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Mode of action of ANG II on ion transport in guinea pig distal colon

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Hosoda, Yutaka, Adi Winarto, Toshihiko Iwanaga, and Atsukazu Kuwahara. Mode of action of ANG II on ion transport in guinea pig distal colon. *Am J Physiol Gastrointest Liver Physiol* 278: G625–G634, 2000.—The effect of ANG II on mucosal ion transport and localization of ANG type 1 receptor (AT_1R) in the guinea pig distal colon was investigated. Submucosal/mucosal segments were mounted in Ussing flux chambers, and short-circuit current (I_{sc}) was measured as an index of ion transport. Serosal addition of ANG II produced a concentration-dependent (10^{-9}–10^{-5} M) increase in I_{sc}. The maximal response was observed at 10^{-6} M; the increase in I_{sc} was 164.4 ± 11.8 μA/cm^2. The ANG II (10^{-6} M)-evoked response was mainly due to Cl^- secretion. Tetrodotoxin, atropine, the neurokinin type 1 receptor antagonist FK-888, and piroxicam significantly reduced the ANG II (10^{-6} M)-evoked response to 28, 45, 58, and 16% of control, respectively. Pretreatment with prostaglandin E_2 (10^{-5} M) resulted in a threefold increase in the ANG II-evoked response. The AT_1R antagonist FR-130739 completely blocked ANG II (10^{-6} M)-evoked responses, whereas the ANG type 2 receptor antagonist PD-123319 had no effect. Localization of AT_1R was determined by immunohistochemistry. In the immunohistochemical study, AT_1R-immunopositive cells were distributed clearly in enteric nerves and moderately in surface epithelial cells. These results suggest that ANG II-evoked electrogenic Cl^- secretion may involve submucosal cholinergic and tachykinergic neurons and prostanoid synthesis pathways through AT_1R on the submucosal plexus and surface epithelial cells in guinea pig distal colon.

substance P; prostaglandin; local renin-angiotensin system; angiotensin type 1 receptor; neurokinin type 1 receptor

THE RENIN-ANGIOTENSIN SYSTEM is a major regulatory mechanism for plasma electrolyte concentrations and blood pressure control. The system is activated by extracellular volume depletion resulting from hyponatremia, dehydration, or hemorrhage. These stimuli trigger renin release from the kidneys, which cleaves the circulating angiotensinogen to ANG I. Angiotensin-converting enzyme (ACE) removes two COOH-terminal amino acids from ANG I, producing a biologically active octapeptide, ANG II. ANG II can be further degraded by an aminopeptidase-induced deletion of an NH_2-terminal residue to yield ANG III.

The cloning of renin, angiotensinogen, ACE, and angiotensin receptor genes has demonstrated the widespread presence of the renin-angiotensin system in brain, heart, kidney, adrenal, and adipose tissue (3, 4, 14, 16, 27, 37). These results suggest other functions for ANG II in addition to those in the circulatory system. Two angiotensin receptors, the angiotensin type 1 receptor (AT_1R) and the angiotensin type 2 receptor (AT_2R), have been cloned (23, 24, 29, 39, 45). These reports suggest that AT_1R is widely distributed throughout the body, predominantly regulating body fluid volume, whereas AT_2R is found in minor locations and its function is not well known.

In the gastrointestinal tract, the distribution of ANG II receptors and ACE has been mapped by in vitro autoradiography, and ANG II has been shown to be present in rat intestine (15). Sechi et al. (41) reported that ANG II receptors are present in rat colonic mucosa and that the predominant receptor subtype is AT_1R. Angiotensinogen mRNA was detected in rat mesentery and large intestines (3, 37). The existence of renin in the gastrointestinal tract has not been reported; however, a recent report has shown that serine proteinases, such as kallikrein, tonin, and cathepsin G, have renin activity and directly generate ANG II (37). Kallikrein and kallikrein-like enzyme immunoreactivity has been shown to be localized in goblet (or mucous) cells and mast cells of rat small and large intestines (40). Therefore, it is likely that ANG II is normally produced in intestinal tissues and may act as a local mediator in the control of intestinal functions.

The reported effects of ANG II on epithelial ion transport in the intestine vary. It has been reported that intravenous injection of a low dose of ANG II ($\leq$7.0 ng·kg^{-1}·min^{-1}) stimulated Na^+ and water absorption in rat small and large intestines through extrinsic nerves, whereas a high dose of ANG II (>7.0 ng·kg^{-1}·min^{-1}) inhibited Na^+ absorption or promoted secretory processes by the production of prostaglandin (33, 34). In an in vitro Ussing flux chamber experiment, it was shown that serosal application of ANG II (10^{-10}–10^{-5} M) induced an increase in short-circuit current (I_{sc}) in rat jejunum by stimulating electrogenic Cl^- secretion, whereas the addition of the same concentration of ANG II evoked a decrease in I_{sc} in rat colon (9). Hatch et al. (21, 22) showed that, in rat colon, serosal application of ANG II (10^{-9}–10^{-5} M) induced net K^+ secretion, whereas the concentration of ANG II (10^{-4}

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M) additionally induced net Cl^- secretion, and that these secretory effects were inhibited by AT_1R antagonist. Jin et al. (26) reported that a low dose of ANG II given in intravenous infusion (0.7 $pmol \cdot kg^{-1} \cdot min^{-1}$) stimulates net Na^+ absorption through AT_2R-mediated cGMP activation, whereas a high dose of ANG II (700 $pmol \cdot kg^{-1} \cdot min^{-1}$) inhibits Na^+ absorption and induces prostaglandin E_2 (PGE_2) synthesis in rat small intestine. These results indicate regional differences, concentration dependency, and different receptor-mediated actions of the ANG II effects on ion transport.

Various secretagogues are able to induce Cl^- secretion in colonic mucosa. Secretagogue-induced Cl^- secretion in the distal colon is important, because such Cl^- secretion provides an essential driving force for lubrication or for the flushing of intestinal contents during host defense against microbial invasion or artificial irritants. ACh and substance P (SP) are involved in secretory reflexes (6, 8, 17, 30, 43). Remarkably, a relationship between ANG II and SP in the central nervous system was reported. In rat hypothalamus brain slices, it was observed that ANG II significantly evokes SP release and mediates cardiovascular effects by SP (11–13). Thus it is possible that an ANG II-evoked response involves SP action in the enteric nervous system. However, the effect of ANG II on the enteric nervous system in controlling Cl^- secretion has not been reported.

