

1 Angiotensin II type 1 receptor dependent GLP-1 and PYY secretion in mice and  
2 humans

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30 ***Abstract***

31 Angiotensin II (Ang II) is the key hormone mediator of the renin angiotensin system which  
32 regulates blood pressure and fluid and electrolyte balance in the body. Here we report that in  
33 colonic epithelium the Ang II type 1 receptor (AT<sub>1</sub>R) is highly and exclusively expressed in  
34 enteroendocrine L-cells which produce the gut hormones glucagon-like peptide-1 (GLP-1)  
35 and peptide YY (PYY). Ang II stimulated GLP-1 and PYY release from primary cultures of  
36 mouse and human colon, which was antagonised by the specific AT<sub>1</sub>R receptor blocker  
37 candesartan. Ang II raised intracellular calcium levels in L-cells in primary cultures, recorded  
38 by live-cell imaging of L-cells specifically expressing the fluorescent calcium sensor  
39 GCaMP3. In Ussing chamber recordings, Ang II reduced short circuit currents in mouse distal  
40 colon preparations, which was antagonised by candesartan or a specific NPY1R receptor  
41 inhibitor but insensitive to amiloride. We conclude that Ang II stimulates PYY secretion, in  
42 turn inhibiting epithelial anion fluxes, thereby reducing net fluid secretion into the colonic  
43 lumen. Our findings highlight an important role of colonic L-cells in whole body fluid  
44 homeostasis by controlling water loss through the intestine.

45 ***Introduction***

46 The prime functions of the gut are the digestion and absorption of ingested food. These are  
47 regulated by intestinal hormones, such as glucagon-like peptide-1 (GLP-1) and peptide YY  
48 (PYY), which are co-secreted from enteroendocrine L-cells found predominantly in the ileum  
49 and colon (1). Both hormones underlie the ileal brake, slowing gastric emptying when  
50 nutrient delivery exceeds the absorptive capacity of the duodenum/jejunum, and control food  
51 intake and appetite (2). These effects beyond the confines of the intestine have raised interest  
52 in the exploitation of gut hormones for the treatment of diabetes and obesity. GLP-1 augments  
53 glucose induced insulin secretion (3), and has been exploited in the form of GLP-1 mimetics  
54 for the treatment of diabetes and obesity.

55 An additional action of PYY is to inhibit intestinal water and anion secretion. This is achieved  
56 through a direct action on enterocyte Y1 receptors and an indirect effect on Y2 receptors  
57 located on enteric neurons(4). This paracrine effect of PYY is important for body fluid and  
58 electrolyte homeostasis. We showed previously that Arginine Vasopressin (AVP) stimulates  
59 GLP-1 and PYY release from mouse and human colonic L-cells and suggested that this forms  
60 part of a mechanism that reduces water loss through the intestine (5). Another important  
61 regulator of water and electrolyte balance and blood pressure is the renin angiotensin system  
62 (RAS) (reviewed in (6)), which exhibits both systemic and local regulation. Sympathetic  
63 stimulation, renal artery hypotension or reduced blood volume (e.g. dehydration or  
64 haemorrhage) initiate the release of renin from renal juxtaglomerular cells which converts  
65 circulating angiotensinogen to angiotensin I (Ang I). Ang I in turn is hydrolysed by  
66 angiotensin converting enzyme (ACE) to form the biologically active octapeptide  
67 Angiotensin II (Ang II). Ang II causes arterial vasoconstriction and renal retention of sodium  
68 and fluid, and stimulates the release of aldosterone and AVP from the adrenal cortex and  
69 posterior pituitary, respectively.

70 Several studies have identified different components of the RAS, including angiotensinogen,  
71 renin, ACE, Ang II and angiotensin receptors in the mucosal and muscular layers of the  
72 gastro-intestinal tract (7-10). Angiotensin receptors, particularly AT<sub>1</sub>, have been implicated in

73 gut motility (11,12) and electrolyte absorption (13-15). Here we report that AT<sub>1</sub> is highly and  
74 selectively expressed in colonic L-cells, and is linked to the stimulation of PYY and GLP-1  
75 secretion and colonic fluid balance.

76

## 77 ***Methods***

78

### 79 ***Solutions and compounds***

80 All compounds were purchased from Sigma Aldrich (Poole, U.K.) unless otherwise stated.  
81 BIBP 32267 trifluoroacetate was purchased from Bioquote (York, U.K.) and Angiotensin (1-  
82 7) from Bio-Techne (Abingdon, U.K.). The composition of the standard bath solution used in  
83 secretion and imaging experiments was: 4.5 mmol/L KCl, 138 mmol/L NaCl, 4.2 mmol/L  
84 NaHCO<sub>3</sub>, 1.2 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 2.6 mmol/L CaCl<sub>2</sub>, 1.2 mmol/L MgCl<sub>2</sub> and 10 mmol/L  
85 HEPES (adjusted to pH 7.4 with NaOH). For experiments where CoCl<sub>2</sub> was used, carbonates  
86 and phosphates were omitted from the saline buffer and the osmolarity was compensated with  
87 additional NaCl (143 mmol/L total). The composition of Ringer's solution used in Ussing  
88 chamber experiments was: 120 mmol/L NaCl, 3 mmol/L KCl, 0.5 mmol/L MgCl<sub>2</sub>, 1.25  
89 mmol/L CaCl<sub>2</sub>, 23 mmol/L NaHCO<sub>3</sub> and 10 mmol/L glucose.

90

### 91 ***Animals and ethical approval***

92 All animal procedures were approved by the University of Cambridge Animal Welfare and  
93 Ethical Review Body and conformed to the Animals (Scientific Procedures) Act 1986  
94 Amendment Regulations (SI 2012/3039). The work was performed under the UK Home  
95 Office Project License 70/7824. Male and female mice, aged 3-6 months on a C57BL6  
96 background were housed in individually-ventilated cages on a 12h dark/light cycle with ad  
97 libitum access to water and chow. Mice were euthanized by cervical dislocation and intestinal  
98 tissue used in the experiments. For *in vivo* experiments, only male mice, aged 11-12 weeks  
99 were used. Mice were fasted overnight for a maximum of 16 hours before receiving an intra-  
100 peritoneal injection of either Ang II (100 µg/kg) or PBS (vehicle). Ten minutes after the  
101 injection, each animal was anaesthetised (isoflurane) and a terminal blood sample taken.

102 Blood was collected in tubes containing EDTA and protease inhibitors (10  $\mu\text{mol/L}$  amastatin  
103 hydrochloride, 100  $\mu\text{mol/L}$  diprotinin A, 18  $\mu\text{mol/L}$  aprotinin), centrifuged at 13,000 g for 90  
104 s, and plasma collected and used for active GLP-1 and total PYY analysis.

105

#### 106 ***Transgenic Mice***

107 GLU-Venus and GLU-Cre mice have been previously described (16) (17) and express the  
108 fluorescent protein Venus and *Cre* recombinase under the control of the proglucagon  
109 promoter, respectively. To monitor calcium fluctuations in L-cells, GLU-Cre mice were  
110 crossed with ROSA26-GCaMP3 reporter mice (18) (Jax stock 014538) to generate L-cell  
111 specific expression of the genetically encoded  $\text{Ca}^{2+}$  sensor.

112

#### 113 ***Primary murine colonic crypt cultures***

114 Colonic crypts were isolated and cultured as previously described (16). Briefly, mice 3-6  
115 months old were sacrificed by cervical dislocation and the colon was excised. Luminal  
116 contents were flushed thoroughly with PBS and the outer muscle layer removed. Tissue was  
117 minced and digested with Collagenase Type XI (0.4 mg/ml) and the cell suspension plated  
118 onto Matrigel (BD Bioscience, Oxford, UK) pre-coated 24-well plates for GLP-1 secretion  
119 experiments or on 35mm glass bottomed dishes (Mattek Corporation, MA, USA) for live cell  
120 calcium imaging.

