages but particularly
34 children providing protein, minerals and vitamins for growth and development
35 (Coulter, 2002). Although the main constituent of milk is water (~87%) an important

36 and highly nutritious component is the protein (~3.3%), of which approximately 80%
37 is casein (CN) and 20% is whey.

38

39 Epidemiological evidence suggests that there is an inverse relationship between the
40 consumption of dairy foods and the development of metabolic syndrome and/or
41 type 2 diabetes (T2DM) (Lacroix & Li-Chan, 2012). Lifestyle intervention strategies are
42 employed in the management of T2DM (Mirmiran, Bahadoran, & Azizi, 2014), but
43 pharmacotherapy is often necessary when these strategies fail. Harnessing the
44 bioactive properties of food to enhance the effectiveness of current T2DM
45 treatments remains an attractive and realistic prospect (Mirmiran et al., 2014).

46

47 In recent years the whey fraction of milk has received a lot of attention for its
48 potential anti-diabetic effects (Drucker, 2006; Jakubowicz & Froy, 2012; Gillespie et
49 al., 2015). Whey protein consumption has been shown to act through a number of
50 mechanisms to curb diet-induced obesity and prevent the onset of T2DM. By
51 comparison CN proteins are much less studied. Whey and CN differ quite markedly in
52 their amino acid composition and rate of absorption, but some anti-diabetic
53 properties have been attributed to CN. Rats fed a high cheese diet (high in CN) have
54 lower levels of VLDL- and LDL-cholesterol, reduced accumulation of abdominal
55 adipose and increased serum adiponectin levels (Higurashi et al., 2007). Furthermore,
56 a randomised, controlled, cross-over study in overweight or moderately obese men
57 found that both CN and hydrolysed CN exert positive effects on energy expenditure,
58 appetite regulation, plasma insulin and plasma levels of glucagon-like peptide-1 (GLP-
59 1) (Bendtsen et al., 2014). It is believed that modulating the secretion of gut
60 hormones such as GLP-1 is one way in which CN could mediate anti-diabetic actions.
61 Geraedts et al. (2011) have demonstrated that CN potently stimulates the secretion
62 of both GLP-1 and also cholecystokinin (CCK). The study also demonstrated that
63 intact CN (referred to hereon in as whole CN (WCN)) is much more effective than
64 hydrolysed CN. However, the study was somewhat limited as it did not confirm
65 whether the effects were concentration-dependant, nor did it provide any
66 information regarding the degree of CN hydrolysis nor the proteolytic enzymes used.
67 Rafferty et al. (2011) found that intact beta-CN (β -CN) stimulated GLP-1 secretion but
68 did not perform concentration-dependent studies, and they did not compare its
69 activity to WCN. Uenishi et al. (2012) reported that peptides derived from β -CN may
70 indirectly modulate incretin hormone activity by inhibiting dipeptidyl-peptidase 4
71 (DPP-4), an enzyme which physiologically inactivates GLP-1. They isolated an 8 amino

72 acid β -CN peptide from water-soluble extract of Gouda cheese which had significant
73 DPP-4 inhibitory activity.

74

75 Although the above evidence indicates that dietary CN stimulates incretin hormone
76 secretion the contribution that each CN protein makes to the incretin hormone
77 response has not been elucidated. More important still, it is not yet understood what
78 influence CN proteins have on the enteroendocrine (EE) cells which produce these
79 hormones. The present study undertook a holistic examination of CN protein actions
80 on an established EE cell line. We examined not just the effects of WCN but also α -
81 CN, β -CN and κ -CN, as well as, hydrolysates of α -CN and β -CN across a range of
82 concentrations. We employed highly sensitive high-content analysis (HCA) to
83 accurately monitor cellular changes across multiple parameters. This reported
84 changes in cellular health including cell number, mitochondrial membrane potential
85 (MMP) and mitochondrial area (MA). Furthermore, we combined HCA with
86 immunohistochemistry to observe changes in the levels of intracellular GLP-1. In
87 addition to these morphometric parameters we also assessed the GLP-1 secretory
88 responses of EE cells to each CN. Finally, to assess food-related relevance we
89 examined how the duration of cheese maturation affects the levels of each CN
90 protein and what effect this has on GLP-1 secretory responses.

91

92 **2. Methods and materials**

93 *2.1 Materials*

94 pGIP/Neo STC-1 cells were a gift from Dr. B. Wice (Washington University of St. Louis)
95 (Ramshur, Rull & Wice, 2002) with permission from Dr D. Hanahan (University of
96 California, San Francisco, CA). Culture medium contained DMEM containing 4.5 g/L
97 with L-glutamine, without sodium pyruvate (Life Technologies, Paisley, UK) and
98 supplemented with 10% foetal bovine serum, 100 U/ mL penicillin, 100 mg/L
99 streptomycin and geneticin - G418, 400 μ g/mL were purchased from Sigma (Dorset,
100 UK). Isolated CN proteins α -CN, β -CN and κ -CN, trypsin, α -chymotrypsin and pepsin,
101 as well as the reagents for the OPA method to determine degree of hydrolysis and for
102 Urea PAGE gel electrophoresis - di-sodium-tetraborate decahydrate, sodium-dodecyl-
103 sulphate, *o*-phthaldialdehyde, glycine, acrylamide suitable for electrophoresis $\geq 99\%$,
104 N,N'-Methylenebis(acrylamide) powder for electrophoresis $\geq 99.5\%$, N,N,N',N'-
105 Tetramethylethylenediamine BioReagent (TEMED) suitable for electrophoresis,
106 ammonium persulfate for electrophoresis $\geq 98\%$, Tris(hydroxymethyl)aminomethane
107 ACS reagent $\geq 99.8\%$, urea, DL-Dithiothreitol $\geq 98\%$ (DDT) were all purchased from

108 Sigma-Aldrich Ltd (Dorset, UK). Commercial cheese curd samples were supplied by
109 Dale Farm (Belfast, UK). Mitochondrial Membrane Permeability Dye and Hoechst
110 nuclear dye were purchased from Thermo Scientific (Hampstead, UK). Alamar Blue
111 was purchased from Invitrogen (Grand Island, NY, USA). GLP-1 antibody (FITC) was
112 purchased from Biorbyt (Cambridge, UK). GLP-1 and glucose-dependent
113 insulinotropic peptide (GIP) ELISA kits were purchased from Millipore (Billerica, MA,
114 USA). GLP-1 assay detected only active forms of GLP-1 (7–36 amide) and GLP-1 (7–
115 37) while the GIP assay detected total GIP (GIP (1–42) and GIP (3–42)).

