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

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Abbreviated Title: Control of L-cells by Angiotensin II

Key terms: Renin-Angiotensin System (RAS); Angiotensin II; glucagon-like peptide-1 (GLP-1); L-cells; Peptide YY (PYY)

Word count: 4652

No of figures and tables: 6 figures, 0 tables

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Disclosure statement: The authors have nothing to disclose.

Abstract

Angiotensin II (Ang II) is the key hormone mediator of the renin angiotensin system which regulates blood pressure and fluid and electrolyte balance in the body. Here we report that in colonic epithelium the Ang II type 1 receptor (AT_1R) is highly and exclusively expressed in enteroendocrine L-cells which produce the gut hormones glucagon-like peptide-1 (GLP-1) and peptide YY (PYY). Ang II stimulated GLP-1 and PYY release from primary cultures of mouse and human colon, which was antagonised by the specific AT_1R receptor blocker candesartan. Ang II raised intracellular calcium levels in L-cells in primary cultures, recorded by live-cell imaging of L-cells specifically expressing the fluorescent calcium sensor GCaMP3. In Ussing chamber recordings, Ang II reduced short circuit currents in mouse distal colon preparations, which was antagonised by candesartan or a specific NPY1R receptor inhibitor but insensitive to amiloride. We conclude that Ang II stimulates PYY secretion, in turn inhibiting epithelial anion fluxes, thereby reducing net fluid secretion into the colonic lumen. Our findings highlight an important role of colonic L-cells in whole body fluid homeostasis by controlling water loss through the intestine.

Introduction

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

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

Several studies have identified different components of the RAS, including angiotensinogen, renin, ACE, Ang II and angiotensin receptors in the mucosal and muscular layers of the gastro-intestinal tract (7-10). Angiotensin receptors, particularly AT_1, have been implicated in

gut motility (11,12) and electrolyte absorption (13-15). Here we report that AT_1 is highly and selectively expressed in colonic L-cells, and is linked to the stimulation of PYY and GLP-1 secretion and colonic fluid balance.

Methods

Solutions and compounds

All compounds were purchased from Sigma Aldrich (Poole, U.K.) unless otherwise stated. BIBP 32267 trifluoroacetate was purchased from Bioquote (York, U.K.) and Angiotensin (1-7) from Bio-Techne (Abingdon, U.K.). The composition of the standard bath solution used in secretion and imaging experiments was: 4.5 mmol/L KCl, 138 mmol/L NaCl, 4.2 mmol/L $NaHCO_3$, 1.2 mmol/L NaH_2PO_4, 2.6 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$ and 10 mmol/L HEPES (adjusted to pH 7.4 with NaOH). For experiments where $CoCl_2$ was used, carbonates and phosphates were omitted from the saline buffer and the osmolarity was compensated with additional NaCl (143 mmol/L total). The composition of Ringer's solution used in Ussing chamber experiments was: 120 mmol/L NaCl, 3 mmol/L KCl, 0.5 mmol/L $MgCl_2$, 1.25 mmol/L $CaCl_2$, 23 mmol/L $NaHCO_3$ and 10 mmol/L glucose.

Animals and ethical approval

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

Blood was collected in tubes containing EDTA and protease inhibitors (10 µmol/L amastatin hydrochloride, 100 µmol/L diprotinin A, 18 µmol/L aprotinin), centrifuged at 13,000 g for 90 s, and plasma collected and used for active GLP-1 and total PYY analysis.

Transgenic Mice

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

Primary murine colonic crypt cultures

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

Preparation of crypt cultures from human colons

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

GLP-1 and PYY secretion assays

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

Calcium imaging

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

Microarray analysis and RNA sequencing

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

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

Quantitative RT-PCR

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

this study were from Applied Biosystems, *Agtr1*: Mm01957722_s1 and *Mas1*:Mm00434823_s1. All experiments were performed on at least three cDNAs isolated from one mouse each.

Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde, dehydrated in 15% and 30% sucrose, and frozen in optimal cutting temperature embedding media (CellPath, Newtown, U.K.). Cryostat-cut sections (6–10 μm) were mounted directly onto polylysine-covered glass slides (VWR, Leuven, Belgium). Slides were incubated for 1 h in blocking solution containing PBS/0.05% Triton X-100/10% donkey serum and overnight with primary antibodies (goat anti- GLP-1 (sc-7782) and rabbit anti-AT_1R (sc-579, Santa Cruz Biotechnology Inc, CA, USA) in blocking solution. Sections were rinsed with blocking solution before being incubated for 1 hour at room temperature with Alexa Fluor 488 (1:300) and Alexa Fluor 555 (1:300) secondary antibodies (Invitrogen) and Hoechst (1:1300) for nuclear staining. Control sections were stained with secondary antibodies alone. Sections were mounted with Prolong Gold (Life Technologies) before being imaged by confocal microscopy (Leica TCS SP8, Milton Keynes, U.K.).