121

#### 122 ***Preparation of crypt cultures from human colons***

123 The study was approved by the Research Ethics Committee under license number  
124 09/H0308/24. Fresh surgical specimens of human colon were obtained from Tissue Bank at  
125 Addenbrooke's Hospital, Cambridge, UK, stored at 4°C and processed within a few hours of  
126 surgery. The crypt isolation procedure was similar to that used for mouse tissue with the  
127 exception that a higher concentration of collagenase XI (0.5 mg/ml) was used for digestion  
128 (1).

129

130 ***GLP-1 and PYY secretion assays***

131 18-24 hours after plating, cells were washed and incubated with test agents dissolved in  
132 standard bath solution supplemented with 0.1% BSA for 2 hours at 37°C. At the end of the  
133 incubation, supernatants were collected and centrifuged at 2000 rcf for 5 minutes and snap  
134 frozen on dry ice. Cells were lysed with lysis buffer containing 50 mmol/l Tris-HCl, 150  
135 mmol/L NaCl, 1% IGEPAL-CA 630, 0.5% deoxycholic acid and complete EDTA-free  
136 protease inhibitor cocktail (Roche, Burgess Hill, UK) to extract intracellular peptides,  
137 centrifuged at 10,000 rcf for 10 minutes and snap frozen. GLP-1 and PYY were measured  
138 using total GLP-1 and total PYY assays (MesoScale Discovery (MSD), Gaithersburg, MD,  
139 USA) and supernatant concentrations were expressed as a percent of the total  
140 (secreted+lysate) GLP-1 or PYY content of each well.

141

142 ***Calcium imaging***

143 L-cell cytosolic calcium concentrations were monitored as intensity changes in GCaMP3  
144 fluorescence excited at 488nm using a xenon arc lamp and a monochromator (Cairn Research,  
145 UK) in colonic crypt cultures prepared from GLU-Cre/ROSA26-GCaMP3 mice. Solutions  
146 were perfused continuously at a rate of approximately 1 ml/min. Imaging was performed  
147 using an Olympus IX71 microscope with a 40x oil immersion objective and an OrcaER  
148 camera (Hamamatsu, Japan). Images were acquired at 1 Hz and analysed, after background  
149 subtraction, using MetaFluor software (Molecular Devices, USA). Fluorescence in the  
150 presence of the test agent was normalised to the respective mean background fluorescence of  
151 each cell, measured before the addition and after the washout of the test compound. For  
152 presentation data were smoothed with a sliding average over 20s.

153 ***Microarray analysis and RNA sequencing***

154 Microarray analysis of total RNA from FACS purified L-cells using Affymetrix mouse 430  
155 2.0 expression arrays (Affymetrix UK Ltd, high Wycombe, UK) has been described

156 previously (19). Expression levels of each probe were determined by robust multichip average  
157 (RMA) analysis. For sequencing, total RNA from 2,000 to 10,000 FACS purified L-cells  
158 from the upper small intestine (top 10 cm), lower small intestine (bottom 10 cm) or  
159 colon/rectum from GLU-Venus mice was extracted using an RNeasy Micro Plus kit  
160 (QIAGEN) according to the manufacturer's instructions. RNA was amplified using Ovation  
161 RNA-seq System V2 (NuGEN), using 1 ng of RNA for each sample (3 replicates each were  
162 used for L-cells and non-fluorescent control cells for each segment of the gastrointestinal  
163 tract, totalling 18 samples). To prepare the RNAseq library, the amplified cDNA (1µg per  
164 sample) was fragmented to 200 bp using a Bioruptor Sonicator (Diagenode), and barcode-  
165 ligation and end repair were achieved using the Ovation Rapid DR Multiplex System 1-96  
166 (NuGEN). Barcoded libraries were combined and sent for SE50 sequencing using an Illumina  
167 HiSeq 2500 system at the Genomics Core Facility, Cancer Research UK Cambridge Institute.  
168 Sequence reads were demultiplexed using the Casava pipeline (Illumina) and then aligned to  
169 the mouse genome (GRCm38) using Tophat version 2.1.0 ([http://](http://ccb.jhu.edu/software/tophat/index.shtml)  
170 [ccb.jhu.edu/software/tophat/index.shtml](http://ccb.jhu.edu/software/tophat/index.shtml)). Differential gene expression was determined using  
171 Cufflinks version 2.2.1 (<http://cole-trapnell-lab.github.io/cufflinks/>).

172

### 173 ***Quantitative RT-PCR***

174 Populations of Venus-positive cells (L-cells) or Venus-negative cells (non-L cells) of purity  
175 >90% were separated from the tissues of GLU-Venus mice using a BD Influx cell sorter  
176 running BD FACS Software as previously described (16). Laser alignment was performed  
177 using eight-peak rainbow beads (Spherotech), and drop delay was determined using BD  
178 Accudrop beads. RNA was extracted from FACS-sorted cells by a microscale RNA isolation  
179 kit (Ambion, Austin, TX, USA) and reverse transcribed to cDNA according to standard  
180 protocols. First-strand cDNA template was mixed with specific TaqMan primers (Applied  
181 Biosystems, Foster City, CA, USA), water and PCR Master Mix (Applied Biosystems), and  
182 quantitative RT-PCR was conducted using a 7900HT Fast Real-Time PCR system (Applied  
183 Biosystems).  $\beta$ -Actin was used as the normalisation control. The primer/probe pairs used in

184 this study were from Applied Biosystems, *Agtr1*: Mm01957722\_s1 and  
185 *Mas1*:Mm00434823\_s1. All experiments were performed on at least three cDNAs isolated  
186 from one mouse each.

187

### 188 ***Immunohistochemistry***

189 Tissues were fixed in 4% paraformaldehyde, dehydrated in 15% and 30% sucrose, and frozen  
190 in optimal cutting temperature embedding media (CellPath, Newtown, U.K.). Cryostat-cut  
191 sections (6–10  $\mu\text{m}$ ) were mounted directly onto polylysine-covered glass slides (VWR,  
192 Leuven, Belgium). Slides were incubated for 1 h in blocking solution containing PBS/0.05%  
193 Triton X-100/10% donkey serum and overnight with primary antibodies (goat anti- GLP-1  
194 (sc-7782) and rabbit anti-AT<sub>1</sub>R (sc-579, Santa Cruz Biotechnology Inc, CA, USA) in  
195 blocking solution. Sections were rinsed with blocking solution before being incubated for 1  
196 hour at room temperature with Alexa Fluor 488 (1:300) and Alexa Fluor 555 (1:300)  
197 secondary antibodies (Invitrogen) and Hoechst (1:1300) for nuclear staining. Control sections  
198 were stained with secondary antibodies alone. Sections were mounted with Prolong Gold  
199 (Life Technologies) before being imaged by confocal microscopy (Leica TCS SP8, Milton  
200 Keynes, U.K.).