Therefore, the aim of this study was to examine whether the effect of ANG II on mucosal Cl^- secretion includes an effect on the enteric nervous system and prostanoid synthesis. The involvement of cholinergic and/or tachykinergic pathways and ANG II receptors was also tested. Furthermore, interaction between ANG II and PGE_2 was examined. Immunohistochemistry was used to identify the distribution of AT_1Rs that may be involved in ANG II-evoked Cl^- secretion.

MATERIALS AND METHODS

Ussing Flux Chamber Experiment

Male albino guinea pigs (Hartley-Hazleton, Nippon, Hamamatsu, Japan) ranging in weight from 414 to 840 g were allowed food and water ad libitum before the experiments. The animals were stunned and exsanguinated according to the method approved by the *Guide for Animal Experimentation* of the National Institute for Physiological Sciences of Japan. Segments of distal colon 5–10 cm proximal to the anus were removed, flushed with Krebs-Ringer solution, and cut along the mesenteric border. The tissues were pinned flat with the mucosal side down in a Sylgard-lined petri dish. The entire muscular layer including myenteric plexus was removed by blunt dissection, and the submucosal plexus was reserved intact. Four of these stripped mucosal preparations were obtained from one animal. Flat sheets of distal colon with intact submucosal ganglia were mounted between halves of Ussing flux chambers in which the total cross-sectional area was 0.64 cm^2.

The mucosal and serosal surfaces of tissues were bathed with 10 ml of Krebs-Ringer solution by recirculation from a reservoir maintained at 37°C during the experiment. Tissues were left for 0.5–1 h before the addition of drugs. Buffer solutions contained (in mM) 120 NaCl, 6 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 14.4 $NaHCO_3$, 2.5 $CaCl_2$, and 11.5 glucose. The solution was gassed with 95% O_2-5% CO_2 and buffered at pH 7.2. For a Cl^--free solution, Cl^- was replaced by sulfate salts, and mannitol was added to make up the difference in osmolarity. The Cl^--free solution contained (in mM) 2.7 K_2SO_4, 1.1 $MgSO_4$, 1.2 NaH_2PO_4, 54.9 Na_2SO_4, 13 $NaHCO_3$, 1.7 $CaSO_4$, 60.4 mannitol, and 11.5 glucose (7). The chambers were equipped with a pair of Ringer-agar bridges and calomel half cells for the measurement of transmural electrical potential difference (*PD*). A pair of Ag-AgCl disk electrodes was connected to an automatic voltage-clamp apparatus (model SS-1335, Nihon-Kohden, Tokyo, Japan) that automatically compensated for solution resistance between *PD*-sensing bridges. Tissue conductance (G_{ti}) was calculated as the ratio of I_{sc} to open-circuit values of *PD* or by determining the current necessary to change *PD* by 10 mV.

The responses were continuously recorded on a chart recorder (Recit-Horitz-8K, Nihon-Denki Sanei, Tokyo, Japan) and a Macintosh computer (MacLab/8 system, Analog Digital Systems, Castle Hill, Australia) for control and experimental tissues. The ΔI_{sc} was calculated on the basis of the value before and after stimulation. The tissues' currents were compared before the stimulation was begun. The tissues were paired on the basis of similar G_{ti}. A concentration-response curve for ANG II was established by addition of ANG II in a single concentration to the serosal bathing solution. In further experiments, ANG II was added in a single concentration (10^{-6} M) to the serosal bathing solution in the presence or absence of antagonists, blockers, or PGE_2 to assess their effects on baseline or stimulated I_{sc}. The following antagonists or blockers were used: the Na^+-K^+-$2Cl^-$ cotransporter inhibitor bumetanide (5×10^{-4} M), atropine (10^{-6} M), tetrodotoxin (TTX, 10^{-7} M), the neurokinin type 1 (NK_1) receptor antagonist FK-888 (10^{-8}–10^{-6} M), the cyclooxygenase inhibitor piroxicam (10^{-5} M), the EP1 receptor antagonist SC-51089 (10^{-5} M), the AT_1R antagonist FR-130739 (10^{-8}–10^{-5} M), and the AT_2R antagonist PD-123319 (10^{-7}–10^{-5} M). The blocking drugs were added ≥10 min before the addition of ANG II (10^{-6} M). PGE_2 (10^{-5} M) was added before or after the addition of ANG II (10^{-6} M). Changes in I_{sc} during ANG II stimulation were measured as the difference between the peak response and baseline I_{sc} before stimulation.

Immunohistochemistry

Male albino guinea pigs were used in the present immunostaining for AT_1R. The animals were anesthetized with pentobarbital sodium and perfused via the aorta with physiological saline followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Strips of the distal colon were removed and immersed in the same fixative for an additional 6 h. They were dipped in 30% sucrose solution overnight at 4°C and then rapidly frozen in liquid nitrogen. Frozen sections, ~20 μm thick, were prepared in a cryostat (Coldtome CD41, Sakura, Tokyo, Japan). Immunohistochemistry for AT_1R was performed according to the avidin-biotin complex method. After treatment with a normal goat serum, frozen sections were incubated with a rabbit polyclonal antiserum (1:3,200 dilution) raised against amino acids 15–24 mapping at the NH_2 terminus of the human AT_1R. Recently, guinea pig AT_1R cDNA has been cloned, and it has been confirmed that amino acids 15–24 of guinea pig and human AT_1R are identical. The nucleotide sequence data of guinea pig AT_1R have been recorded in the GenBank database (accession no. AF165888). The sites of antigen-antibody reaction were visualized using streptavidin and biotin-peroxidase complex Histfine. For whole mount preparations, the fixed distal colon was longitudinally opened, and the

mucosal and outer muscle layers were separated into sheets under a dissecting microscope. They were then processed, as free-floating sections, with the avidin-biotin complex method, as mentioned above. The specificity of the immunoreaction was checked by preincubation of the antiserum with the corresponding antigen (10 μg/diluted antiserum).