Ussing Chamber recordings

The most distal part of the colon (~1.25 cm) was cut open longitudinally and rinsed in Ringer's solution. Serosa and most of the outer muscular layer were removed by fine forceps. The tissue was mounted in an Ussing chamber (EM-LVSYS-4 system with P2400 chambers and P2404 sliders, all from Physiologic Instruments, San Diego, CA, USA). Only one preparation was used from each animal. The active epithelial surface was 0.25 cm^2. Both parts of the Ussing chambers were filled with 3 ml of Ringer's solution, maintained at 37^oC and continuously bubbled with 5% vol/vol CO_2/ 95% vol/vol O_2. The transepithelial potential

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

Statistics

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

Results

AT1 receptor expression in mouse and human colonic L-cells

Ang II interacts with two seven-transmembrane G-protein coupled receptors, AT_1 and AT_2. Whereas rodents possess two AT_1 receptor isoforms, AT_{1A} and AT_{1B} (encoded by *Agtr1a* and *Agtr1b*, respectively) (20) humans have only one type 1 receptor gene. Microarray analysis was performed to compare the expression of *Agtr1a*, *Agtr1b* and *Agtr2* in primary murine glucose-dependent insulinotropic polypeptide (GIP) secreting K-cells as well as L-cells from the duodenum/jejunum (top 10 cm of the small intestine (LD) or the colon. As shown in Figure 1A, *Agtr1a* expression was ~100-fold higher in colonic (LC) than upper SI L-cells (LDJ), and 14-fold enriched in colonic L-cells (LC) over non-L-cells (CC). *Agtr1b* and *Agtr2* were poorly expressed in all cell populations examined (Figure 1A). RNA-sequencing confirmed the high selective expression of *Agtr1a* in colonic L-cells (LC, Figure 1B). Microarray and RNA-seq results were also validated by quantitative PCR, performed on cDNA prepared from independently FACS-sorted L- and non L-cells from the upper SI (duodenum/jejunum, LDJ), the lower SI (jejunum/ileum, LJI) and colon (LC) as well as K-cells and non K-cells. By q-PCR, *Agtr1a* was highly enriched in colonic L-cells (LC) over colonic control cells (CC), and was found at much lower levels in small intestinal epithelial control (CDJ, CJI) and L-cells (LDJ, LJI) and K- and non-K-cells (CK) (Figure 1C).

In human colon tissue sections, AT_1 immuno-positive cells were found scattered through the epithelium and co-stained with antibodies against GLP-1(Fig 1D). No visible staining for AT_1 was detected in GLP-1 negative cells of the epithelial layer. Some cells in the lamina propria showed AT_1 reactivity, but their identity was not further investigated (data not shown).

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

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

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

GLP-1 and PYY secretion is mediated by AT_1 receptor

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

Ang II induced intracellular calcium responses in colonic L-cells

Previous studies have revealed that AT_1 receptors activation recruits phospholipase C and stimulates the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3), promoting calcium release from internal stores (22,23). Depending on the cell or tissue type, it was also reported that Ang II inhibits adenylate cyclase and lowers intracellular cAMP levels (24,25). To elucidate the mechanistic pathway involved in Ang II-triggered GLP-1 and PYY release, we monitored the changes in intracellular calcium in primary colonic L-cells identified in cultures from GLU-

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

Intracellular calcium can be increased either by opening of plasma membrane calcium channels or by release from intracellular calcium stores. To establish whether the Ang II dependent cytoplasmic calcium rise was due to calcium release from intracellular endoplasmic reticulum stores or the opening of plasma membrane voltage-gated calcium channels, calcium imaging experiments were performed in the presence of cobalt chloride ($CoCl_2$), a general voltage gated calcium channel blocker that impairs L-cell calcium responses to depolarising stimuli such as KCl (26). Cytoplasmic calcium responses to Ang II were still observed in the presence of $CoCl_2$ (5 mmol/l) (Fig 4C and D), suggesting they do not depend on voltage gated calcium channels. This is consistent with the reported Gq coupled nature of AT_1 (27).Further corroborating the results obtained with calcium imaging experiments, the L-type voltage gated calcium channel blocker nifedipine (10µmol/L) did not significantly inhibit Ang II stimulated GLP-1 secretion (Fig4E), but GLP-1 responses to Ang II were blocked by 2-aminoethoxydiphenylborate (2- APB, 100µmol/L), an inhibitor of IP3 receptors (Fig 4E).

Non classical RAS and L-cells

Whereas the classical RAS (ACE-Ang II-AT_1) promotes actions to maintain blood pressure, a 'non-classical' RAS, consisting of ACE2-Ang (1-7)-Mas1 receptor has opposing effects (28). Ang (1-7) is generated by the cleavage of an amino acid from the carboxy-terminus of Ang II by an ACE homologue ACE2 and mediates vasodilatory/diuretic actions through the AT_7/Mas1 receptor (29). Microarray (Fig 5A) and RNA-seq analysis (data not shown) for *Mas1* receptor expression were performed on K- and L-cells from mouse upper SI (LDJ) and colon (LC) and their respective control cells (CK, CDJ, CC). *Mas1* expression was very low