### 201 ***Ussing Chamber recordings***

202 The most distal part of the colon (~1.25 cm) was cut open longitudinally and rinsed in  
203 Ringer's solution. Serosa and most of the outer muscular layer were removed by fine forceps.  
204 The tissue was mounted in an Ussing chamber (EM-LVSY-4 system with P2400 chambers  
205 and P2404 sliders, all from Physiologic Instruments, San Diego, CA, USA). Only one  
206 preparation was used from each animal. The active epithelial surface was 0.25 cm<sup>2</sup>. Both parts  
207 of the Ussing chambers were filled with 3 ml of Ringer's solution, maintained at 37°C and  
208 continuously bubbled with 5% vol/vol CO<sub>2</sub>/ 95% vol/vol O<sub>2</sub>. The transepithelial potential

209 difference was clamped to 0 mV using a DVC 1000 amplifier (WPI, Sarasota, FL, USA) and  
210 the resulting short circuit current was recorded through Ag-AgCl electrodes and 3 mol/L KCl  
211 agarose bridges. The recordings were collected and stored using Digidata 1440A acquisition  
212 system and AxoScope 10.4 software (both from Molecular Devices, Sunnyvale, CA, USA).  
213 The transepithelial resistance and short circuit current (Isc) were allowed to stabilise for at  
214 least 30 minutes before the application of drugs. During this period, transepithelial resistance  
215 was assessed by measuring current changes in response to 2 mV pulses lasting 2.5 seconds,  
216 applied every 100 s. After stabilisation of the electrical parameters, the following drugs were  
217 applied: 5  $\mu\text{mol/L}$  amiloride, 1  $\mu\text{mol/L}$  candesartan, 1  $\mu\text{mol/L}$  BIBP 3226, and 1  $\mu\text{mol/L}$  Ang  
218 II. Forskolin (10  $\mu\text{mol/L}$ ) was applied bilaterally at the end of each experiment to confirm the  
219 responsiveness/viability of the tissue. As Ang II triggered a sustained depression in Isc in all  
220 tissue preparations tested, but a short-lived increase (1-3 min duration) in only ~half the  
221 preparations, the difference between the mean Isc 2-5 min immediately preceding, and the  
222 mean Isc during 30 minutes following Ang II application was used to combine data from  
223 different preparations.

## 224 *Statistics*

225 Results are expressed as mean  $\pm$  SD unless otherwise indicated. Statistical analysis was  
226 performed using GraphPad Prism 5.01 (San Diego, CA, USA). For GLP-1 and PYY secretion  
227 data, one-way ANOVA with post hoc Dunnett's or Bonferroni tests were performed on log-  
228 transformed secretion data, as these data were heteroscedastic. For Ussing chamber  
229 recordings, one-way ANOVA with post hoc Dunnett's test was performed on non-  
230 transformed Isc data normalised for a surface area of 1  $\text{cm}^2$ . For qRT-PCR, one-way ANOVA  
231 with post hoc Bonferroni analysis was done on non-transformed  $\Delta\text{Ct}$  data. Statistical  
232 significance for  $\text{Ca}^{2+}$  imaging data was assessed by Student's t-test.

## 233 *Results*

### 234 *AT1 receptor expression in mouse and human colonic L-cells*

235 Ang II interacts with two seven-transmembrane G-protein coupled receptors, AT<sub>1</sub> and AT<sub>2</sub>.  
236 Whereas rodents possess two AT<sub>1</sub> receptor isoforms, AT<sub>1A</sub> and AT<sub>1B</sub> (encoded by *Agtr1a* and  
237 *Agtr1b*, respectively) (20) humans have only one type 1 receptor gene. Microarray analysis  
238 was performed to compare the expression of *Agtr1a*, *Agtr1b* and *Agtr2* in primary murine  
239 glucose-dependent insulinotropic polypeptide (GIP) secreting K-cells as well as L-cells from  
240 the duodenum/jejunum (top 10 cm of the small intestine (LD) or the colon. As shown in  
241 Figure 1A, *Agtr1a* expression was ~100-fold higher in colonic (LC) than upper SI L-cells  
242 (LDJ), and 14-fold enriched in colonic L-cells (LC) over non-L-cells (CC). *Agtr1b* and *Agtr2*  
243 were poorly expressed in all cell populations examined (Figure 1A). RNA-sequencing  
244 confirmed the high selective expression of *Agtr1a* in colonic L-cells (LC, Figure 1B).  
245 Microarray and RNA-seq results were also validated by quantitative PCR, performed on  
246 cDNA prepared from independently FACS-sorted L- and non L-cells from the upper SI  
247 (duodenum/jejunum, LDJ), the lower SI (jejunum/ileum, LJI) and colon (LC) as well as K-  
248 cells and non K-cells. By q-PCR, *Agtr1a* was highly enriched in colonic L-cells (LC) over  
249 colonic control cells (CC), and was found at much lower levels in small intestinal epithelial  
250 control (CDJ, CJI) and L-cells (LDJ, LJI) and K- and non-K-cells (CK) (Figure 1C).

251 In human colon tissue sections, AT<sub>1</sub> immuno-positive cells were found scattered through the  
252 epithelium and co-stained with antibodies against GLP-1(Fig 1D). No visible staining for AT<sub>1</sub>  
253 was detected in GLP-1 negative cells of the epithelial layer. Some cells in the lamina propria  
254 showed AT<sub>1</sub> reactivity, but their identity was not further investigated (data not shown).

#### 255 ***Ang II stimulated GLP-1 and PYY secretion from mouse and human colon cultures***

256 The functional relevance of the high *Agtr1a* expression in mouse colonic L-cells was  
257 investigated by performing hormone secretion experiments from primary murine colonic  
258 cultures. Cells were incubated for 2 hours with Ang II (10<sup>-10</sup>, 10<sup>-8</sup> and 10<sup>-6</sup> mol/L) or with a  
259 positive control containing a combination of forskolin (10 μmol/L), IBMX (10 μmol/L) and  
260 glucose (10 mmol/L). Ang II stimulated GLP-1 secretion at all concentrations tested. The

261 highest concentration,  $10^{-6}$  mol/L, increased GLP-1 secretion from 3% to 9.5% of the total  
262 GLP-1 content (Figure 2A). Secretion of PYY, which is co-released from colonic L-cells, was  
263 examined with a single concentration of Ang II ( $10^{-8}$  mol/L), and increased from 8.5% to 24%  
264 of the total PYY content (Figure 2B). Consistent with the localisation of  $AT_1$  in human  
265 colonic L-cells, Ang II ( $10^{-6}$  mol/L) also enhanced GLP-1 and PYY secretion by ~1.4-fold  
266 each (Fig 2C and 2D) in human colonic crypt cultures. In mice, acute intra peritoneal  
267 injection of Ang II (100  $\mu$ g/kg) did not, however, significantly increase plasma GLP-1 or  
268 PYY concentrations (Fig 2E and 2F).

### 269 *GLP-1 and PYY secretion is mediated by $AT_1$ receptor*

270 To investigate if other receptors for Ang II or its metabolites play a role in Ang II stimulated  
271 hormone secretion from the colon, we performed secretion experiments in the presence of  
272 Candesartan cilexetil, a prodrug used to treat hypertension, which is converted to the selective  
273  $AT_1$  inhibitor Candesartan by the intestinal wall esterases (21). Candesartan (Can,  $10^{-7}$  mol/L)  
274 had no effect on basal GLP-1 secretion, but abolished Ang II triggered GLP-1 release from  
275 mouse colonic cultures (Figure 3A). Ang II triggered PYY secretion was also blocked by co-  
276 treatment with Candesartan (Figure 3B), thereby establishing the role of  $AT_1$  in mediating  
277 Ang II stimulated GLP-1 and PYY secretion.

### 278 *Ang II induced intracellular calcium responses in colonic L-cells*

279 Previous studies have revealed that  $AT_1$  receptors activation recruits phospholipase C and  
280 stimulates the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) to diacylglycerol  
281 (DAG) and inositol-1,4,5-trisphosphate (IP<sub>3</sub>), promoting calcium release from internal stores  
282 (22,23). Depending on the cell or tissue type, it was also reported that Ang II inhibits  
283 adenylate cyclase and lowers intracellular cAMP levels (24,25). To elucidate the mechanistic  
284 pathway involved in Ang II-triggered GLP-1 and PYY release, we monitored the changes in  
285 intracellular calcium in primary colonic L-cells identified in cultures from GLU-

286 Cre/ROSA26-GCaMP3 mice during Ang II application. As shown in Figure 4A, Ang II  
287 triggered a rapid increase in L-cell GCaMP3 fluorescence, indicative of an increase in the  
288 intracellular calcium concentration. Responses peaked shortly after Ang II addition, were  
289 rapidly reversible, and were reproducible on second application of Ang II (Figure 4A &B).