116

117 *2.2 Secretion studies of GLP-1 and GIP*

118 pGIP/Neo STC-1 cells were cultured in culture medium and incubated in a 5% CO₂
119 humidified atmosphere at 37°C. Cells underwent passage upon reaching 80–90%
120 confluence and were used between passage numbers 15–50. Cells were seeded into
121 12-well plates (2 x 10⁶ per well) and cultured overnight at 37 °C in a humidified
122 atmosphere of 5% CO₂. Medium was removed and cells washed (x3) with HEPES
123 buffer (20mM HEPES, 10mM glucose, 140nM NaCl, 4.5mM KCl, 1.2mM CaCl₂, 1.2 mM
124 MgCl₂) then allowed to incubate for 1 h in HEPES buffer prior to adding appropriate
125 buffer control or buffer supplemented with test agents. Cells were incubated for 3 h
126 after which the buffer control and test agents were removed, centrifuged and stored
127 at -80°C prior to ELISA assays.

128

129 *2.3 Measurement of cell numbers and mitochondrial membrane potential*

130 Cells were seeded into 96-well plates (5 x 10⁴ per well) and cultured overnight at 37
131 °C in a humidified atmosphere of 5% CO₂. After the 3 h incubation period the media
132 and test agents were removed from the wells and pre-heated mitochondrial
133 membrane potential dye (MMPD) (diluted with media 1:2) was added to wells and
134 incubated at 37°C for 30 min, protected from light (at all steps). Cells were fixed
135 with 10% formalin for 10 min at room temperature and washed with PBS (x1).
136 Hoechst nuclear staining solution was added and incubated at room temperature for
137 10 min. After a further wash with PBS (x1), PBS was added to each well and the plate
138 sealed. The plate was read using the ArrayScan[®] HCS Reader (Thermo,
139 Loughborough, UK) and evaluated at absorbance 350-461 nm and 554-576 nm for
140 Hoechst dye and mitochondrial dye, respectively.

141

142 *2.4 Cell viability*

143 Cells were seeded into 96-well plates (5×10^4 per well) and cultured overnight at 37
144 °C in a humidified atmosphere of 5% CO₂. After the 3 h incubation period the media
145 and test agents were removed, Alamar blue added (10 µl), the plate incubated (37°C;
146 1 h) and then absorbance read (570-600 nm) using a microplate reader (Tecan,
147 Safire²).

148

149 2.5 Measurement intracellular GLP-1 levels

150 Cells were seeded into 96-well plates (5×10^4 per well) and cultured overnight at 37
151 °C in a humidified atmosphere of 5% CO₂. Media was removed and fresh
152 media(control) or media supplemented with test agents were added for 3 h after
153 which media was removed and cells fixed with 10% formalin for 10 min at room
154 temperature. Cells were washed (x4) with PBS and permeabilisation solution added
155 and incubated for 30 min at room temperature. Again, cells were washed (x4) with
156 PBS before blocking solution (0.3 M glycine + 10% goat serum in 1% BSA in PBST (PBS
157 + 0.01% Tween 20) was added for a further 1h. The blocking solution was removed
158 and the GLP-1 antibody was added (1:200 dilution) and allowed to incubate
159 overnight. Supernatant was removed and cells were washed with PBS (3 times x 5
160 min) in the dark after which Hoechst staining solution was added for 10 min. Cells
161 were washed with PBS (x1) and PBS added to each well and the plate was sealed
162 prior to reading using the ArrayScan[®] HCS Reader (Thermo, Loughborough, UK). The
163 plate was evaluated at absorbance 350-461nm and 485-520nm for Hoechst dye and
164 antibody fluorescence, respectively.

165

166 2.6 CN extraction

167 Curd was stored at 5°C and approximately 10 g was taken on days 1, 7, 14 and 21 of
168 the maturation process. Samples were covered and stored at -31°C until ready for
169 analysis. Curd samples were then thawed for 12 h at 3-4°C prior to analysis and
170 grated. 2.5 g of each curd sample was homogenised with 15 mL of ddH₂O and the pH
171 reduced to 4.3 using 1M HCl then centrifuged (4°C, 15 min, 3000xg) to recover
172 precipitated CNs. The supernatant was discarded and the pellet disrupted and
173 washed with 15 mL of 1mM ammonia acetate buffer (pH 4.3), vortexed for 30s,
174 centrifuged again for 10 min (4°C, 3000xg). This washing step was repeated twice and
175 supernatant was discarded each time. A final wash with 15 mL of acetone removed
176 fat. The supernatant was discarded and excess acetone was evaporated using
177 nitrogen gas. The pellet was disrupted and allowed to dry overnight in a fume hood
178 at room temperature.

179

180 2.7 Urea-PAGE gel electrophoresis

181 Gels were cast and run in a Mini-PROTEAN 3 system (BIO-RAD, Hertfordshire, UK)
182 with a 15% resolving gel and a 5% stacking gel. Resolving gel was made with 15g
183 acrylamide and 0.625g N,N'-Methylenebis(acrylamide) made to 100 mL Tris-HCL
184 buffer, pH 8.9 (40 g Tris, 260 g Urea and 4 mL concentrated HCL diluted to 1 L with
185 ddH₂O). Immediately before use 10 µl of TEMED and 80 µl of 100 mg/mL ammonium
186 persulphate (APS) were added to 20 mL of the resolving gel solution. The 5% stacking
187 gel was made with 2.5 g acrylamide and 0.125g N, N'-Methylenebis(acrylamide)
188 made to 50 mL in Tris-HCL buffer (7.5 g Tris, 260 g Urea and 4 mL concentrated HCL
189 diluted to 1 L, pH 7.6) and immediately before use 5 µl TEMED and 40 µl APS were
190 added to 10 mL of the stacking gel solution.