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

Antisecretory effect of Ang II in mouse colon

Given the well-known inhibitory effect of PYY on intestinal anion and water secretion (4,5,30-32), we employed Ussing chambers to study the functional relevance of Ang II in mouse colon. In all tissue preparations tested, basolateral addition of Ang II (10^{-6} mol/L) caused a sustained depression in Isc (of mean 15.1 $\mu A/cm^2$) lasting for at least 35 minutes (Fig 6A). In 3/5 preparations we also observed a transient increase in Isc, with a peak increase of 40.6 ± 10.1 $\mu A/cm^2$ (Fig 6A), but this was absent in the other 2 preparations (not shown). Pre-treatment with apically-added amiloride (5 µmol/L) alone decreased Isc, which came to a new plateau 9.6 ± 9.1 $\mu A/cm^2$ lower than the Isc before amiloride addition. Subsequent application of Ang II 10-12 minutes after amiloride pre-treatment caused further Isc depression, which was not different from the response caused by Ang II without any pre-treatment (Fig 6D). These results suggest that the Ang II-related Isc decrease was due to inhibition of electrogenic anion secretion and did not involve ENaC-dependent sodium absorption. Pre-treatment with basolaterally-added BIBP3226 (BIBP, a specific NPY1R antagonist) caused an increase of Isc, with the new plateau being 1.8 ± 2.7 $\mu A/cm^2$ higher than before BIBP addition. The inhibitory Isc response to Ang II applied 10-12 minutes after BIBP was significantly impaired, confirming a role of the PYY receptor NPY1R in Ang II mediated changes of colonic transepithelial ion movement (Fig 6B and 6D). Candesartan (10^{-6} mol/L bilaterally) reduced the basal Isc by1.9 ± 2.8 $\mu A/cm^2$, and abolished any subsequent responses to Ang II application, confirming the role of AT_1 in Ang II stimulated Isc changes (Fig 6C and 6D).

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

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

Discussion

Digestion and absorption of nutrients from the intestine depends on sufficient availability of water in the lumen. Indeed, in addition to the average ingested fluid volume of ~2.5 litres per day in humans, it has been estimated that 5-10 litres of water are secreted into the gut lumen and re-absorbed to aid intestinal processes (33), necessitating a close link between the gut and systems regulating body fluid and electrolyte homeostasis. Here we identified AT_1 in colonic L-cells and demonstrated that its activation by Ang II triggered GLP-1 and PYY secretion and downstream PYY-dependent inhibition of anion secretion. This offers a potential explanation for previous reports that colonic fluid secretion is regulated by the renin angiotensin system (15).

The effect of Ang II on intestinal water and ion absorption has been studied extensively in the rat. At low physiological concentrations Ang II stimulates water absorption in the jejunum and colon, although higher doses were also reported to inhibit absorption (15). In the jejunum the pro-absorptive effect of Ang II was linked to the activation of noradrenergic nerve endings, based on the sensitivity of the response to α-adrenergic antagonists (34). Early investigations concluded that the proabsorptive effects of Ang II are predominantly mediated by electroneutral mechanisms (13), but experiments on rat descending colon mounted in Ussing chambers revealed a reduction of I_{SC} over a wide Ang II concentration range (10^{-9} – 10^{-5} mol/L) (14). This was sensitive to the chloride channel blocker, diphenylamine-2-carboxylate (DPC) but not to amiloride, suggesting that the action of Ang II on Isc is mediated through inhibition of anion secretion rather than stimulation of electrogenic sodium absorption (14). Our observed decrease in Isc in the colon is in agreement with these results and is clearly mediated via AT_1 as it was sensitive to candesartan. In about half the

preparations, we also observed a transient increase in Isc – this might also be downstream of AT_1 as it was never observed in the presence of candesartan, but in contrast to the sustained Isc reduction, it was not affected by the Y1-receptor antagonist BIBP3226. Our finding that the sustained reduction in Isc by Ang II was sensitive to BIBP3226 and insensitive to amiloride suggests that this effect lies downstream of PYY secretion. PYY, in addition to slowing gastric emptying and reducing hunger, is well recognised as an inhibitor of anion and electrolyte secretion (35), exerting its inhibitory action mainly via Y1 receptors on enterocytes and to some extent by Y2 receptors on enteric neurons (31). Activation of the Gi/Go coupled Y1 receptor lowers intracellular enterocyte cAMP levels, subsequently inhibiting CFTR channels, and thereby reduces anion secretion into the gut lumen (4).

Previous studies have shown that AT_1 is the predominant Ang II receptor in the muscularis of rat ileum and colon (7), submucosal plexus in guinea pig distal colon (36), vessel walls, myofibroblasts, and macrophages in the lamina propria, crypt bases and surface epithelium in human colon (9), as well as a subset of human jejunal cells resembling enteroendocrine cells (37). Our data contrast with the previously-reported detection of AT_1 in jejunal enterocytes (37), as we found only very low mRNA expression in the non-L-cell population of the mouse small intestine, which would be dominated by enterocytes. While this might reflect species differences, we also observed clear AT_1 staining in human colonic L-cells but not enterocytes. The fact that we were able to block the sustained drop of short circuit current observed in Ussing chamber mounted colonic tissue in response to Ang II with the Y1R-blocker BIBP3226 is consistent with the observed restriction of AT_1 to L-cells in the murine colon and an important role of L-cells in the secretory responses of the colon to Ang II.