290 Intracellular calcium can be increased either by opening of plasma membrane calcium  
291 channels or by release from intracellular calcium stores. To establish whether the Ang II  
292 dependent cytoplasmic calcium rise was due to calcium release from intracellular  
293 endoplasmic reticulum stores or the opening of plasma membrane voltage-gated calcium  
294 channels, calcium imaging experiments were performed in the presence of cobalt chloride  
295 ( $\text{CoCl}_2$ ), a general voltage gated calcium channel blocker that impairs L-cell calcium  
296 responses to depolarising stimuli such as KCl (26). Cytoplasmic calcium responses to Ang II  
297 were still observed in the presence of  $\text{CoCl}_2$  (5 mmol/l) (Fig 4C and D), suggesting they do  
298 not depend on voltage gated calcium channels. This is consistent with the reported Gq  
299 coupled nature of  $\text{AT}_1$  (27). Further corroborating the results obtained with calcium imaging  
300 experiments, the L-type voltage gated calcium channel blocker nifedipine ( $10\mu\text{mol/L}$ ) did not  
301 significantly inhibit Ang II stimulated GLP-1 secretion (Fig4E), but GLP-1 responses to Ang  
302 II were blocked by 2-aminoethoxydiphenylborate (2- APB,  $100\mu\text{mol/L}$ ), an inhibitor of IP3  
303 receptors (Fig 4E).

#### 304 *Non classical RAS and L-cells*

305 Whereas the classical RAS (ACE-Ang II- $\text{AT}_1$ ) promotes actions to maintain blood pressure, a  
306 'non-classical' RAS, consisting of ACE2-Ang (1-7)-Mas1 receptor has opposing effects (28).  
307 Ang (1-7) is generated by the cleavage of an amino acid from the carboxy-terminus of Ang II  
308 by an ACE homologue ACE2 and mediates vasodilatory/diuretic actions through the  
309  $\text{AT}_7/\text{Mas1}$  receptor (29). Microarray (Fig 5A) and RNA-seq analysis (data not shown) for  
310 *Mas1* receptor expression were performed on K- and L-cells from mouse upper SI (LDJ) and  
311 colon (LC) and their respective control cells (CK, CDJ, CC). *Mas1* expression was very low

312 or undetectable in all cell populations examined. This was confirmed by qPCR (Fig 5B).  
313 Consistent with these findings, application of Ang (1-7) to primary murine colonic crypt  
314 cultures had no significant effect on GLP-1 release (Fig 5C).

### 315 *Antisecretory effect of Ang II in mouse colon*

316 Given the well-known inhibitory effect of PYY on intestinal anion and water secretion  
317 (4,5,30-32), we employed Ussing chambers to study the functional relevance of Ang II in  
318 mouse colon. In all tissue preparations tested, basolateral addition of Ang II ( $10^{-6}$  mol/L)  
319 caused a sustained depression in Isc (of mean  $15.1 \mu\text{A}/\text{cm}^2$ ) lasting for at least 35 minutes  
320 (Fig 6A). In 3/5 preparations we also observed a transient increase in Isc, with a peak increase  
321 of  $40.6 \pm 10.1 \mu\text{A}/\text{cm}^2$  (Fig 6A), but this was absent in the other 2 preparations (not shown).  
322 Pre-treatment with apically-added amiloride ( $5 \mu\text{mol}/\text{L}$ ) alone decreased Isc, which came to a  
323 new plateau  $9.6 \pm 9.1 \mu\text{A}/\text{cm}^2$  lower than the Isc before amiloride addition. Subsequent  
324 application of Ang II 10-12 minutes after amiloride pre-treatment caused further Isc  
325 depression, which was not different from the response caused by Ang II without any pre-  
326 treatment (Fig 6D). These results suggest that the Ang II-related Isc decrease was due to  
327 inhibition of electrogenic anion secretion and did not involve ENaC-dependent sodium  
328 absorption. Pre-treatment with basolaterally-added BIBP3226 (BIBP, a specific NPY1R  
329 antagonist) caused an increase of Isc, with the new plateau being  $1.8 \pm 2.7 \mu\text{A}/\text{cm}^2$  higher  
330 than before BIBP addition. The inhibitory Isc response to Ang II applied 10-12 minutes after  
331 BIBP was significantly impaired, confirming a role of the PYY receptor NPY1R in Ang II  
332 mediated changes of colonic transepithelial ion movement (Fig 6B and 6D). Candesartan ( $10^{-6}$   
333 mol/L bilaterally) reduced the basal Isc by  $1.9 \pm 2.8 \mu\text{A}/\text{cm}^2$ , and abolished any subsequent  
334 responses to Ang II application, confirming the role of  $\text{AT}_1$  in Ang II stimulated Isc changes  
335 (Fig 6C and 6D).

336 The above-mentioned initial short-lived (1-3mins) Isc increase after Ang II addition was  
337 observed in 2/4 preparations pre-treated with amiloride, 2/4 preparations pre-treated with

338 BIBP and 0/4 preparations pre-treated with Candesartan. When considering all preparations  
339 together, there was no significant difference in the early peak magnitude between the groups  
340 (data not shown).

#### 341 *Discussion*

342 Digestion and absorption of nutrients from the intestine depends on sufficient availability of  
343 water in the lumen. Indeed, in addition to the average ingested fluid volume of ~2.5 litres per  
344 day in humans, it has been estimated that 5-10 litres of water are secreted into the gut lumen  
345 and re-absorbed to aid intestinal processes (33), necessitating a close link between the gut and  
346 systems regulating body fluid and electrolyte homeostasis. Here we identified AT<sub>1</sub> in colonic  
347 L-cells and demonstrated that its activation by Ang II triggered GLP-1 and PYY secretion and  
348 downstream PYY-dependent inhibition of anion secretion. This offers a potential explanation  
349 for previous reports that colonic fluid secretion is regulated by the renin angiotensin system  
350 (15).

351 The effect of Ang II on intestinal water and ion absorption has been studied extensively in the  
352 rat. At low physiological concentrations Ang II stimulates water absorption in the jejunum  
353 and colon, although higher doses were also reported to inhibit absorption (15). In the jejunum  
354 the pro-absorptive effect of Ang II was linked to the activation of noradrenergic nerve  
355 endings, based on the sensitivity of the response to  $\alpha$ -adrenergic antagonists (34). Early  
356 investigations concluded that the proabsorptive effects of Ang II are predominantly mediated  
357 by electroneutral mechanisms (13), but experiments on rat descending colon mounted in  
358 Ussing chambers revealed a reduction of I<sub>SC</sub> over a wide Ang II concentration range ( $10^{-9}$  –  
359  $10^{-5}$  mol/L) (14). This was sensitive to the chloride channel blocker, diphenylamine-2-  
360 carboxylate (DPC) but not to amiloride, suggesting that the action of Ang II on I<sub>sc</sub> is  
361 mediated through inhibition of anion secretion rather than stimulation of electrogenic sodium  
362 absorption (14). Our observed decrease in I<sub>sc</sub> in the colon is in agreement with these results  
363 and is clearly mediated via AT<sub>1</sub> as it was sensitive to candesartan. In about half the

364 preparations, we also observed a transient increase in Isc – this might also be downstream of  
365 AT<sub>1</sub> as it was never observed in the presence of candesartan, but in contrast to the sustained  
366 Isc reduction, it was not affected by the Y1-receptor antagonist BIBP3226. Our finding that  
367 the sustained reduction in Isc by Ang II was sensitive to BIBP3226 and insensitive to  
368 amiloride suggests that this effect lies downstream of PYY secretion. PYY, in addition to  
369 slowing gastric emptying and reducing hunger, is well recognised as an inhibitor of anion and  
370 electrolyte secretion (35), exerting its inhibitory action mainly via Y1 receptors on  
371 enterocytes and to some extent by Y2 receptors on enteric neurons (31). Activation of the  
372 Gi/Go coupled Y1 receptor lowers intracellular enterocyte cAMP levels, subsequently  
373 inhibiting CFTR channels, and thereby reduces anion secretion into the gut lumen (4).