191 The extracted CNs were dissolved in sample buffer (0.75 g Tris, 49 g of Urea, 0.441 g
192 DDT and in ddH₂O to a final volume of 100 mL), sonicated (20 min, 30 °C) and protein
193 concentration assessed on Nanodrop 8000 to ensure all samples were at a
194 concentration of 10 mg/mL. Samples were further diluted to contain 15 µg of protein
195 in a total volume of 21 µl of sample buffer. Bromophenol blue was added and
196 samples were heated at 55°C for 15 min and allowed to cool. Running buffer (15 g
197 Tris, 73 g of glycine in 5 L of ddH₂O) was added and the gel was pre-run at 280 V for
198 35 min prior to sample loading. 20 µl of the sample was added to their designated
199 wells and run at 25 V/cm until tracking dye was approximately 1 cm from the bottom
200 of the gel. Gels were stained in 0.25% (w/v) Coomassie blue G250 in 50% methanol
201 containing 12.5% TCA for 1 h and destained in 7% Acetic Acid overnight.

202

203 2.8 Hydrolysis of α-CN and β-CN

204 Chymotrypsin or trypsin was added to α-CN or β-CN (5 mg/mL) in sodium phosphate
205 buffer (0.1M, pH 7.4) at E/S ratio 1:25. Pepsin digestion was carried out in an E/S
206 ratio of 1:25 in a 50 mM HCl buffer at a protein concentration of 10 mg/mL which
207 allowed for a post-hydrolysis increase in pH (pH 7.4) using NaOH and dilution to 5
208 mg/mL using ddH₂O. Control sample (buffer) was treated in the same way. Solutions
209 were pre-warmed to 37°C prior to incubation. Sample (T1) was produced when the
210 enzyme was added to the protein solution, mixed well, an aliquot removed and
211 heated at 95°C for 10 min to stop the action of the enzyme. Subsequent samples
212 were removed every 30 min and sequentially labelled (T30, T60... etc.). The sample
213 was cooled and stored at -20°C until required for secretion study assays. Samples of

214 'enzyme only' or 'protein only' before addition of enzyme were treated the same as
215 hydrolysed samples.

216

217 2.9 Determination of the degree of hydrolysis (DH)

218 The DH was measured using the method of o-phthaldialdehyde (OPA) described by
219 Nielsen, Petersen, and Dambmann (2001) with some modifications as described by
220 Gonzalez-Gonzalez, Tuohy & Jauregi (2011) below. The OPA reagent was prepared as
221 follows: 7.620 g di-sodium-tetraborate decahydrate and 200 mg sodium-dodecyl-
222 sulphate (SDS) were dissolved in 150 mL ddH₂O. 160 mg o-phthaldialdehyde (OPA)
223 was dissolved in 4 mL ethanol. The OPA solution was added by rinsing with ddH₂O.
224 176 mg dithiothreitol (DTT) was added to the solution by rinsing with ddH₂O. The
225 solution was made up to 200 mL with deionized water.

226 The serine standard was prepared as follows: 50 mg serine was diluted in 500 mL
227 ddH₂O water (0.9516 meqv/L).

228 Samples were diluted in ddH₂O to reach a concentration of 0.1 – 1 mg/mL. 50µl of
229 diluted protein sample was added to 375µl of OPA reagent, after 2 min incubation
230 the absorbance was immediately read at 340 nm in a spectrophotometer. DdH₂O was
231 used as a blank and serine (0.9516 meqv/L) was used as standard. The DH was
232 calculated using the following equation:

233 (1)

$$\text{DH \%} = \frac{h}{h_{\text{tot}}} \times 100\%$$

234 Where DH % is the percentage of degree of hydrolysis and h is obtained from the
235 equation below:

236 (2)

$$h = \frac{\text{serine} - \text{NH}_2 - \beta}{\alpha \text{ meqv /g}}$$

237 Where α , β and h_{tot} with the values 1.039, 0.383 and 8.2 respectively, are constants
238 for CN described by Nielsen et al. (2001).

239 To calculate Serine-NH₂ formula (3) was used:

240 (3)

$$\text{Serine} - \text{NH}_2 = \frac{(\text{OD sample} - \text{OD blank})}{(\text{OD standard} - \text{OD blank})} \times 0.9516 \text{ meqv/L} \times \frac{(S \times D)}{(P)}$$

241

242 Where Serine-NH₂ = meqv serine NH₂/g protein; S = sample volume (L); D = dilution
243 factor; P = protein concentration of sample (g/L). Samples were tested in duplicate.

244

245 2.10 Statistical analysis

246 Results were expressed as mean $\pm$ SEM. Data were compared using one-way ANOVA
247 with Tukey's post-hoc test or using two-way ANOVA with Bonferroni post-hoc tests.
248 Groups were considered significant if $P < 0.05$.

249

250 3. Results

251 3.1 Effects of CN on cell numbers

252 WCN increased cell numbers compared with the control 1.8 – 3.2-fold (Figure 1A;
253 0.3125 – 5 mg/mL; $P < 0.05$ – $P < 0.01$) while β -CN significantly increased cell numbers
254 at lower concentrations 2.8 – 3.4-fold (Figure 1B; 0.15625 – 1.25 mg/mL; $P < 0.01$ –
255 $P < 0.001$). As the concentration increased to 2.5 – 5 mg/mL cell number was similar to
256 the control. α -CN significantly increased cell number at 0.625 – 5 mg/mL (Figure 1B;
257 2.4 – 2.6-fold; $P < 0.01$) compared with the control. κ -CN significantly increased cell
258 numbers at higher concentrations (Figure 1B; 2.5 – 5 mg/mL; $P < 0.01$ – $P < 0.001$) but
259 did not have such an affect at 0.15625 – 0.625 mg/mL.

260 β -CN also increased cell numbers significantly when compared with α -CN 1.7 – 3.4-
261 fold at lower concentrations (Figure 1B; 0.15625 – 0.3125 mg/mL; $P < 0.01$ – $P < 0.001$)
262 but this effect was reversed at higher concentrations where α -CN significantly
263 increased cell number compared to β -CN (2.5 – 5 mg/mL; $P < 0.05$). There appeared
264 to be no significant difference in cell number between α -CN and κ -CN at all
265 concentrations. β -CN increased cell number significantly compared with κ -CN at
266 lower concentrations (Figure 1B; 0.15625 – 0.625 mg/mL; $P < 0.01$ – $P < 0.001$) and
267 again, this was reversed at higher concentrations where κ -CN had significantly higher
268 cell numbers compared with β -CN at 2.5 – 5 mg/mL; $P < 0.05$ – $P < 0.001$.