Although Ang II could in principle also exert some of its effects through other receptors, we were unable to demonstrate a role of other angiotensin receptors in L-cells. mRNAs encoding both AT_2, which has a similar affinity for Ang II as AT_1, and the MAS1 receptor were only expressed at very low levels, barely detectable by RT-PCR. Ang (1-7), the ligand for MAS1,

had no effect in GLP-1 secretion. The effects on gut hormone secretion of other angiotensin derived peptides such as Ang III and Ang IV have not been studied and a possible function cannot be ruled out. However, candesartan, a specific antagonist for AT_1, completely abolished Ang II-triggered GLP-1 and PYY secretion, emphasising the predominant role of this receptor for Ang II stimulated gut hormone release. In keeping with the known Gq-coupling of AT_1 in heterologous expression systems, we observed Ang II triggered Ca^{2+}-responses that were maintained in the presence of extracellular Co^{2+} - a treatment that eliminates Ca^{2+}-rises downstream of voltage gated Ca^{2+}-channels in L-cells (38). Consistent with these results, nifedipine, which blocks L-type voltage gated Ca^{2+}-channels and inhibits GLP-1 secretion from L-cells (39), had no significant effect on Ang II stimulated secretion. Sensitivity of the secretory-response in L-cells to 2-APB, an inhibitor of IP_3-receptors, is consistent with the recruitment of ER-stores, although we cannot exclude additional contributions from plasma membrane channels such as TRP-channels, a number of which are expressed in L-cells (38) sensitive to 2-APB.

Physiological relevance

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

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

Despite the enrichment of AT_1 receptors in colonic L-cells and the finding that AT_1 receptor activation triggered GLP-1 and PYY release, these receptors would not seem a promising target for drug discovery in the field of L-cell secretagogues. Although it has been proposed that local AT_1 agonism in the jejunum might beneficially reduce SGLT1-mediated glucose absorption (37,44), the potential benefits of targeting intestinal AT_1 receptors do not weigh favourably against the evident clinical cardiovascular benefits of ACE inhibitors and angiotensin receptor blockers. Nevertheless, the finding that AVP and angiotensin receptors are highly enriched in colonic L-cells raises the concept of an important cross-talk between colonic enteroendocrine cells and fluid balance regulatory pathways, and raises interesting questions about the physiological control and functional roles of colonic hormones.

Funding

This work was funded by grants from the Wellcome Trust (106262/Z/14/Z, 106263/Z/14/Z), the MRC Metabolic Diseases Unit (MRC_MC_UU_12012/3, MRC_MC_UU_12012/5) and Full4Health (FP7/2011-2015, grant agreement no 266408).

Acknowledgments

GLP-1 and PYY immuno-assays were performed by the Core Biochemical Assay Laboratory. Cell sorting was conducted by Reiner Schulter and colleagues at the Cambridge Institute for Medical Research. The Human Research Tissue Bank is supported by the NIHR Cambridge Biomedical Research Centre.

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Figure Legends

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

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

Figure 2: Angiotensin II stimulates GLP-1 and PYY secretion from mouse and human colon cultures. (A) GLP-1 secretion was measured from mouse mixed colon cultures incubated for 2 h in saline solution alone (Control; Con) or containing increasing concentrations of Ang II. (B) PYY secretion was measured from mixed cultures incubated with Ang II (10 nmol/L) or forskolin (10 μmol/L) plus IBMX (10 μmol/L) plus glucose (10 mmol/L) (F/I/G). GLP-1 and PYY secretion is expressed as a percentage of total hormone content in each well. Similarly, GLP-1 (C) and PYY (D) secretion was measured from human colon cultures incubated with Ang II (10 nmol/L) or F/I/G. Results are shown as the mean + SEM of (A) n = 12, (B) n = 13-14, (C) n= 11-14, (D) n=11-15 wells with 3 or 4 wells originating from a single mouse or human tissue sample. *$P<0.05$, **$P<0.01$, ***$P < 0.001$ compared to controls using one-way ANOVA followed by post hoc Bonferroni analysis on log10 transformed data. Active GLP-1 (E) and total PYY (F) levels were measured in plasma of mice that received a single intra-peritoneal injection of either Ang II (100 μg/kg) or PBS (vehicle). Mean ± SEM from 6-7 mice per group are depicted.

Figure 3: Antagonism of AT1 receptor reduces GLP-1 and PYY secretion from mouse colon cultures. GLP-1 (A) and PYY (B) secretion was measured from colon cultures treated with Ang II (10 nmol/L) in the presence or absence of Candesartan cilexetil (Can. 1 μmol/L), a selective AT1 receptor antagonist. Where applicable, wells were pre-treated with Can. 30

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

Figure 4: Ang II elevates intracellular calcium responses in colonic L-cells. (A) A representative trace showing calcium response to Ang II (10 nmol/L) in a L-cell from a mixed colon culture imaged by GCaMP3 fluorescence. (B) Mean normalised GCaMP3 fluorescence changes in L-cells exposed to two successive applications of Ang II, recorded as in (A). n = 6 cells and results are the mean + SEM. ***P < 0.001 compared to baseline by one-sample Student's t test. (C) A representative trace showing calcium response to Ang II (10 nmol/L) in the presence of cobalt chloride (CoCl2; 5 mmol/L) to block voltage-gated calcium channels and (D) mean GCaMP3 fluorescence changes in L-cells in response to Ang II (10 nmol/L) in the presence of CoCl2 (n = 12 cells). Results are shown as the mean + SEM. ***P < 0.001 compared to baseline by a one-sample Student's t test. (E) GLP-1 secretion from mouse mixed colon cultures stimulated with Ang II (10 nmol/L) in the presence or absence of nifedipine (Nif, 10 µmol/L) or 2-APB (100 µmol/L). Where applicable, wells were pre-treated with nifedipine or 2-APB 30 min before the administration of Ang II. GLP-1 secretion is expressed as a percentage of total content. Results are shown as the mean + SEM; n = 10–12 wells with 3 or 4 wells originating from a single mouse. ***P < 0.001 compared to controls or ##P <0.01 compared to Ang II alone as indicated, using one-way ANOVA followed by post hoc Dunnett's test or Bonferroni analysis on log10 transformed data.