374 Previous studies have shown that AT<sub>1</sub> is the predominant Ang II receptor in the muscularis of  
375 rat ileum and colon (7), submucosal plexus in guinea pig distal colon (36), vessel walls,  
376 myofibroblasts, and macrophages in the lamina propria, crypt bases and surface epithelium in  
377 human colon (9), as well as a subset of human jejunal cells resembling enteroendocrine cells  
378 (37). Our data contrast with the previously-reported detection of AT<sub>1</sub> in jejunal enterocytes  
379 (37), as we found only very low mRNA expression in the non-L-cell population of the mouse  
380 small intestine, which would be dominated by enterocytes. While this might reflect species  
381 differences, we also observed clear AT<sub>1</sub> staining in human colonic L-cells but not enterocytes.  
382 The fact that we were able to block the sustained drop of short circuit current observed in  
383 Ussing chamber mounted colonic tissue in response to Ang II with the Y1R-blocker  
384 BIBP3226 is consistent with the observed restriction of AT<sub>1</sub> to L-cells in the murine colon  
385 and an important role of L-cells in the secretory responses of the colon to Ang II.

386 Although Ang II could in principle also exert some of its effects through other receptors, we  
387 were unable to demonstrate a role of other angiotensin receptors in L-cells. mRNAs encoding  
388 both AT<sub>2</sub>, which has a similar affinity for Ang II as AT<sub>1</sub>, and the MAS1 receptor were only  
389 expressed at very low levels, barely detectable by RT-PCR. Ang (1-7), the ligand for MAS1,

390 had no effect in GLP-1 secretion. The effects on gut hormone secretion of other angiotensin  
391 derived peptides such as Ang III and Ang IV have not been studied and a possible function  
392 cannot be ruled out. However, candesartan, a specific antagonist for AT<sub>1</sub>, completely  
393 abolished Ang II-triggered GLP-1 and PYY secretion, emphasising the predominant role of  
394 this receptor for Ang II stimulated gut hormone release. In keeping with the known Gq-  
395 coupling of AT<sub>1</sub> in heterologous expression systems, we observed Ang II triggered Ca<sup>2+</sup>-  
396 responses that were maintained in the presence of extracellular Co<sup>2+</sup> - a treatment that  
397 eliminates Ca<sup>2+</sup>-rises downstream of voltage gated Ca<sup>2+</sup>-channels in L-cells (38). Consistent  
398 with these results, nifedipine, which blocks L-type voltage gated Ca<sup>2+</sup>-channels and inhibits  
399 GLP-1 secretion from L-cells (39), had no significant effect on Ang II stimulated secretion.  
400 Sensitivity of the secretory-response in L-cells to 2-APB, an inhibitor of IP<sub>3</sub>-receptors, is  
401 consistent with the recruitment of ER-stores, although we cannot exclude additional  
402 contributions from plasma membrane channels such as TRP-channels, a number of which are  
403 expressed in L-cells (38) sensitive to 2-APB.

#### 404 *Physiological relevance*

405 Our results suggest that physiological activation of the renin angiotensin system will be  
406 accompanied by increased colonic GLP-1 and PYY secretion, and are in keeping with our  
407 previous report that colonic L-cells are also activated by AVP. Whereas PYY likely exerts  
408 local actions on fluid secretion, it is not known whether Ang II-dependent stimulation of  
409 colonic L-cells would be sufficient to elevate circulating GLP-1 and PYY levels and trigger  
410 anorexigenic and insulinotropic responses. Chronic infusion of Ang II at a rate of 1.5  
411 µg/(kg\*min) has been shown to reduce food intake in C57B6 mice in a candesartan sensitive  
412 manner (40). While no changes in intestinal hormone mRNA expression were observed,  
413 circulating hormone levels were not reported. However, we failed to detect significant  
414 changes in plasma GLP-1 or PYY in response to intraperitoneal Ang II injection (100 µg/kg),  
415 a supraphysiological dose chosen ~3-20-fold in excess of doses previously reported to affect

416 taste behaviour (41) and blood pressure (42) in mice. While this might support the view that  
417 the well documented anorexic effects of Ang II are downstream of direct action in the central  
418 nervous system (43), it is also well known that anorexic effects of enteroendocrine hormones  
419 are at least in part mediated via afferent neuronal fibers, which could be stimulated by local  
420 elevations of gut hormones insufficient to raise plasma levels (2).

421 Despite the enrichment of AT<sub>1</sub> receptors in colonic L-cells and the finding that AT<sub>1</sub> receptor  
422 activation triggered GLP-1 and PYY release, these receptors would not seem a promising  
423 target for drug discovery in the field of L-cell secretagogues. Although it has been proposed  
424 that local AT<sub>1</sub> agonism in the jejunum might beneficially reduce SGLT1-mediated glucose  
425 absorption (37,44), the potential benefits of targeting intestinal AT<sub>1</sub> receptors do not weigh  
426 favourably against the evident clinical cardiovascular benefits of ACE inhibitors and  
427 angiotensin receptor blockers. Nevertheless, the finding that AVP and angiotensin receptors  
428 are highly enriched in colonic L-cells raises the concept of an important cross-talk between  
429 colonic enteroendocrine cells and fluid balance regulatory pathways, and raises interesting  
430 questions about the physiological control and functional roles of colonic hormones.

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432

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575 ***Figure Legends***

576 ***Figure 1: Angiotensin II type 1 receptor (AT1R) is highly and exclusively present in***  
577 ***colonic L-cells.*** Gene expression of Agtr1a, Agtr1b and Agtr2 was examined by (A)  
578 microarray analysis from FACS-sorted mouse K-cells (K), upper small intestinal  
579 (duodenal/jejunal) L-cells (LDJ) and colonic L-cells (LC), together with corresponding non-  
580 fluorescent control cells collected in parallel (CK, CDJ, CC, respectively) and by (B) RNA-

581 sequencing on FACS- sorted L-cells and controls from mouse duodenum/jejunum (LDJ,  
582 CDJ), jejunum/ileum (LJI, CJI) and colon (LC, CC). (C) Agtr1a expression was validated by  
583 q-RT PCR in mouse K-, L- and control cells. Data are presented as the geometric mean +  
584 upper SEM of the  $2\Delta\text{Ct}$  data ( $n \geq 3$  each). Comparisons between L-cells and controls were  
585 assessed on non-transformed  $\Delta\text{Ct}$  data using one-way ANOVA and post hoc Bonferroni  
586 analysis. \*\*\* $P < 0.001$ . (D) Representative photomicrograph demonstrating co-localisation of  
587 GLP-1 (green) and AT1R (red) in 4% PFA fixed human colon tissue section. Nuclei were  
588 visualised with Hoechst staining (blue). Scale bar is 10  $\mu\text{m}$ .