Chemicals

Ussing chamber experiments. ANG II was purchased from the Peptide Institute (Osaka, Japan); atropine sulfate, bumetanide, DMSO, and TTX from Sigma Chemical; PD-123319 from Research Biochemicals International; piroxicam and SC-51089 from Biomol Research Laboratories; and PGE_2 from Cayman Chemical. FK-888 and FR-130739 were a gift from Fujisawa Pharmaceutical (Osaka, Japan). ANG II, atropine, TTX, SC-51089, FR-130739, PD-123319, and PGE_2 were dissolved in distilled water or Krebs solution unless otherwise stated. Bumetanide, FK-888, and piroxicam were dissolved in DMSO. The volume of dissolved drugs in water or Krebs solution and DMSO added to the bath solutions did not exceed 100 μl and 10 μl/10 ml, respectively.

Immunohistochemistry. AT_1 (N-10) antiserum (rabbit polyclonal IgG) and AT_1 (N-10) were purchased from Santa Cruz Biotechnology; pentobarbital sodium from Abbot; and Histfine from Nichirei (Tokyo, Japan).

Statistics

Values are means ± SE. Unpaired Student's *t*-test was used to determine the statistical significance between control and experimental tissues. ANOVA in conjunction with the Bonferroni test was used to determine significant differences among multiple comparison groups. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of ANG II on Baseline I_{sc}

The transmural *PD* in the guinea pig distal colon was 2.4 ± 0.2 mV ($n = 220$) and was oriented luminal side positive (with respect to the serosa). The corresponding I_{sc} was negative and averaged −24.7 ± 1.4 μA/cm² ($n = 220$). G_{ti} was 10.3 ± 0.3 mS/cm² ($n = 220$).

After the addition of ANG II, I_{sc} gradually increased to a peak value within 1–2 min and then declined toward baseline within 5–10 min. The increases in I_{sc} were concentration dependent (Fig. 1*A*). The maximal response for ANG II was achieved at 10^{-6} M; 10^{-6} M ANG II evoked an increase in I_{sc} of 164.4 ± 11.8 μA/cm² from baseline (Fig. 1*B*), and this concentration was used in all subsequent studies.

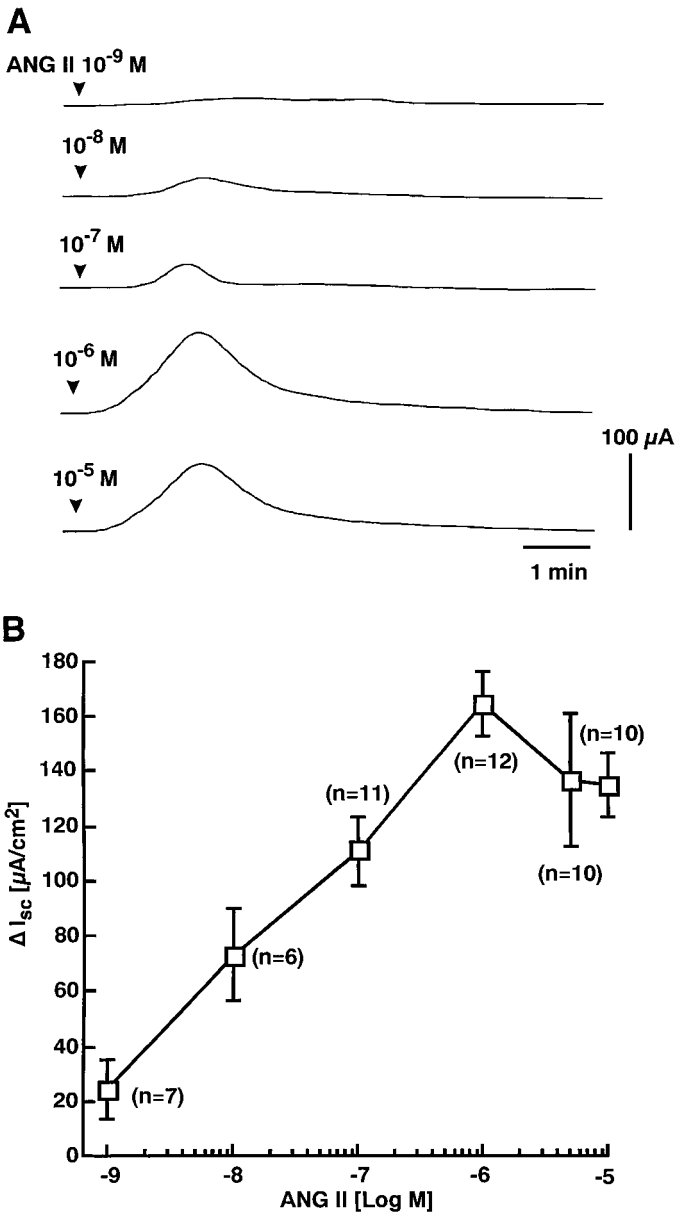

Fig. 1. Effect of ANG II on basal short-circuit current (I_{sc}) in guinea pig distal colonic mucosa. *A*: representative traces of ANG II-evoked changes in I_{sc} (μA/0.64 cm²). Arrowheads, time of addition of ANG II. *B*: concentration-response curve for ANG II-evoked response. Values are means ± SE of maximal increase in I_{sc}.

Effects of Bumetanide or Cl^--Free Solution on ANG II-Evoked Increase in I_{sc}

To determine the ionic basis for the increases in I_{sc} induced by ANG II, bumetanide and Cl^--free solution were used. The concentration of bumetanide (5×10^{-4} M) was chosen on the basis of the previous data (31). *PD* and I_{sc} changed significantly, to −2.9 ± 2.3 mV ($P < 0.05$) and 24.2 ± 21.6 μA/cm² ($P < 0.05$), respectively, after addition of bumetanide to the serosal bathing solution. There was no significant change in G_{ti} in the presence of bumetanide. Bumetanide significantly reduced the ANG II-evoked increase in I_{sc} to 50.0 ± 27.5 μA/cm² from the control value of 160.6 ± 15.0 μA/cm² (Fig. 2). In Cl^--free solution, *PD* and I_{sc} changed significantly to −0.5 ± 1.4 mV ($P < 0.05$) and −1.6 ± 6.4 μA/cm² ($P < 0.05$), respectively. G_{ti} also changed significantly to 3.5 ± 1.2 mS/cm² ($P < 0.05$). When Cl^- was removed from mucosal and serosal solution, the ANG II-evoked increase in I_{sc} decreased to 22.3 ± 7.9 μA/cm² (Fig. 2).