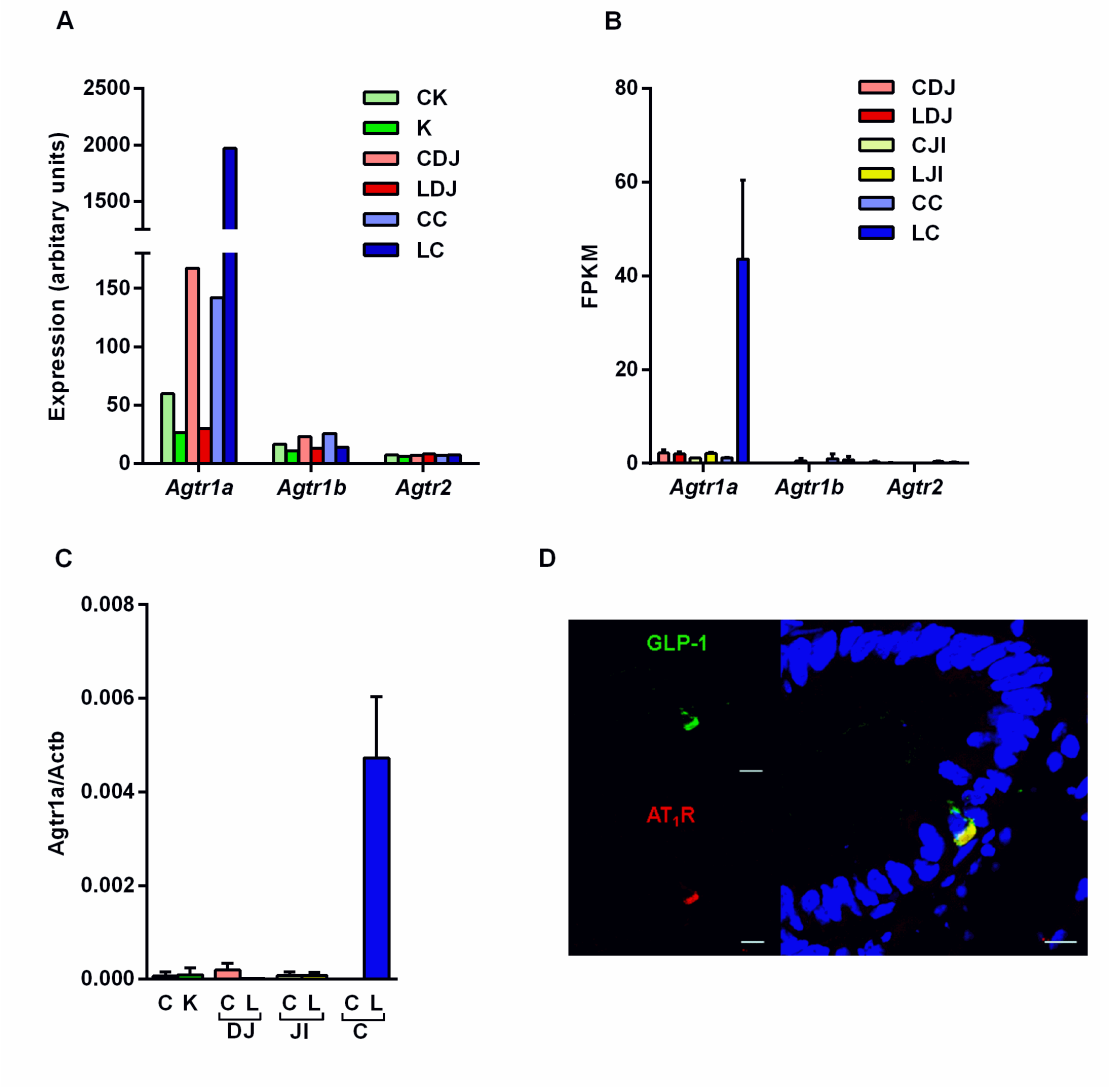
Figure 5: Ang (1-7) and the Mas-1 receptor are not involved in GLP-1 secretion. Mas1 receptor expression was analysed by (A) Microarray analysis on FACS-sorted K-, and L-cells from duodenum/jejunum (LDJ) and colon (LC) and respective control cells (CDJ, CC) and by (B) qRT-PCR on colonic L- and control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

633 geometric mean + upper SEM of the 2ΔCt data (n ≥ 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 log10 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 µmol/L). (B) Isc
642 changes from colon tissue as in (A), but in the additional presence of basolateral NPY1R
643 antagonist BIBP3226 (1 µmol/L) (C) Isc changes from colon tissue as in (A), but in the
644 additional presence of bilateral AT1R antagonist candesartan (1 µmol/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 µmol/L), BIBP3226 (BIBP) or Candesartan (Can). ΔIsc 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 cm2. *$p < 0.05$, **$p < 0.01$
650 compared with Ang II application alone using one-way ANOVA followed by post hoc
651 Bonferroni analysis on non-transformed data.