589 **Figure 2: Angiotensin II stimulates GLP-1 and PYY secretion from mouse and human**  
590 **colon cultures.** (A) GLP-1 secretion was measured from mouse mixed colon cultures  
591 incubated for 2 h in saline solution alone (Control; Con) or containing increasing  
592 concentrations of Ang II. (B) PYY secretion was measured from mixed cultures incubated  
593 with Ang II (10 nmol/L) or forskolin (10  $\mu\text{mol/L}$ ) plus IBMX (10  $\mu\text{mol/L}$ ) plus glucose (10  
594 mmol/L) (F/I/G). GLP-1 and PYY secretion is expressed as a percentage of total hormone  
595 content in each well. Similarly, GLP-1 (C) and PYY (D) secretion was measured from human  
596 colon cultures incubated with Ang II (10 nmol/L) or F/I/G. Results are shown as the mean +  
597 SEM of (A)  $n = 12$ , (B)  $n = 13-14$ , (C)  $n = 11-14$ , (D)  $n = 11-15$  wells with 3 or 4 wells  
598 originating from a single mouse or human tissue sample. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$   
599 compared to controls using one-way ANOVA followed by post hoc Bonferroni analysis on  
600  $\log_{10}$  transformed data. Active GLP-1 (E) and total PYY (F) levels were measured in plasma  
601 of mice that received a single intra-peritoneal injection of either Ang II (100  $\mu\text{g/kg}$ ) or PBS  
602 (vehicle). Mean  $\pm$  SEM from 6-7 mice per group are depicted.

603 **Figure 3: Antagonism of AT1 receptor reduces GLP-1 and PYY secretion from mouse**  
604 **colon cultures.** GLP-1 (A) and PYY (B) secretion was measured from colon cultures treated  
605 with Ang II (10 nmol/L) in the presence or absence of Candesartan cilexetil (Can. 1  $\mu\text{mol/L}$ ),  
606 a selective AT1 receptor antagonist. Where applicable, wells were pre-treated with Can. 30

607 min before the administration of Ang II. GLP-1 and PYY secretion is expressed as a  
608 percentage of total content. Results are shown as the mean + SEM; n = 9–12 wells with 3 or 4  
609 wells originating from a single mouse. \*\*\*P < 0.001 compared to controls or as indicated  
610 using one-way ANOVA followed by post hoc Dunnett's test or Bonferroni analysis on log10  
611 transformed data.

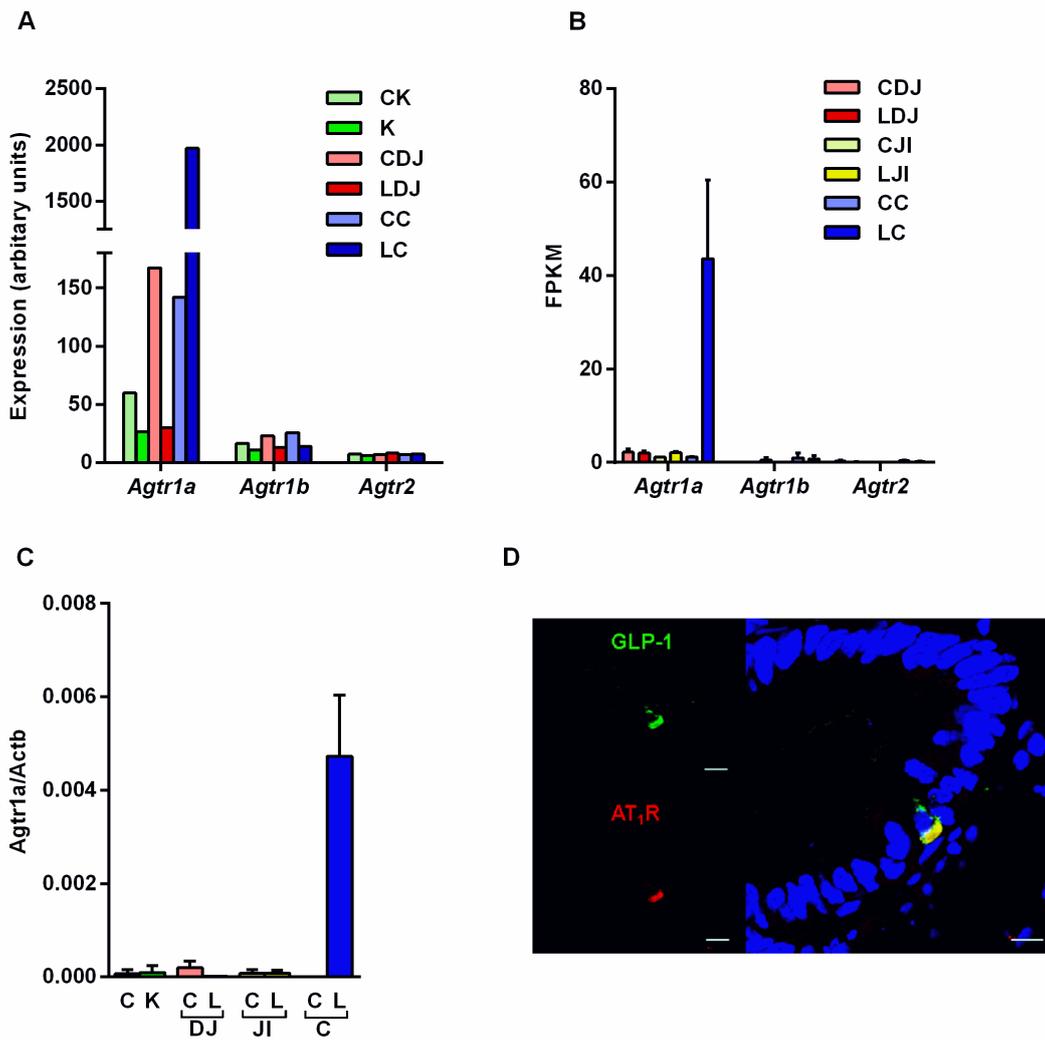
612 **Figure 4: Ang II elevates intracellular calcium responses in colonic L-cells.** (A) A  
613 representative trace showing calcium response to Ang II (10 nmol/L) in a L-cell from a mixed  
614 colon culture imaged by GCaMP3 fluorescence. (B) Mean normalised GCaMP3 fluorescence  
615 changes in L-cells exposed to two successive applications of Ang II, recorded as in (A). n = 6  
616 cells and results are the mean + SEM. \*\*\*P < 0.001 compared to baseline by one-sample  
617 Student's t test. (C) A representative trace showing calcium response to Ang II (10 nmol/L) in  
618 the presence of cobalt chloride (CoCl<sub>2</sub>; 5 mmol/L) to block voltage-gated calcium channels  
619 and (D) mean GCaMP3 fluorescence changes in L-cells in response to Ang II (10 nmol/L) in  
620 the presence of CoCl<sub>2</sub> (n = 12 cells). Results are shown as the mean + SEM. \*\*\*P < 0.001  
621 compared to baseline by a one-sample Student's t test. (E) GLP-1 secretion from mouse mixed  
622 colon cultures stimulated with Ang II (10 nmol/L) in the presence or absence of nifedipine  
623 (Nif, 10 μmol/L) or 2-APB (100 μmol/L). Where applicable, wells were pre-treated with  
624 nifedipine or 2-APB 30 min before the administration of Ang II. GLP-1 secretion is expressed  
625 as a percentage of total content. Results are shown as the mean + SEM; n = 10–12 wells with  
626 3 or 4 wells originating from a single mouse. \*\*\*P < 0.001 compared to controls or ##P < 0.01  
627 compared to Ang II alone as indicated, using one-way ANOVA followed by post hoc  
628 Dunnett's test or Bonferroni analysis on log10 transformed data.

629 **Figure 5: Ang (1-7) and the Mas-1 receptor are not involved in GLP-1 secretion.** Mas1  
630 receptor expression was analysed by (A) Microarray analysis on FACS-sorted K-, and L-  
631 cells from duodenum/jejunum (LDJ) and colon (LC) and respective control cells (CDJ, CC)  
632 and by (B) qRT-PCR on colonic L- and control cells. qRT-PCR data are presented as the

633 geometric mean + upper SEM of the  $2\Delta Ct$  data ( $n \geq 3$  each). (C) GLP-1 secretion was  
634 measured from mouse colon cultures in the presence of two concentrations of Ang (1-7).  
635 GLP-1 secretion is expressed as a percentage of total content. Results are shown as the mean  
636 + SEM;  $n = 9-12$  wells with 3 or 4 wells originating from a single mouse. Statistics were  
637 performed using one-way ANOVA followed by post hoc Dunnett's on  $\log_{10}$  transformed  
638 data.