Effect of TTX on ANG II-Evoked Increase in I_{sc}

To test whether the response to ANG II in the distal colon was mediated by the enteric nervous system, TTX

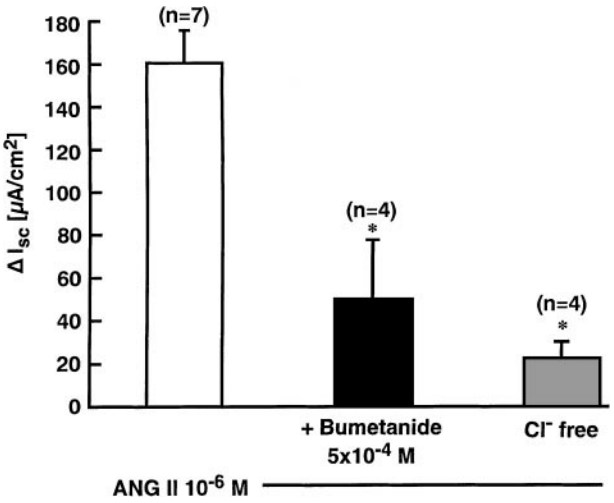

Fig. 2. Effects of bumetanide and Cl^--free solution on ANG II-evoked increase in I_{sc} in guinea pig distal colonic mucosa. Values are means ± SE of maximal increase in I_{sc} evoked by ANG II. *Significantly different from control response to ANG II, $P < 0.05$.

was added to the serosal bath solution before the administration of ANG II (10^{-6} M). The concentration of TTX (10^{-7} M) was chosen on the basis of the previous data (6). Serosal addition of TTX did not significantly alter *PD*, baseline I_{sc}, or G_{ti}. TTX significantly reduced the ANG II-evoked increase in I_{sc} to 40.9 ± 9.0 μA/cm² from the control value of 144.2 ± 13.5 μA/cm² ($n = 5$, $P < 0.05$).

Effects of NK_1 Receptor Antagonist and Atropine on ANG II-Evoked Increase in I_{sc}

To estimate the neural mediators on the ANG II-evoked response, FK-888 and atropine were used. Serosal addition of FK-888 did not significantly alter *PD*, baseline I_{sc}, or G_{ti}. Our preliminary experiment has shown that 90% of the maximal increase in I_{sc} to SP (10^{-7} M) was blocked by FK-888 (10^{-6} M), whereas FK-888 (10^{-7} M) inhibited the SP (10^{-7} M)-evoked increase in I_{sc} to only 50% of the control response (data not shown). Only the highest concentration (10^{-6} M) of FK-888 significantly reduced the ANG II-evoked increase in I_{sc}. FK-888 (10^{-6} M) reduced the ANG II-evoked increase in I_{sc} to 84.7 ± 10.5 μA/cm² from the control value of 145.1 ± 12.9 μA/cm² (Fig. 3*A*).

The concentration of atropine (10^{-6} M) was chosen on the basis of previous data (30). Serosal addition of atropine did not significantly alter *PD*, baseline I_{sc}, or G_{ti}. Atropine reduced the ANG II-evoked increase in I_{sc} to 58.5 ± 12.4 μA/cm² from the control value of 128.4 ± 11.6 μA/cm² (Fig. 3*B*).

The combination of FK-888 and atropine further reduced the ANG II-evoked increase in I_{sc} to 26.0 ± 6.5 μA/cm² (Fig. 3*B*). This inhibition was significantly larger than that resulting from FK-888 alone (Fig. 3*B*), whereas there was no difference in the inhibiting effect between the FK-888-atropine combination and atropine alone.

Effects of Piroxicam and EP1 Receptor Antagonist on ANG II-Evoked Increase in I_{sc}

To determine the involvement of prostanoid synthesis, the cyclooxygenase inhibitor piroxicam and the EP1 receptor antagonist SC-51089 were used. The concentration of piroxicam (10^{-5} M) was chosen on the basis of previous data (17). Serosal addition of piroxicam did not significantly alter *PD*, baseline I_{sc}, or G_{ti}. Piroxicam significantly reduced the ANG II-evoked increase in I_{sc} to 22.6 ± 5.7 μA/cm² from the control value of 133.5 ± 35.5 μA/cm² (Fig. 4). The combination of piroxicam and TTX reduced the ANG II-evoked response to 10.1 ± 1.3 μA/cm² (Fig. 4). However, the effect of piroxicam and TTX was not significantly different from that of piroxicam treatment alone.

The concentration of SC-51089 (10^{-5} M) was chosen on the basis of previous data (44). Serosal addition of SC-51089 did not alter *PD*, baseline I_{sc}, or G_{ti}. SC-51089 reduced the ANG II-evoked increase in I_{sc} to 66.9 ± 21.2 μA/cm² from the control value of 162.0 ± 18.9 μA/cm² ($n = 5$, $P < 0.05$).