652

Figure 1


A
Expression (arbitary units)
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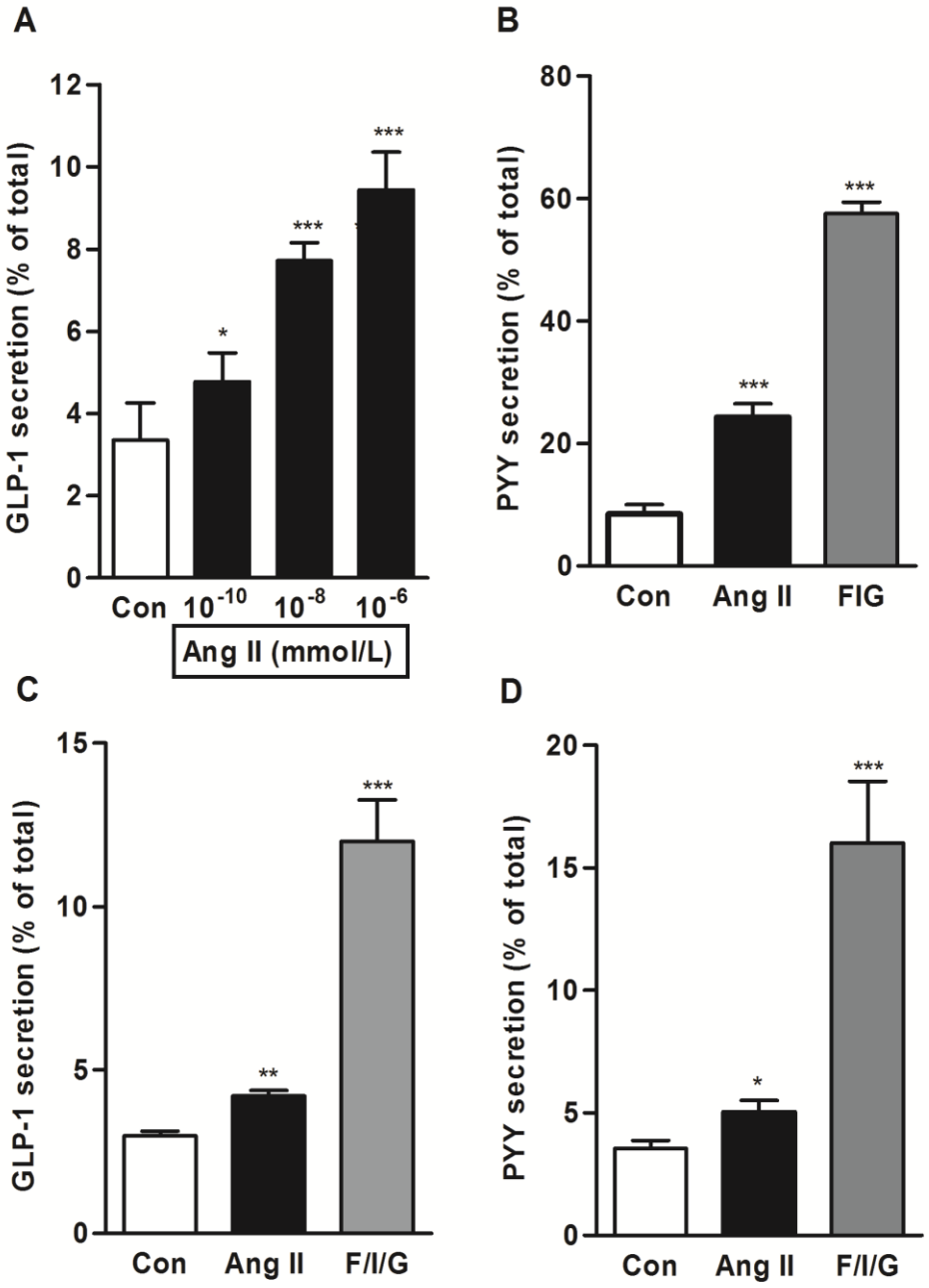
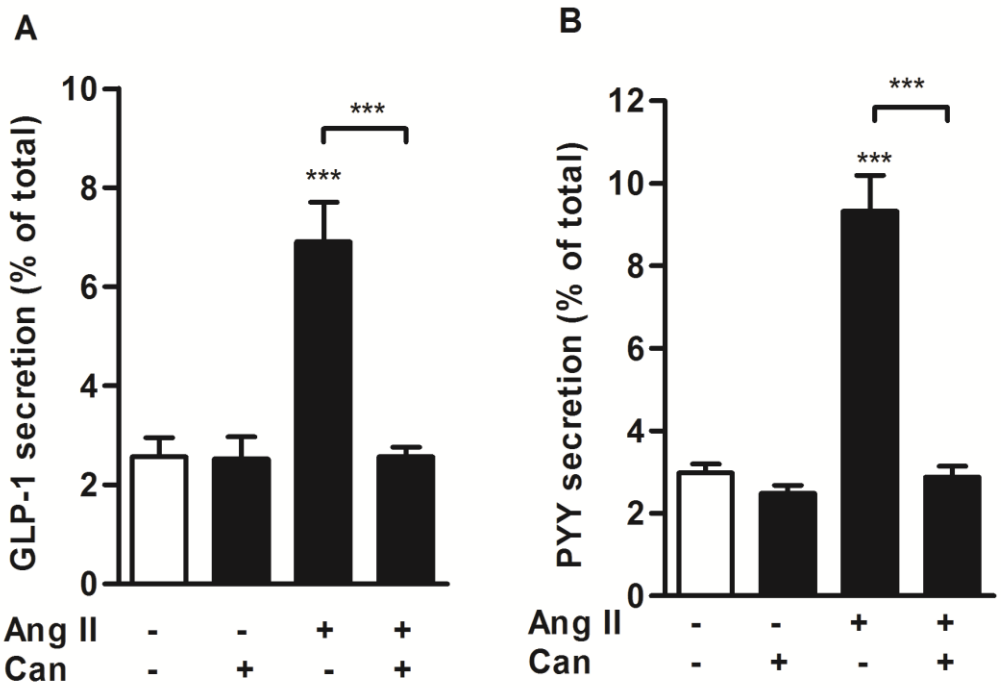
2000
1500
150
100
50
0
CK
K
CDJ
LDJ
CC
LC
Agtr1a
Agtr1b
Agtr2
B
FPKM
80
60
40
20
0
CDJ
LDJ
CJI
LJI
CC
LC
Agtr1a
Agtr1b
Agtr2
C
Agtr1a/Actb
0.008
0.006
0.004
0.002
0.000
C K
C L
DJ
C L
JI
C L
C
D
GLP-1
AT1R