269

270 3.2 Cell Viability

271 WCN and individual CN proteins appear to have no significant effect on cell viability
272 when assessed using Alamar Blue (Figure 2A & B). The mitochondrial membrane
273 potential was measured using HCA. Mitochondria energy production involves the
274 creation of a membrane potential within the inner membrane. The mitochondrial
275 fluorescence intensity (MFI) represents the membrane potential of all the
276 mitochondria of the cell, hence a reduced MFI represents a reduced membrane
277 potential signalling a reduction in cell health. The 'Total MFI' value (Figures 2G-2H)
278 represents the MFI from a well as a whole. The 'Average MFI' (Figures 2E-2F) takes
279 account for the number of cells in a well. 'Mitochondrial area' (Figure 2C-2D)
280 represents the area within a cell that mitochondria reside (measured in pixels).

281 Mitochondria may swell when the cell is under stress and the membrane potential
282 has been uncoupled.

283 The mitochondrial membrane potential did not appear to be effected by WCN but
284 individual CN proteins significantly decreased membrane potential at higher
285 concentrations by 1.2 – 1.5-fold (Figure 2F; 1.25 – 5 mg/mL; $P<0.05$ – $P<0.001$) with
286 the greatest reduction in cells exposed to κ -CN at 5 mg/mL (1.5-fold; $P<0.001$)
287 reduction in average MFI.

288 WCN significantly increased total MFI at concentrations 0.3125 – 2.5 mg/mL (Figure
289 2G; 2.4 - 3.1-fold; $P<0.05$ – $P<0.01$). Individual CN proteins also significantly
290 increased total MFI compared with the control. β -CN significantly increased total
291 MFI (Figure 2H; 3.1 – 3.5-fold) at 0.15625 – 1.25 mg/mL but at higher concentrations
292 this effect was close to control levels. α -CN showed no significant differences at all
293 concentrations. κ -CN had significantly increased total MFI (Figure 2H; 2.9 – 3-fold) at
294 1.25 – 5 mg/mL ($P<0.05$ – $P<0.001$), which appears to be concentration dependant.

295 Significant effects were also seen when α -CN and β -CN were compared. β -CN elicited
296 a 1.9 – 3.3-fold increase in total MFI compared with α -CN at the same concentration
297 (Figure 2H; 0.15625 – 0.3125 mg/mL). The only significant difference between α -CN
298 and κ -CN appeared at 5 mg/mL with a 1.7-fold increase with κ -CN. β -CN significantly
299 increased total MFI compared with κ -CN at 0.15625 – 0.6 mg/mL (Figure 2H; 1.9 -2.6-
300 fold) while the reverse was true at 5 mg/mL where κ -CN significantly increased total
301 MFI compared with β -CN (2.5-fold).

302

303 *3.3 Degree of hydrolysis*

304 Chymotrypsin appears to hydrolyse α -CN significantly more than either trypsin or
305 pepsin at all time points (Figure 3A; T1: 10.9 – 65.5-fold, T150: 1.54 – 3.41-fold
306 respectively, $P<0.001$). There was no significant difference in hydrolysis of α -CN with
307 trypsin or pepsin until T150 (2.2-fold, $P<0.001$).

308 The trend was similar for β -CN, chymotrypsin hydrolysed the protein significantly
309 more than either trypsin or pepsin at nearly all time points (Figure 3B; T1: 4.3 - 6-
310 fold; $P<0.01$, T150: 1.8 - 2.4-fold, $P<0.001$). There was no significant difference
311 between hydrolysis with trypsin and pepsin at all concentrations, although with both
312 α -CN and β -CN trypsin hydrolysis was greater than that of pepsin at all time points.

313

314 *3.4 Stimulatory effects of CN on GLP-1 secretion*

315 WCN stimulated a significant (Figure 3C; 2.6 – 4.5-fold) increase in GLP-1 secretion at
316 0.3125 – 10 mg/mL ($P<0.001$) compared with the control but at 10 mg/mL there was

317 a significant decrease compared with lower concentrations (1.5 –1.7-fold change;
318 $P<0.001$). α -CN also stimulated a significant increase in GLP-1 secretion at all
319 concentrations (Figure 3D; 1.3 – 2.3-fold; $P<0.01$ – $P<0.001$) and this appears to be a
320 concentration dependant effect. β -CN also stimulated a significant increase in GLP-1
321 secretion at all concentrations (Figure 3D; 1.4 – 2.1-fold; $P<0.05$ – $P<0.001$) although
322 this was significantly less than α -CN 0.625, 2.5 - 5 mg/mL (Figure 3D; $P<0.01$ –
323 $P<0.001$). κ -CN increased GLP-1 secretion (Figure 3D; 1.25 – 1.3-fold; $P<0.01$ –
324 $P<0.01$) although this was significantly less than α -CN or β -CN from 0.3125 - 5 mg/mL
325 ($P<0.001$).

326 Chymotrypsin hydrolysates of α -CN and β -CN (T1 – T150) did not show any significant
327 difference when compared with the control. Intact α -CN significantly increased GLP-
328 1 secretion compared with control (Figures 3E; 4.4-fold; $P<0.001$) and also compared
329 with all hydrolysates (Figure 3E; 4.6 - 5.2-fold; $P<0.001$). The same trend occurred
330 with β -CN (Figure 3E). The intact protein stimulated a significant 6.3-fold increase in
331 GLP-1 secretion compared with the control ($P<0.001$) and when compared with all
332 hydrolysates (6.8 – 7.4-fold; $P<0.001$).

333 When α -CN was hydrolysed with trypsin the intact protein significantly increased
334 GLP-1 secretion compared with control (Figure 3G; 4.4-fold; $P<0.001$) as did the
335 hydrolysates T1 – T90 (3.2 – 4.1-fold; $P<0.001$) and T120 – T150 (1.9 – 2-fold; $P<0.05$).
336 T1 appeared to have significantly less of an effect on GLP-1 secretion than the intact
337 CN (1.4-fold; $P<0.05$), hydrolysates T30 – T90 were not significantly different than the
338 intact α -CN while T120 – T150 (2.2 – 2.3-fold; $P<0.001$) also had significantly reduced
339 effect on GLP-1 secretion compared with the intact α -CN. The intact β -CN
340 significantly increased GLP-1 secretion compared with the control (Figures 3H; 6.3-
341 fold; $P<0.001$) and when compared with all hydrolysates (T1 – T150; 4.1 – 5.7-fold;
342 $P<0.001$). T1 hydrolysate also stimulated more GLP-1 compared with T30-T150 (2.1 –
343 2.7-fold; $P<0.001$).