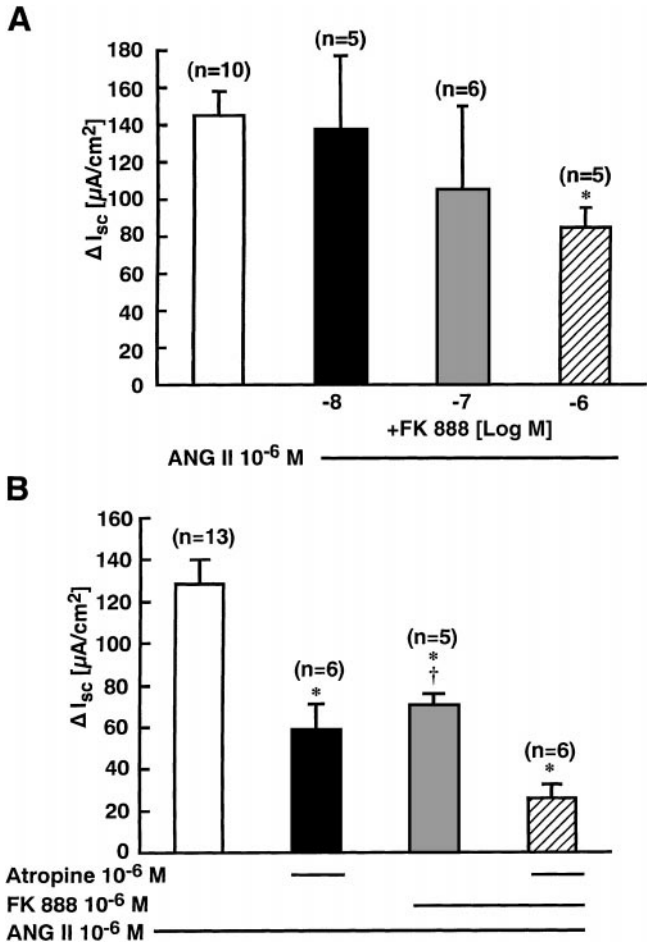

Fig. 3. Effects of neurokinin type 1 receptor antagonist FK-888 (*A*) and effects of FK-888, muscarinic receptor antagonist atropine, and combination of FK-888 and atropine (*B*) on ANG II-evoked increase in I_{sc} in guinea pig distal colonic mucosa. Values are means ± SE of maximal increase in I_{sc} evoked by ANG II. *Significantly different from control response to ANG II, $P < 0.05$. †Significantly different from combination of atropine and FK-888 pretreatment, $P < 0.05$.

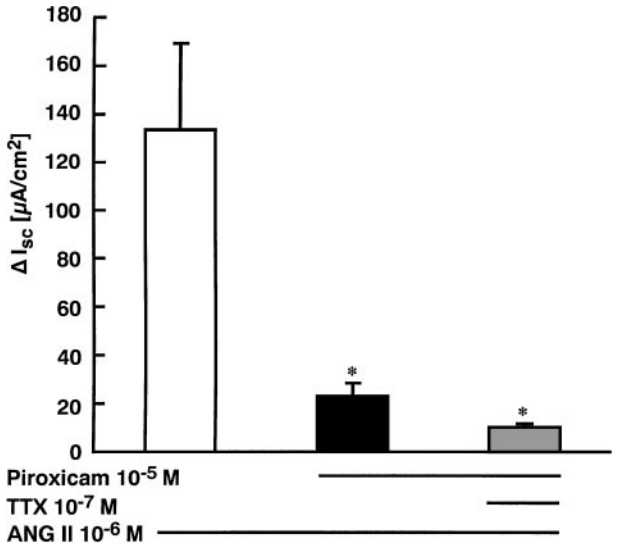

Fig. 4. Effects of cyclooxygenase inhibitor piroxicam and combination of piroxicam and tetrodotoxin (TTX) on ANG II-evoked increase in I_{sc} in guinea pig distal colonic mucosa. Values are means ± SE of maximal increase in I_{sc} evoked by ANG II (n = 4). *Significantly different from control response to ANG II, $P < 0.05$.

Effects of PGE_2 on ANG II-Evoked Increase in I_{sc} and ANG II on PGE_2-Evoked Increase in I_{sc}

To determine the involvement of prostaglandins, exogenous PGE_2 (10^{-5} M) was added to the serosal bath solution 10 min before or after the addition of ANG II (10^{-6} M).

Serosal addition of PGE_2 (10^{-5} M) evoked a biphasic and long-lasting increase in I_{sc} (Fig. 5*A*). The increase in I_{sc} was 114.2 ± 2.1 μA/cm² in the first phase and 83.5 ± 16.3 μA/cm² in the second phase (Fig. 5*B*). Pretreatment with PGE_2 enhanced the ANG II-evoked increase in I_{sc} to 341.9 ± 33.2 μA/cm² (Fig. 5, *C* and *D*). The PGE_2-evoked response was also enhanced by pretreatment with ANG II (10^{-6} M; Fig. 5*A*). ANG II enhanced the second phase of the PGE_2-evoked increase in I_{sc} to 179.4 ± 23.0 μA/cm² (Fig. 5*B*).

Effects of AT_1R and AT_2R Antagonists on ANG II-Evoked Increase in I_{sc}

To determine which ANG II receptor subtypes contribute to the ANG II-evoked increase in I_{sc}, ANG II receptor antagonists were used. Serosal addition of the AT_1R antagonist FR-130739 (10^{-8}–10^{-5} M) or the AT_2R antagonist PD-123319 (10^{-7}–10^{-5} M) did not change *PD*, baseline I_{sc}, or G_{ti}. FR-130739 (10^{-8}–10^{-5} M) reduced the ANG II-evoked increase in I_{sc} in a concentration-dependent manner, and in the presence of the highest concentration of FR-130739 (10^{-5} M) the ANG II-evoked increase in I_{sc} was completely blocked (Fig. 6*A*). On the other hand, PD-123319 (10^{-7}–10^{-5} M) did not affect the ANG II-evoked increase in I_{sc} (Fig. 6*B*).

Immunohistochemistry

Immunostaining for the AT_1R demonstrated the intense and specific immunoreactivity in neuronal soma of the submucous and myenteric nerve plexuses (Fig. 7, *A* and *B*). In whole mount preparations of the mucosal layer, less than half of all cell bodies in the submucous nerve plexus were positive in reaction and were intermingled with weakly positive or negative cell bodies. The intensely positive cell bodies tended to gather at the peripheral portion of the nerve plexus. Immunostaining of the outer muscle layer showed that only a limited number of nerve cells were immunoreactive in the myenteric nerve plexus and were dispersed throughout the nerve plexus. In both nerve plexuses, AT_1R immunoreactivity appeared mainly in the cell bodies.