Figure 2


A
GLP-1 secretion (% of total)
12
10
8
6
4
2
0
*
***
***
Con
10-10
10-8
10-6
Ang II (mmol/L)
B
PYY secretion (% of total)
80
60
40
20
0
***
***
Con
Ang II
FIG
C
GLP-1 secretion (% of total)
15
10
5
0
**
***
Con
Ang II
F/I/G
D
PYY secretion (% of total)
20
15
10
5
0
*
***
Con
Ang II
F/I/G

660 **Figure 3**



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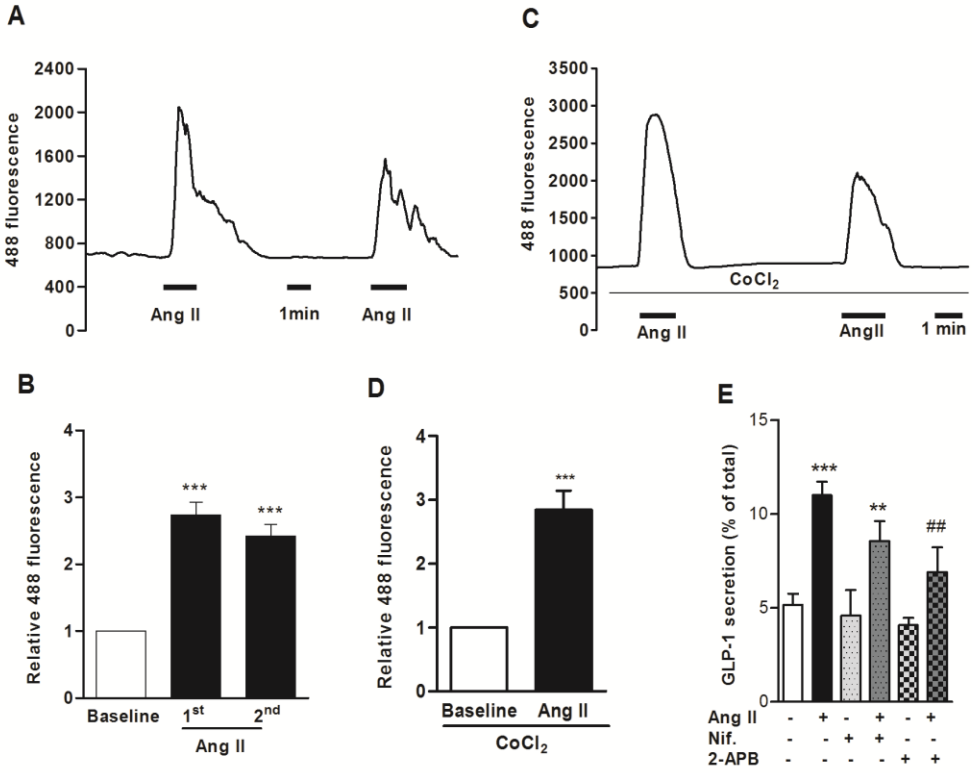
663 **Figure 4**