344 When α -CN was hydrolysed with pepsin the intact protein produced a significant
345 increase in GLP-1 secretion when compared with the control (Figure 3I; 4.6-fold:
346 $P<0.001$) as did all hydrolysates (2.6 – 4.1-fold; $P<0.01$ – $P<0.001$). There was no
347 significant difference between GLP-1 secretion with exposure to intact α -CN or
348 hydrolysates apart from T30 and T60, where GLP-1 secretion was reduced by 1.5 –
349 1.8-fold ($P<0.05$ – $P<0.01$). The intact β -CN significantly increased GLP-1 secretion
350 compared with the control (Figure 3J; 3.3-fold; $P<0.001$) and when hydrolysed with
351 pepsin at all time points (T1 –T150, 3.4 – 4.8-fold; $P<0.001$). There was no significant
352 difference between the intact protein and T1 hydrolysate although there was a

353 significant increase in GLP-1 secretion between the intact protein and T30 – T120
354 hydrolysates (1.4 – 1.5-fold; $P<0.01$ – $P<0.001$) which was lost with hydrolysates
355 produced at T150.

356

357 *3.5 Effects of CN on GLP-1 intracellular storage*

358 The intracellular content of GLP-1 was not significantly altered with exposure to WCN
359 or individual CN proteins (Figure 4 A & B) although κ -CN increased intracellular GLP-1
360 significantly more compared with α -CN at 0.3125 and 1.25 mg/mL (1.1 - 1.3-fold;
361 $P<0.05$) and β -CN at 0.15625 and 1.25 mg/mL (1.2 – 1.3-fold; $P<0.05$). The area of
362 GLP-1 fluorescence did not alter significantly with WCN or individual CN proteins
363 (Figure 4 C & D).

364

365 *3.6 Effect of maturation time of curd on CN profile and on GLP-1 secretion*

366 Day 1 CN produced four distinct peaks consisting of gamma (γ -CN)/ κ -CN, β -CN, α -S₂
367 and α -S₁CN (Figure 5A). Day 7 CN produced six distinct peaks with the appearance of
368 breakdown products from α -S₁ (peaks five and six). Day 14 and day 21 also had six
369 peaks with an increase in intensity and volume of peaks five and six which
370 correspond to the α -S₁ breakdown products (Figure 5A).

371 At all days (day 1 – day 21) and at all concentrations (0.3 – 10 mg/mL) the curd
372 samples increased GLP-1 secretion significantly (Figure 5B; 2.6 – 4.7-fold; $P<0.001$).
373 Day 7 was only significantly different at 0.6 mg/mL compared with day 1 (Figure 5B;
374 1.2-fold; $P<0.05$). The most pronounced differences were at day 14 at concentrations
375 2.5 and 5 mg/mL compared with day 1 (Figure 5B; 1.3 – 1.8-fold; $P<0.01$ – $P<0.001$),
376 day 7 (1.3 – 1.8-fold; $P<0.001$) and day 21 (1.3 – 1.8-fold; $P<0.01$ – $P<0.001$).

377

378 *3.7 Effects on GIP secretion*

379 CN did not alter GIP secretion significantly at any concentration (Figure 6A) while
380 individual CN proteins α -CN (1.25 and 2.5 mg/mL; 1.2-fold; $P<0.05$ – $P<0.01$) and κ -CN
381 (0.15625 – 5 mg/mL; 1.1 – 1.5-fold; $P<0.01$ – $P<0.001$) significantly reduced GIP
382 secretion compared with the control (Figure 6B). α -CN was only significantly different
383 to κ -CN at 1.25 mg/mL (1.2-fold; $P<0.05$) but there was an overall concentration
384 dependant decrease in GIP secretion with both proteins.

385 Although β -CN produced no significant change in GIP secretion compared with
386 control it did have a significantly greater effect compared with α -CN at 0.15625, 2.5
387 and 5 mg/mL (1.2-fold; $P<0.01$ – $P<0.001$) and compared with κ -CN at all
388 concentrations excluding 0.625 mg/mL (1.1 – 1.7-fold; $P<0.05$, $P<0.001$).

389 None of α -CN and β -CN hydrolysates tested had any significant effect on GIP
390 secretion (Figure 6C-6H), although intact β -CN appears to have an increased effect
391 (Figure 6D-6F) after being held at 37°C for the hydrolysis period ($P<0.01$ - $P<0.001$).

393 4. Discussion

394 This study examined in detail the influence which CN have on enteroendocrine (EE)
395 cells. The evidence suggests that these proteins are generally beneficial enhancing
396 cell proliferation, health and function. Both WCN and β -CN significantly increased cell
397 numbers compared with the control. Interestingly, this proliferative effect was often
398 greater at low protein concentrations. Although alpha (α -CN) and kappa casein (κ -
399 CN) possessed some proliferative properties their activity profile and their potency
400 were very different to β -CN. If we consider that WCN contains approximately 24-34%
401 β -CN (Coulter, 2002) approximately 0.11 mg/mL of the 0.3125 mg/mL concentration
402 of WCN sample was comprised of β -CN. This is a similar concentration to the lowest
403 β -CN sample (0.15625 mg/mL). The data suggest that β -CN is the major contributor
404 to the cell proliferating activity of WCN. Plaisancié et al. (2013) reported that a β -CN
405 peptide (β -CN (94-125) increased goblet cell numbers in the intestine of rats.
406 Furthermore, this effect was greatest at lower concentrations which are similar to
407 the WCN and β -CN findings in this study. Our recent work (Gillespie et al., 2015)
408 demonstrated that the whey protein beta-lactoglobulin (BLG) significantly increased
409 numbers of EE cells possibly due to its mitogenic nature. Bovine colostrum whey has
410 also been associated with cell growth in intestinal epithelial cells (Blais et al. 2014)
411 with various growth factors attributed to the activity. Augmenting the capacity of the
412 intestine by altering the EE cell numbers to increase incretin hormones production is
413 a potentially useful approach for improving the self-management of T2DM. This
414 could potentially increase postprandial insulin secretion and improve glucose
415 homeostasis. Taken together these reports indicate that it may be possible to exploit
416 dietary proteins to modulate gut cell number and function.