Additional immunoreactivity to AT_1R was observed on the surface epithelial cells of the distal colon, although it was less intense than that of the nerve plexuses (Fig. 7*C*). Furthermore, crypt cells lacked significant immunoreactivity to AT_1R.

DISCUSSION

It is known that the effect of ANG II on ion transport in the intestine depends on its concentration. At picomolar concentrations, ANG II stimulates Na^+ and water absorption, whereas above nanomolar concentrations, ANG II inhibits Na^+ and water absorption and stimulates secretory processes in rat jejunum and colon (33). The present study has shown that ANG II (10^{-9}–10^{-5} M) evokes an increase in I_{sc} in guinea pig distal colon (Fig. 1). The ANG II (10^{-6} M)-evoked increase in I_{sc} was reduced by the Na^+-K^+-$2Cl^-$ cotransporter blocker bumetanide or Cl^--free solution (Fig. 2). These results suggest that the ANG II-evoked increase in I_{sc} is mainly due to Cl^- secretion in guinea pig distal colon. However, Hatch et al. (21, 22) reported that, in rat colon, ANG II (10^{-9}–10^{-6} M) evokes a decrease in I_{sc}, which is due to K^+ secretion, whereas ANG II (10^{-4} M) evokes an increase in I_{sc} by Cl^- secretion. In the present study we have used concentrations (10^{-9}–10^{-5} M) of ANG II similar to those previously reported, but ANG II did not evoke the decrease in I_{sc}. However, we did not perform a flux experiment using isotopes, so we could not confirm the ANG II-evoked K^+ secretion observed in the rat colon.

In the present study, TTX significantly reduced but did not abolish the ANG II (10^{-6} M)-evoked increase in I_{sc}. This result suggests that ANG II-evoked Cl^- secretion is partially mediated by submucosal neurons. In contrast, Cox et al. (9) reported that ANG II (10^{-10}–10^{-5} M)-evoked changes in I_{sc} are not affected by TTX in rat small intestine. This report suggests that ANG II-evoked Cl^- secretion is not regulated by submucosal neurons in rat small intestine. The discrepancy may reflect a segmental difference between small and large intestines or a species difference.

In the present study we have further analyzed the contribution of muscarinic and NK_1 receptors to ANG II-evoked Cl^- secretion. The muscarinic receptor antagonist atropine and the NK_1 receptor antagonist FK-888 reduced the ANG II (10^{-6} M)-evoked increases in I_{sc} to 45 and 58% of control, respectively (Fig. 3). These results suggest that ANG II may activate cholinergic and tachykinergic neurons to evoke Cl^- secretion in guinea pig distal colon. The results are supported by