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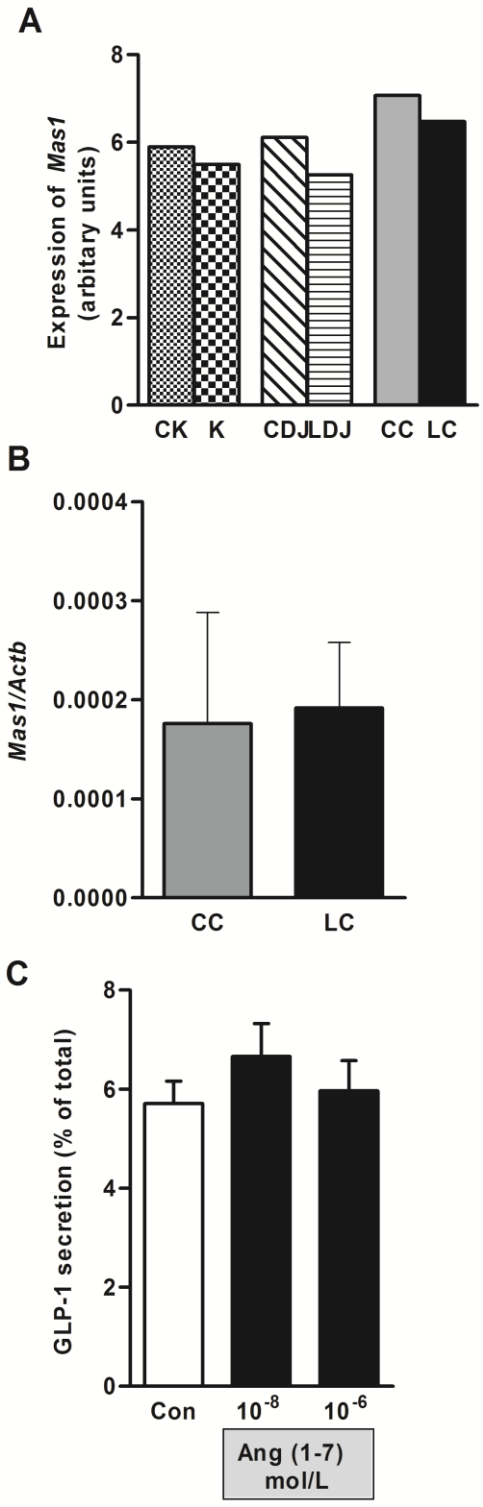
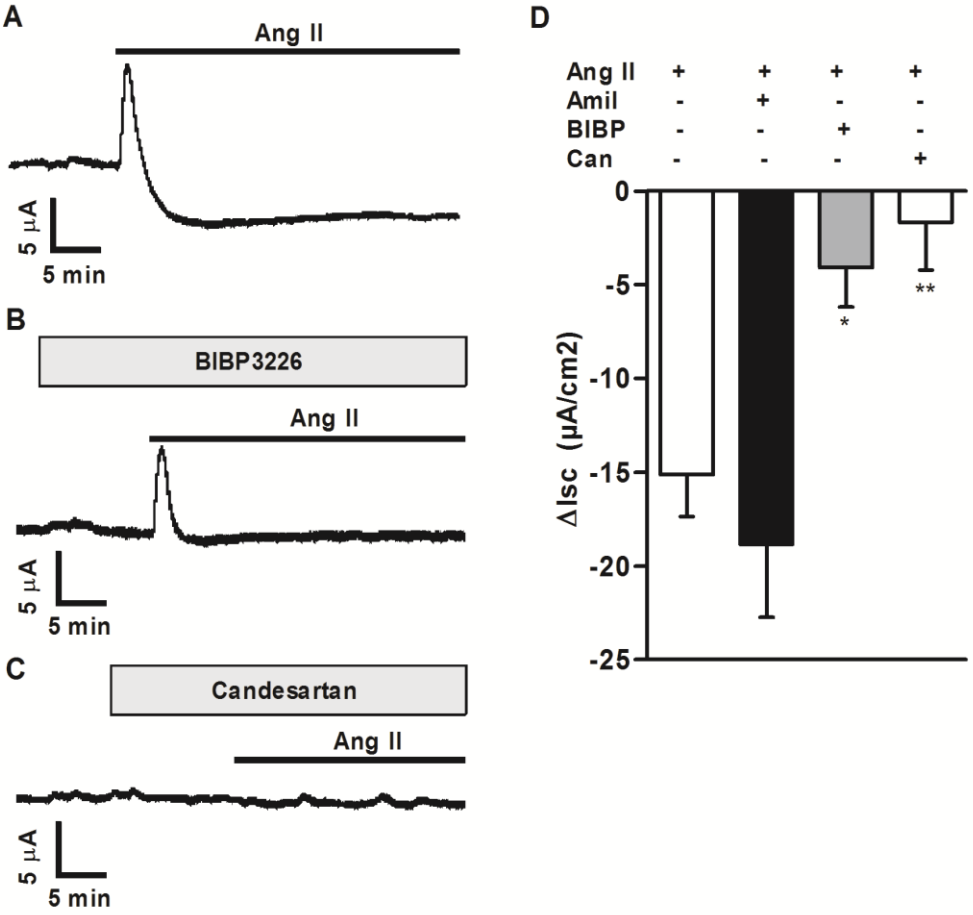


667 **Figure 5**
668

669 **Figure 6**



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671

Antibody Table

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 Biotrechnology, sc-7782	Goat, polyclonal	1:100
Angiotenin II type 1 receptor	Not available	AT_1 Antibody (306)	Santa Cruz Biotrechnology, sc-579	Rabbit, polyclonal	1:100