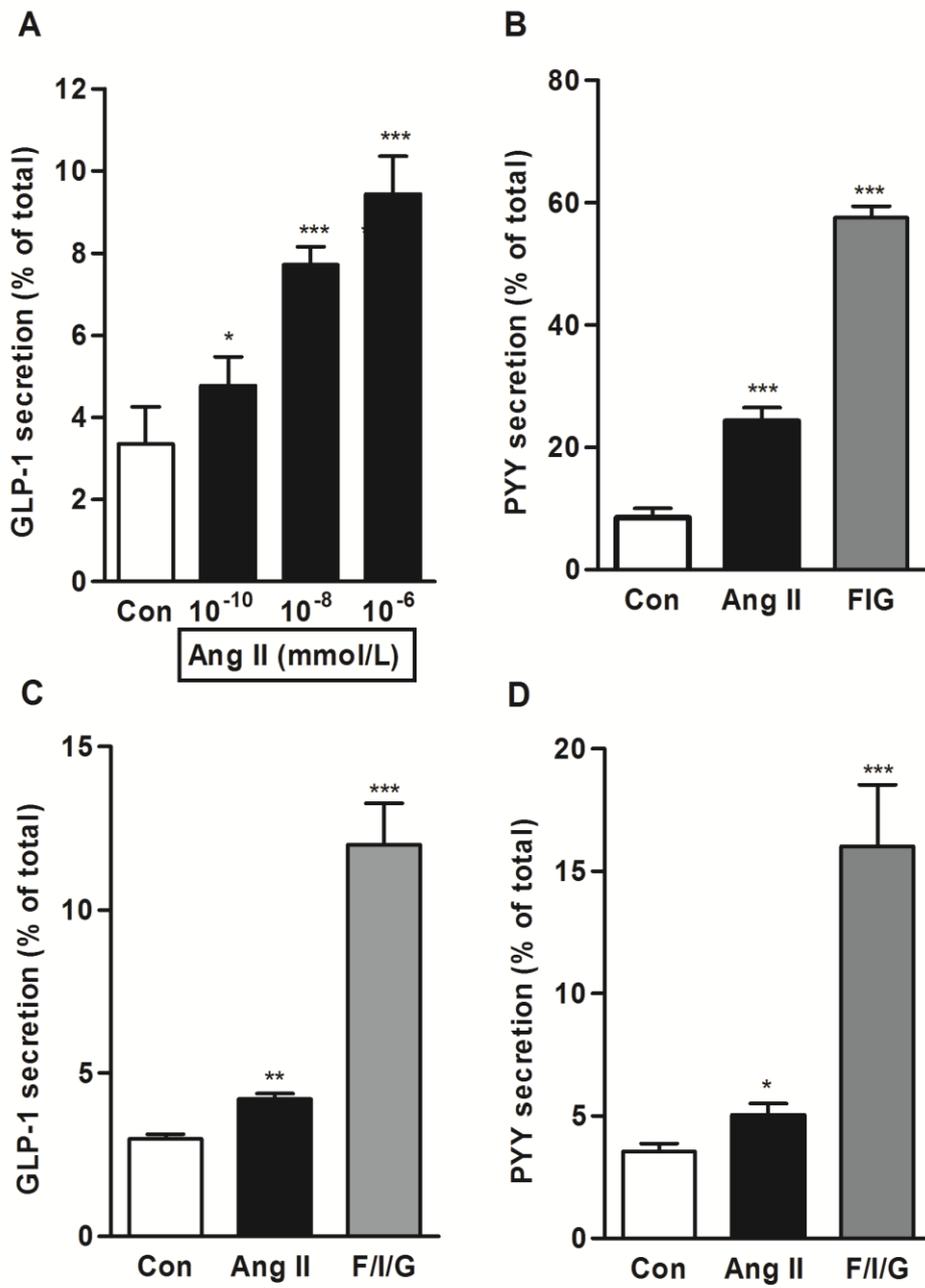
639 ***Figure 6: Ang II induced effect on short circuit current in mouse distal colon.*** (A) Example  
640 traces showing changes in short circuit current recordings (Isc) from mouse distal colon  
641 mounted in Ussing chambers after basolateral application of Ang II (1  $\mu\text{mol/L}$ ). (B) Isc  
642 changes from colon tissue as in (A), but in the additional presence of basolateral NPY1R  
643 antagonist BIBP3226 (1  $\mu\text{mol/L}$ ) (C) Isc changes from colon tissue as in (A), but in the  
644 additional presence of bilateral AT1R antagonist candesartan (1  $\mu\text{mol/L}$ ). (D) Mean changes  
645 in Isc, recorded as in A-C, after application of Ang II alone or in the presence of Amiloride  
646 (Amil. 5  $\mu\text{mol/L}$ ), BIBP3226 (BIBP) or Candesartan (Can).  $\Delta\text{Isc}$  was calculated as the  
647 difference between the means of short circuit currents from the 2-5 min period before and 30  
648 min period after the application of Ang II. Data are the mean + SEM from 4-5 tissue  
649 preparations for each condition, normalised for a surface area of 1  $\text{cm}^2$ . \* $p < 0.05$ , \*\* $p < 0.01$   
650 compared with Ang II application alone using one-way ANOVA followed by post hoc  
651 Bonferroni analysis on non-transformed data.

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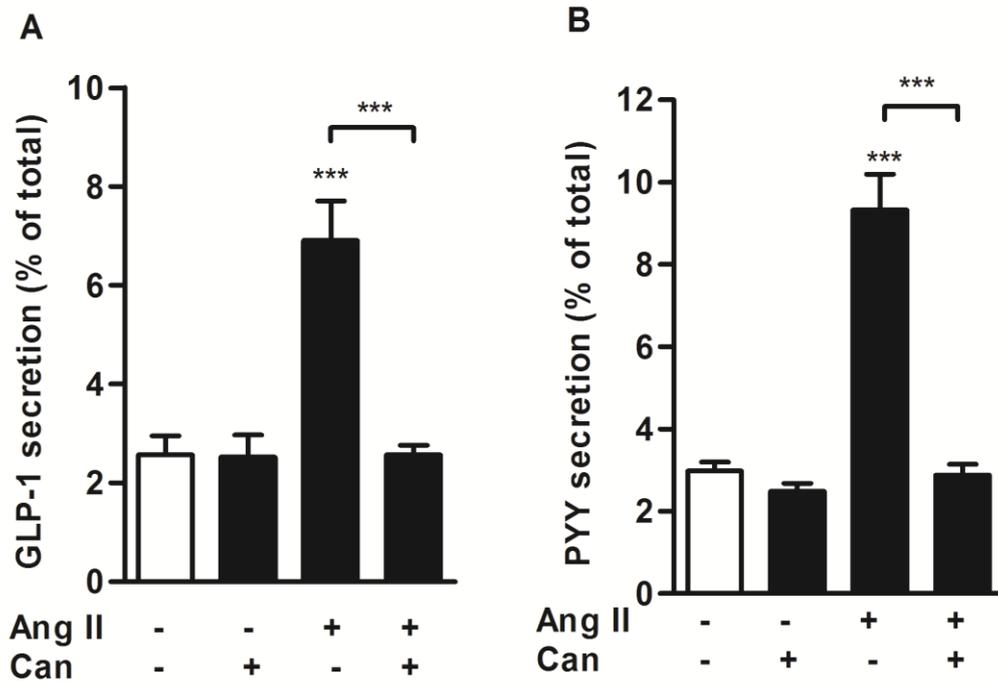
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657 **Figure 2**