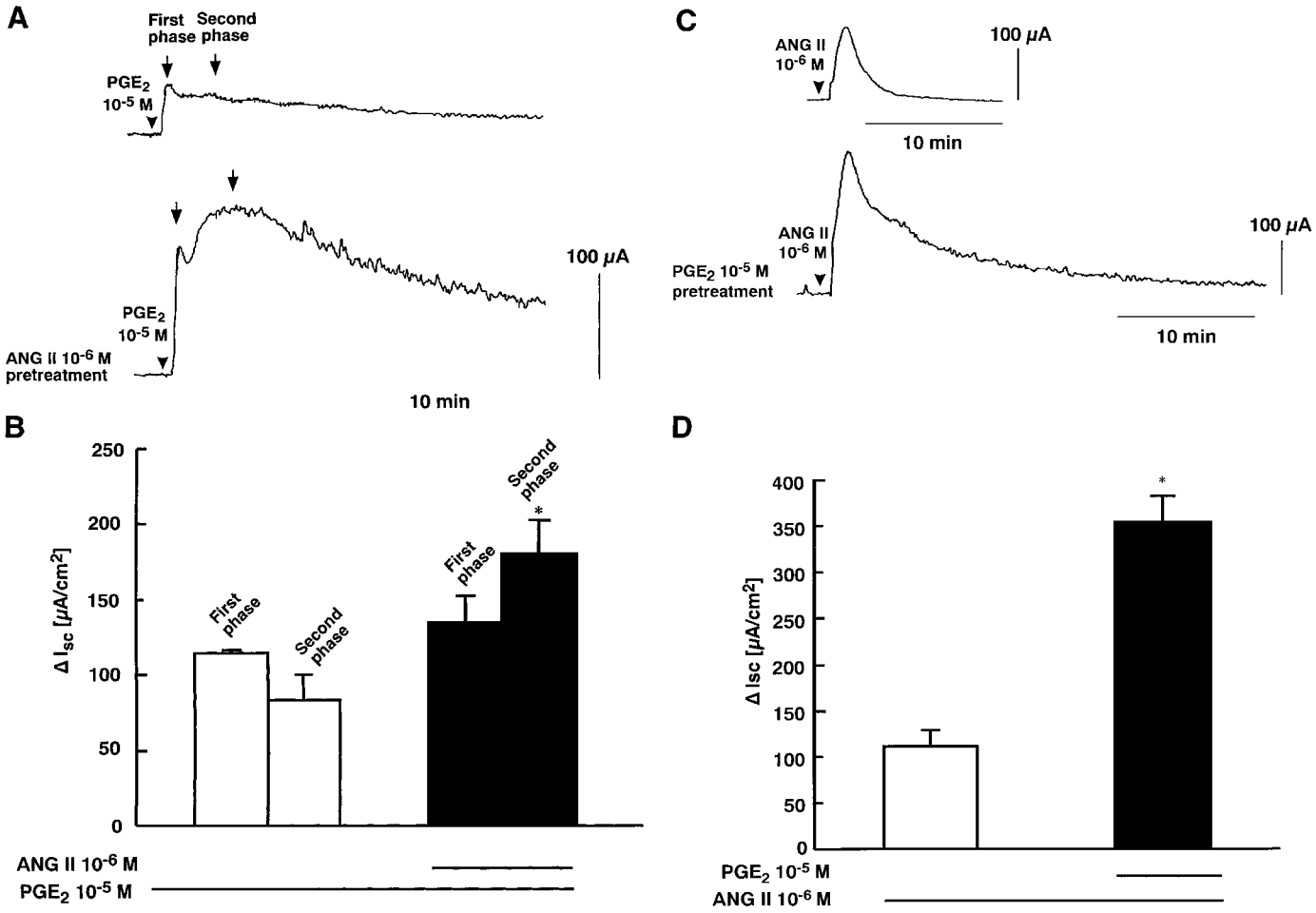

Fig. 5. Effects of ANG II on prostaglandin E_2 (PGE_2)-evoked increase in I_{sc} and of PGE_2 on ANG II-evoked increase in I_{sc} in guinea pig distal colonic mucosa. *A*: representative traces of PGE_2-evoked changes in I_{sc} (μA/0.64 cm^2) in presence of ANG II. Arrowheads, time of addition of PGE_2. *B*: effect of ANG II on PGE_2-evoked increase in I_{sc}. Values are means ± SE of each of 1st and 2nd phases of increases in I_{sc} evoked by PGE_2 ($n = 5$). *C*: representative traces of ANG II-evoked changes in I_{sc} (μA/0.64 cm^2) in presence of PGE_2. Arrowheads, time of addition of ANG II. *D*: effect of ANG II on PGE_2-evoked increase in I_{sc}. Values are means ± SE of maximal increase in I_{sc} evoked by ANG II ($n = 4$). * Significantly different from control response to ANG II, $P < 0.05$.

previous observations that SP and ACh can evoke Cl^- secretion in the distal colon (6, 8, 17, 30, 43) and that SP-immunoreactive neurons in the submucosal plexus in guinea pig small intestine are also immunoreactive to choline acetyltransferase (18). The combination of FK-888 and atropine further reduced the ANG II-evoked increase in I_{sc} to 20% of control, and the reduction was significantly greater than with FK-888 alone. Thus another possible pathway for ANG II-evoked Cl^- secretion is ANG II-induced SP release. SP may then evoke ACh release, which in turn evokes Cl^- secretion. This hypothesis is supported by the observation that SP-evoked Cl^- secretion is partly inhibited by atropine in guinea pig distal colon (30).

Failure of TTX to completely block the response to ANG II suggests that ANG II also acts directly on epithelial or subepithelial cells in the mucosa. It has been reported that ANG II-evoked Cl^- secretion is mediated by prostaglandin synthesis in rat small intestine (9). In the present study, piroxicam reduced the ANG II-evoked increase in I_{sc} to 16% of control (Fig. 4). The result suggests that the ANG II-evoked Cl^- secretion is linked with the prostaglandin synthesis pathway in guinea pig distal colon as it is in rat small intestine. Furthermore, Jin et al. (26) showed that ANG II (700 $ng \cdot kg^{-1} \cdot min^{-1}$) induces production of PGE_2 in rat small intestine. EP1, EP3, and EP4 receptor mRNAs are reported to be localized in rat small and large intestines (10). However, only the EP1 receptor antagonist was commercially available at the time of the experiment. The EP1 receptor antagonist SC-51089 reduced the ANG II-evoked response to 41% of control. This result suggests that ANG II-evoked Cl^- secretion is partially mediated by EP1 receptor activation.

Prostaglandin synthesis is involved in the inflammatory process. Zipser et al. (46) reported that the basal release of PGE_2 is two times greater in an artificial colitis condition than in normal tissues and that ANG II increases the release of PGE_2 in normal and inflammatory conditions. It was reported that the colonic mucosal level of ANG II was three times higher in Crohn's disease patients than in healthy humans (25). These

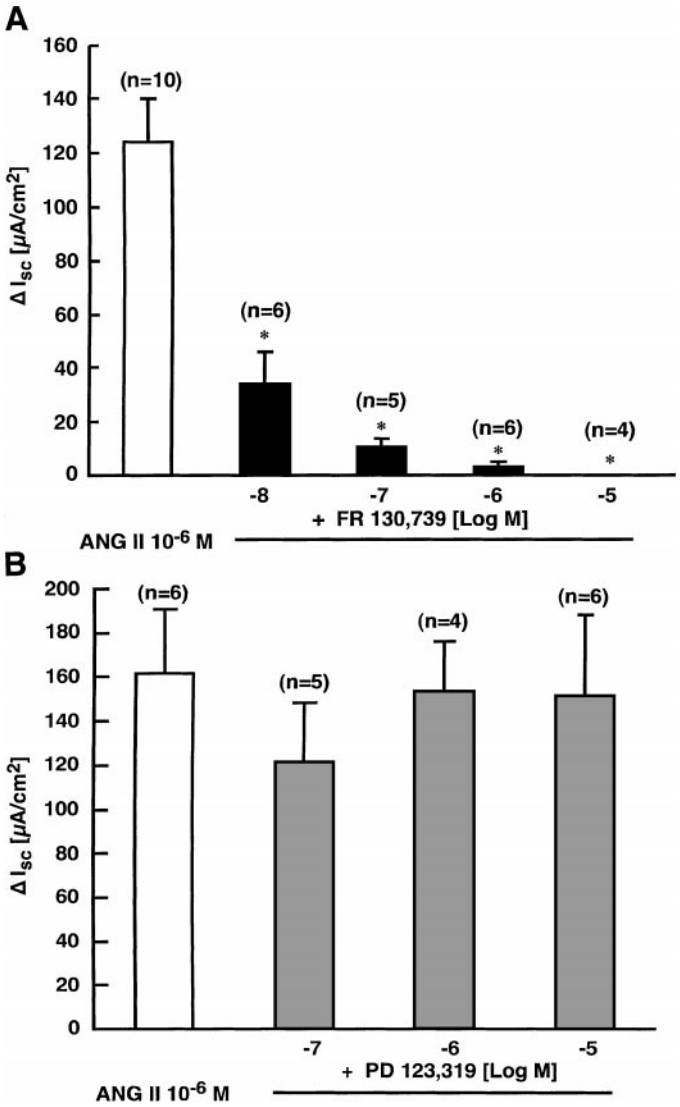

Fig. 6. Effects of ANG type 1 receptor antagonist FR-130739 (*A*) or ANG type 2 receptor antagonist PD-123319 (*B*) on ANG II-evoked increase in I_{sc} in guinea pig distal colonic mucosa. Values are means ± SE of maximal increase in I_{sc} evoked by ANG II. *Significantly different from control response to ANG II, $P < 0.05$.

reports suggest that, in an inflammatory state, ANG II may also play an important role in the modification of electrolyte transport in colonic mucosa. In the present study we have demonstrated the involvement of PGE_2 in the ANG II response. When the tissues were pretreated with PGE_2 (10^{-5} M), the ANG II (10^{-6} M)-evoked increase in I_{sc} was approximately three times higher and longer than when the tissues were treated with ANG II alone (Fig. 5*D*). Moreover, after application of ANG II (10^{-6} M), the PGE_2 (10^{-5} M)-evoked increase in I_{sc} in the second phase was approximately two times higher than that with PGE_2 alone (Fig. 5*B*). These results suggest the possibility that the presence of PGE_2 and ANG II in colonic mucosa may enhance Cl^- secretion in the inflammatory state.