417 We also examined effects of CN on cellular health. Intact CN and individual CN
418 proteins did not significantly affect cell viability (as assessed with Alamar Blue) at any
419 concentrations tested. Viability of EE cells was assessed in more detail by measuring
420 mitochondrial membrane potential with HCA (reported as mitochondrial
421 fluorescence Intensity (MFI)). It appears individual CN proteins reduced the average
422 MFI at their highest concentrations, κ -CN most significantly. Interestingly total MFI
423 followed the same pattern as the cell number results indicating these are functional

424 energy-producing cells despite their rapid proliferation. In this study mitochondrial
425 area did not change, which may indicate that although the average MFI was reduced
426 the cells were still functioning normally. This study also indicates the usefulness and
427 sensitivity of HCA for assessing cell viability compared with more traditional methods
428 such as Alamar blue.

429

430 CNs demonstrated an ability to acutely stimulate GLP-1 secretion from EE cells. At all
431 concentrations tested WCN significantly increased GLP-1 secretion although at the
432 highest concentration there was less secretion than at lower concentrations. This
433 indicates an optimal concentration range for CN to act as a GLP-1 secretagogue. α -
434 CN and β -CN both concentration-dependently affected GLP-1 secretion, although α -
435 CN appears to be the most potent GLP-1 secretagogue. However, the GLP-1
436 secretory actions of α -CN and β -CN do not fully account for the activity observed with
437 WCN. For example, at the lowest concentration of WCN tested approximately 0.14
438 mg/mL of the protein present was comprised of α -CN and approximately 0.11 mg/mL
439 was comprised of β -CN. Despite the fact that these are very similar to the lowest
440 concentrations of α -CN and β -CN tested, they did not trigger GLP-1 secretion of a
441 similar magnitude. Even combining the individual responses of α -CN and β -CN would
442 not account for the secretory activity of WCN. It may be possible that α -CN and β -CN
443 act synergistically but another possibility is that other minor milk proteins not
444 characterised here could play a role (e.g. γ -CN which is formed during milk processing
445 (Reimerdes & Herlitz, 1979). The WCN sample was extracted from immature curd
446 and therefore may have contained small amounts of other components such as fat
447 which may also have contributed to the secretagogue effect seen at low
448 concentrations. It is worth noting that whey proteins BLG and alpha lactalbumin
449 (ALA) have been suggested to act in an additive manner to stimulate GLP-1 secretions
450 (Gillespie et al., 2015). Interestingly, when comparing our previous study (Gillespie
451 et al., 2015) to this current study, WCN appears to induce the greatest GLP-1
452 secretory responses with a 4.5-fold increase (compared with control) compared with
453 a 3.3-fold increase when cells were exposed to cheese whey. Furthermore, although
454 both individual CN and whey proteins were quite similar in their potency, the whey
455 protein α -LA increased GLP-1 secretion to the greatest extent (3.6-fold). In contrast,
456 Geraedts et al. (2011) reported whey protein to be a greater stimulator of GLP-1
457 compared with CN although there were differences in experimental design
458 (incubation times, concentration of protein) which could account for the differences.
459 Intracellular storage of GLP-1 was not significantly affected by CN but our previous

460 study with whey (Gillespie et al., 2015) found significant increases (with the
461 exception of BLG), which suggests that CN and whey protein effect EE cells differently
462 in terms of GLP-1 biosynthesis.

463 When interpreting the concentration-dependant actions of WCN on GLP-1 secretion
464 there is a need to also consider changes which occurred in the overall cell number. As
465 WCN concentrations increase, cell numbers appear to decline and this is one
466 contributing factor for the observed plateauing of GLP-1 secretion. It could also
467 explain the similar plateauing effect at higher concentrations of β -CN and the fact
468 that cellular energy may have redirected towards rapid proliferation rather than
469 hormone biosynthesis. Furthermore, the highly potent effect of α -CN on GLP-1
470 secretion caused a concomitant and concentration-dependant rise in cell numbers.
471 This is not the first time intact CN has been reported as a GLP-1 secretagogue.
472 Rafferty et al. (2011) reported that intact α -CN and β -CN stimulated GLP-1 secretion
473 and concentration dependant secretagogue effects were reported in the human EE
474 cell line NCI-H716 (Chen & Reimer, 2009). Geraedts et al. (2011) found intact CN to
475 be a more potent GLP-1 and cholecystokinin (CCK) secretagogue than hydrolysed CN.

476
477 α -CN and β -CN were hydrolysed with pepsin, trypsin and chymotrypsin for a total
478 period of 150 min with samples removed every 30 min. Regardless of which
479 proteolytic enzyme was used intact α -CN was an equally potent or significantly
480 greater secretagogue than any it's derived hydrolysates. β -CN studies with
481 chymotrypsin and trypsin yielded similar results to α -CN, although pepsin hydrolysis
482 of β -CN yielded the only hydrolysates with significantly greater secretagogue potency
483 than the intact protein. The secretory ability of α -CN is almost immediately lost when
484 hydrolysed by chymotrypsin. Theoretical cleavage sites generated using ExPASy
485 Peptide cutter for α -S₁CN (the most abundant α -CN) and α -S₂CN hydrolysed by
486 chymotrypsin indicates that there are up to 47 and 45 cleavage sites, respectively.
487 Furthermore degree of hydrolysis data does show a significant increase in degree of
488 hydrolysis (DH) even at T1 (< 2 min hydrolysis time). Similarly, β -CN (37 potential
489 cleavage sites) also lost its secretory activity immediately after chymotrypsin
490 hydrolysis. Trypsin hydrolysis of β -CN produced similar results to those of
491 chymotrypsin, although the effect was less immediate taking 30 min or more to
492 reduce GLP-1 secretion to levels similar to control. Interestingly, when α -CN was
493 hydrolysed with trypsin or pepsin both the hydrolysates and the intact protein
494 induced significantly greater GLP-1 secretion than the control. The intact α -CN
495 appeared more potent than hydrolysates.