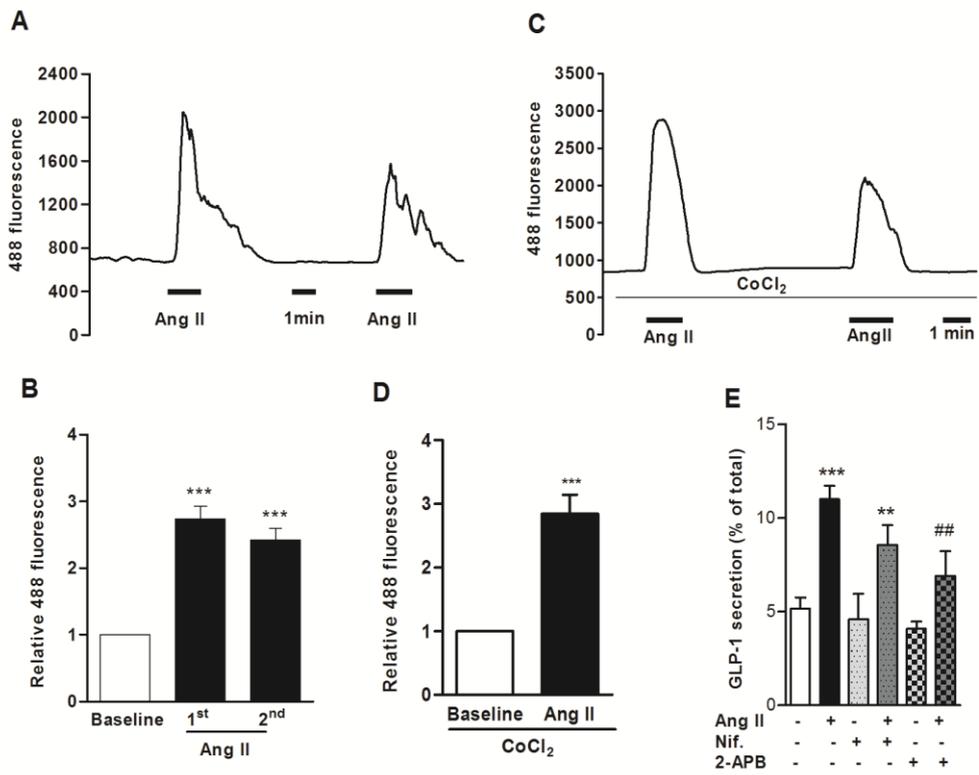


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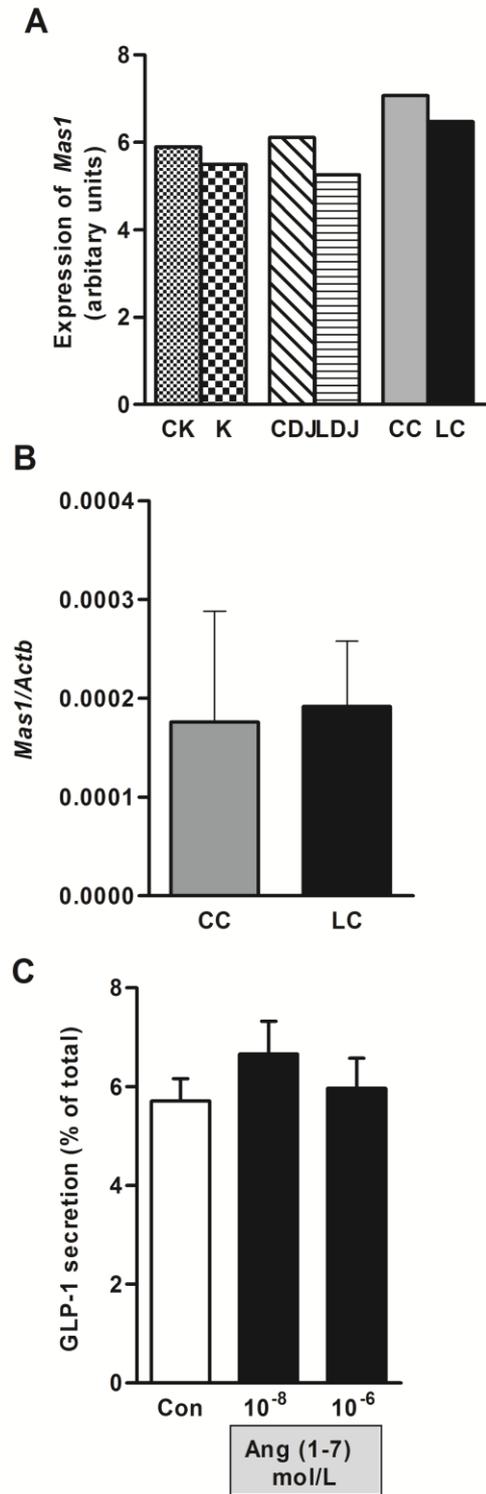
660 **Figure 3**



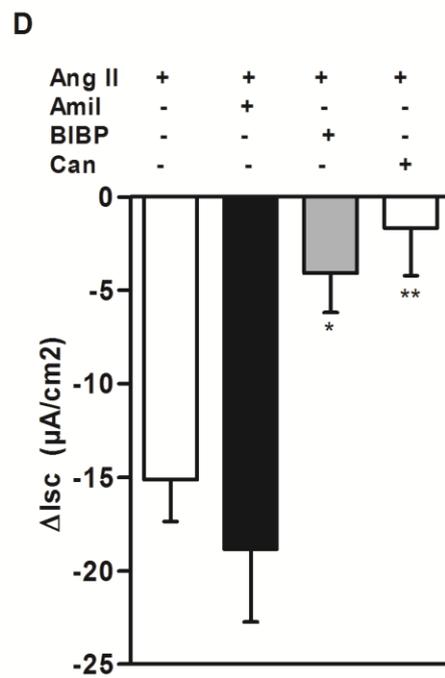
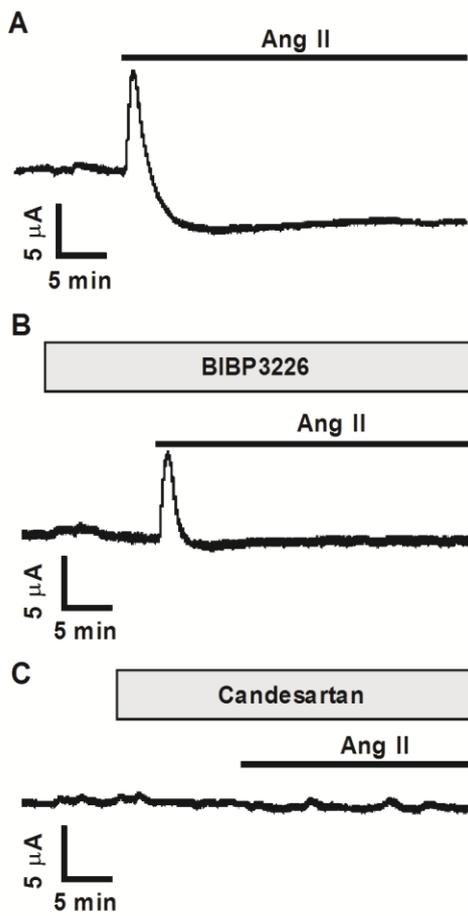
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667 **Figure 5**  
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672 **Antibody Table**  
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Peptide/protein target	Antigen sequence (if known)	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used
GLP-1	Not available	GLP-1 Antibody (C-17)	Santa Cruz Biotechnology, sc-7782	Goat, polyclonal	1:100
Angiotensin II type 1 receptor	Not available	AT <sub>1</sub> Antibody (306)	Santa Cruz Biotechnology, sc-579	Rabbit, polyclonal	1:100