496

497 **β -CN hydrolysates produced by pepsin had greater GLP-1 secretory potency than the**
498 **intact protein, making them unique in this regard. However,** prolonged hydrolysis
499 (150 min) did eventually lead to GLP-1 secretion of similar magnitude to intact β -CN.
500 All β -CN samples induced significantly greater GLP-1 secretion than control. For β -CN
501 there **are** 40 potential cleavage sites of pepsin which is comparably more than either
502 trypsin or chymotrypsin. Despite this the degree of hydrolysis was the lowest of all
503 enzymes tested indicating that cleavage rate was lower and there was likely to be
504 comparably more intact protein present. This provides a reason for the sustained
505 potency of hydrolysates but may suggest that there is an optimal pepsin hydrolysis
506 time for β -CN to maximize GLP-1 secretory activity. Similarly, when WCN was
507 extracted from cheese curd at different stages of maturation, day 14 produced
508 significantly greater GLP-1 secretory responses compared with day 1, 7 and 21 at the
509 two highest concentrations. Furthermore, this effect appeared to be concentration-
510 dependant whereas the other days were not. The proteolysis by bacterial
511 fermentation during the cheese ripening process has some effect on GLP-1 secretory
512 activity but overall CN from curd increased GLP-1 secretion significantly so at all
513 concentrations and at all stages of the maturation and the relatively intact CN (day 1)
514 elicited a similar response than the more mature and degraded (day 21). It is
515 possible that upon cleavage of the protein the 'inducing' site is lost, reducing the
516 ability of the protein to act as a secretagogue (Geraedts et al., 2011). CN have
517 limited secondary structure but Wang et al. (2013) reported increased α -helical and
518 β -turn content upon trypsin hydrolysis of CN, which may indicate that CN structure
519 (or lack thereof) is important for secretory activity.

520

521 Relatively little is known about how dietary proteins stimulate incretin hormone
522 release although it may involve mechanisms such as membrane depolarisation, Ca^{2+}
523 entry through voltage-gated Ca^{2+} channels, activation of G-protein-coupled receptors
524 which stimulate cAMP production (Chen, Li, Yang, & Zhang, 2012; Reimann, Ward, &
525 Gribble, 2006) or PepT1 in di- or tripeptide transportation (Matsumura et al., 2005)
526 and recently Pais, Gribble, & Reimann (2015) revealed the importance of transient
527 receptor potential (TRP) channels and the calcium sensing receptor (CaSR).
528 Interestingly, Hira et al. (2003) reported that CN could bind to the STC-1 cell
529 membrane components and increase intracellular calcium. Furthermore, α -CN did so
530 to a greater extent while bovine serum albumin (BSA) had no effect prompting the
531 authors to speculate that STC-1 cells recognise some proteins but not others and that

532 activation is reasonably specific. This current study supports this hypothesis. We
533 found that κ -CN had little effect compared with either α -CN or β -CN, and we have
534 recently shown that BSA is inactive (Gillespie *et al.*, 2015). Furthermore, the
535 frequently observed reductions in GLP-1 secretory activity brought about by enzyme
536 hydrolysis are also supportive.

537

538 This study is the first detailed examination of how WCN and individual CN proteins
539 interact with EE cells. As far as we are aware this is the first time HCA has been used
540 to examine the effects of CN on intracellular GLP-1 content of EE cells. Interestingly,
541 for nearly all samples tested there was no significant change in either
542 immunofluorescence intensity or the area of GLP-1 secretory vesicles. At some
543 concentrations κ -CN did significantly increase immunofluorescence intensity
544 compared with α -CN or β -CN indicating that κ -CN affects GLP-1 and EE cells
545 differently. Finally, this study has demonstrated that CN like many other proteins
546 (Yoder, Yang, Kindel, & Tso, 2010), are ineffective in stimulating GIP secretion from
547 EE cells, although there may be some indication that incubation (37°C; pH 7.4) for an
548 extended period could improve activity of individual CN proteins.

549

550 Conclusion

551 Overall, CN do not appear to be detrimental to the viability of EE cells and HCA
552 appears to be a highly sensitive and useful tool for studying the effect of food
553 molecules on incretin hormone biology. This study demonstrates that CN can
554 stimulate EE cell proliferation, and β -CN is the major contributor to this activity. CNs
555 demonstrated an ability to acutely stimulate GLP-1 secretion from EE cells and
556 although α -CN appears the most potent GLP-1 secretagogue, it does not fully account
557 for the activity observed with WCN, suggesting other contributing factors.
558 Importantly, proteolysis with digestive enzymes reduced the GLP-1 secretagogue
559 potency of α -CN and β -CN, except for pepsin hydrolysis of β -CN where a significant
560 increase was observed although the low hydrolysis rate could indicate much of the
561 protein remained intact. Proteolysis via bacterial fermentation during cheese
562 ripening had some positive effects on the GLP-1 secretory activity of CN. GIP appears
563 to be generally unaffected by intact or hydrolysed CN. Augmenting the capacity of
564 the intestine by altering the EE cell numbers and increasing incretin hormone
565 secretion is a potentially useful approach for improving the self-management of
566 T2DM. It may be possible to exploit CN proteins to modulate gut cell number and
567 function.

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569

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1 Figure 1.

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5 **Figure 1. Changes in cell number following exposures with intact whole casein (WCN) and**
6 **casein (CN) proteins**

7 Graphs show the number of pGIP/Neo STC-1 cells (mean $\pm$ SEM, n=3) following 3 h
8 incubations with: (A) WCN (0.3125 – 10 mg/mL) and (B) α -CN, β -CN and κ -CN (0.15625 – 5
9 mg/mL). Number of cells was determined by high content analysis following nuclear
10 staining with Hoechst. Statistical significance compared with control (one way ANOVA with
11 Tukey's post-hoc test) is indicated by *P<0.05; **P<0.01; ***P<0.001). Group comparisons
12 (two way ANOVA with Bonferroni post-hoc test) is indicated as follows: α -CN vs β -CN:
13 † P<0.05, †† P<0.005, ††† P<0.001; β -CN vs κ -CN: § P<0.05, §§ P<0.01, §§§ P<0.001

14 Figure 2.

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17 Figure 2. The effect of intact whole casein (WCN) and casein (CN) proteins on cell
18 viability

Graphs show cell viability and mitochondrial membrane potential in pGIP/Neo STC-1 cells (mean $\pm$ SEM; n=6) following 3 h incubations with WCN (A, C, E, G) and CN proteins – α -CN, β -CN κ -CN (B, D, F, H). Cell viability was initially assessed using Alamar Blue (A & B) and subsequently mitochondrial membrane potential using both average intensity (E & F) and total intensity (G & H), as well as mitochondrial area (number of pixels) (C & D) were determined using high content analysis. Statistical significance compared with control (one way ANOVA with Tukey's post-hoc test) is indicated by *P<0.05, **P<0.01, ***P<0.001. Group comparisons (two way ANOVA with Bonferroni post-hoc test) is indicated as follows: α -CN vs β -CN: [†]P<0.05, ^{†††}P<0.001; α -CN vs κ -CN: [†]P<0.05, ^{†††}P<0.001; β -CN vs κ -CN: [§]P< 0.05, ^{§§} P<0.01, ^{§§§}P<0.001

Figure 3.

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Figure 3. Degree of hydrolysis of α casein (α -CN) and β casein (β -CN) with proteolytic enzymes, and the effect of intact whole casein (WCN), individual casein (CN) proteins and enzyme-hydrolysed α -CN and β -CN on GLP-1 secretion.

Degree of hydrolysis (DH) relative to the intact protein was measured by the o-phthalaldehyde (OPA) method. α -CN (A) and β -CN (B) were hydrolysed separately using chymotrypsin, trypsin and pepsin over 150 min period at 37°C pH 7.4 (chymotrypsin and trypsin) or pH 2.3 (pepsin). Samples were removed every 30 mins and DH assessed. Error bars indicate SEM (n=3). Statistical analysis was carried out using a two way ANOVA with Bonferroni post-hoc test and is indicated as follows: Trypsin vs Pepsin:***P<0.001; Chymotrypsin vs Pepsin: **P<0.01, ***P<0.001; Chymotrypsin vs Trypsin: §§§P<0.001.

GLP-1 secretion from pGIP/Neo STC-1 cells (mean $\pm$ SEM, n = 2–4) following 3 h incubations with: (C) WCN (0.3 – 10 mg/mL), (D) α -CN, β -CN and κ -CN (0.15625 – 5 mg/mL), (E) α -CN (5 mg/mL) derived hydrolysates from chymotrypsin, (F) β -CN (5 mg/mL) derived hydrolysates from chymotrypsin, (G) α -CN (5 mg/mL) derived hydrolysates from trypsin, (H) β -CN (5 mg/mL) derived hydrolysates from trypsin, (I) α -CN (5 mg/mL) derived hydrolysates from pepsin, (J) β -CN (5 mg/mL) derived hydrolysates from pepsin (< 2 – 150 min). GLP-1 concentrations were determined by ELISA. Statistical significance compared with control (one way ANOVA with Tukey's post hoc test) is indicated by *P < 0.05, **P < 0.01, ***P < 0.001. Group comparisons (two way ANOVA with Bonferroni post hoc test) is indicated as follows: α -CN vs β -CN: †P<0.05, ††P<0.01, †††P<0.001; α -CN vs κ -CN: †††P<0.001; β -CN vs κ -CN: §§P<0.01, §§§P<0.001.

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90 Figure 4

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92 **Figure 4. Effect of intact whole casein (WCN) and casein (CN) proteins on**
 93 **intracellular levels of GLP-1.**

94 High content analysis of GLP-1 intracellular content of pGIP/Neo STC-1 cells exposed
 95 to WCN and individual CN proteins (mean $\pm$ SEM; $n = 6$) following 3 h incubations
 96 with (A & C) WCN (0.3125 – 10 mg/mL) and (B & D) α -CN, β -CN and κ -CN (0.15625 – 5
 97 mg/mL). Intracellular GLP-1 levels were recorded as the average GLP-1
 98 immunofluorescence intensity (A, B) and area of GLP-1 immunofluorescence
 99 (number of pixels of the target immunofluorescence).

100 Statistical significance compared with control (one way ANOVA with Tukey's post hoc
 101 test) and no significance was found. Group comparisons (two way ANOVA with
 102 Bonferroni post hoc test) is indicated as follows: α -CN vs κ -CN: [‡]P < 0.05; β -CN vs κ -
 103 CN: [§]P < 0.05.

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121 labelled (1) γ -CN (2) β -CN (3) α S₂-CN (4) α S₁-CN(5) hydrolysis product of α S₁-CN(6)
122 hydrolysis product of α S₂-CN. Gels were stained using coomassie blue (G250)
123 overnight and scanned using Epson Perfection v 700 Scanner with Silver Fast launcher
124 v 6.6 and saved in JPEG format and image analysis was performed using GelAnalyzer
125 2010a.

126 **Panel B:** GLP-1 secretion from pGIP/Neo STC-1 cells (mean $\pm$ SEM, $n = 2$) following 3 h
127 incubation with WCN (0.3 – 10 mg/mL). GLP-1 concentrations were determined by
128 ELISA. Statistical significance compared with control (one way ANOVA with Tukey's
129 post hoc test) is indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Group comparisons
130 (two way ANOVA with Bonferroni post hoc test) is indicated as follows: Day 1 vs Day
131 7: † $P < 0.05$; Day 1 vs Day 14: †† $P < 0.01$, ††† $P < 0.001$; Day 14 vs Day 21: §§ $P < 0.01$, §§§ $P <$
132 0.001; Day 14 vs Day 21: °° $P < 0.01$, °°° $P < 0.001$.

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159 **Figure 6.**The effect of **intact whole casein (WCN)**, **individual casein (CN) proteins**,
160 **enzyme-hydrolysed α-CN and β-CN on GIP secretion.**
161 GIP secretion from pGIP/Neo STC-1 cells (mean ± SEM, n = 2–4) following 3 h
162 incubations with: (A) WCN (0.3 – 10 mg/**mL**), (B) α-CN, β-CN and κ-CN (0.15625 – 5
163 mg/mL), (C) α-CN (5 mg/mL) derived hydrolysates from chymotrypsin, (D) β-CN (5
164 mg/mL) derived hydrolysates from chymotrypsin, (E) α-CN(5 mg/**mL**) derived

165 hydrolysates from trypsin, (F) β -CN (5 mg/mL) derived hydrolysates from trypsin, (G)
166 α -CN (5 mg/mL) derived hydrolysates from pepsin, (H) β -CN (5 mg/mL) derived
167 hydrolysates from pepsin (< 2 – 150 min). GIP concentrations were determined by
168 ELISA. Statistical significance compared with control (one way ANOVA with Tukey's
169 post hoc test) is indicated by *P < 0.05, **P < 0.01, ***P < 0.001. Group comparisons
170 (two way ANOVA with Bonferroni post hoc test) is indicated as follows: α -CN vs β -CN:
171 [†]P < 0.05, ^{††}P < 0.01, ^{†††}P < 0.001; α -CN vs κ -CN: [†]P < 0.05, ^{†††}P < 0.001; β -CN vs κ -CN:
172 [§]P < 0.05, ^{§§§}P < 0.001.