The ANG II-evoked Cl^- secretory mechanism may involve increases in the concentration of intracellular cAMP and cytosolic free Ca^{2+} ($[Ca^{2+}]_i$). It has been reported that PGE_2 activates the apical cAMP-activated Cl^- channels and basolateral cAMP-activated K^+ channels in colonic epithelial cells (35, 42). Strabel and Diener (43) suggested that in rat colonic epithelia a carbachol-evoked increase in I_{sc} is enhanced by pretreat-

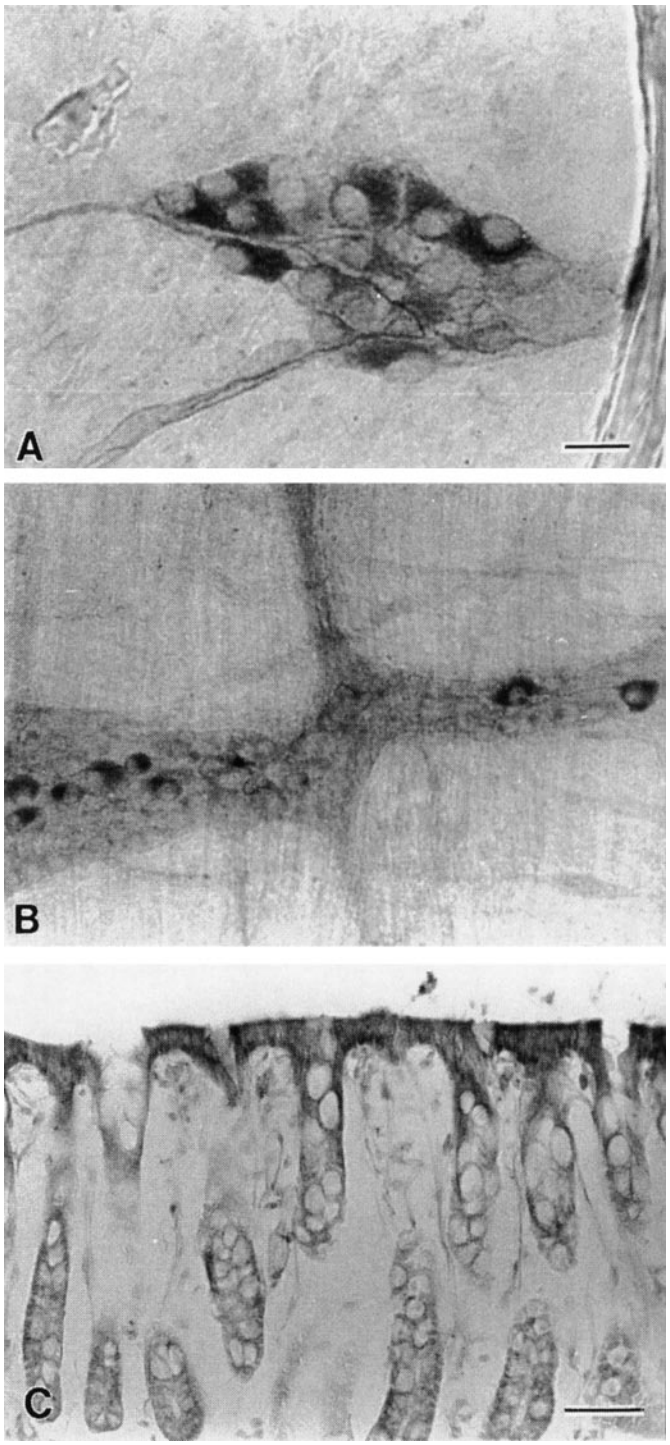

Fig. 7. Immunostaining for ANG type 1 receptor by use of whole mount preparations (*A* and *B*) and a cryostat section (*C*) from guinea pig distal colon. Immunoreactivity is localized in populating neuronal cell bodies in submucous (*A*) and myenteric nerve plexuses (*B*). Scale bar, 20 μm. Positive reactions are also recognizable in surface epithelium (*C*). Scale bar, 50 μm.

ment with cAMP-activating drugs [e.g., PGE_2, forskolin, or 8-(4-chlorophenylthio)-cAMP] and that the dominant effect of carbachol is activation of basolateral Ca^{2+}-activated K^+ channels. It is known that the activation of these channels induces Cl^- secretion in colonic mucosa (19). Romero et al. (38) suggested that ANG II induces an increase in $[Ca^{2+}]_i$ through AT_1R in guinea pig ileum myocytes and activates the Ca^{2+}-activated K^+ channels. Jin et al. (26) and Zipser et al. (46) showed that ANG II induces production of PGE_2 in rat small intestine and rabbit distal colon. In the present study the ANG II-evoked response was blocked by inhibition of the prostaglandin synthesis pathway. Thus these results suggest that the ANG II response and the PGE_2-enhanced ANG II synergistic response may be regulated by the interaction of cAMP concentration and $[Ca^{2+}]_i$ in colonic epithelial cells. However, the exact mechanism of the ANG II-enhanced PGE_2 response is not clear. Calderaro et al. (1) reported that PGE_2-evoked Cl^- secretion in rabbit distal colon is regulated by $[Ca^{2+}]_i$, which regulates adenylate cyclase and phosphodiesterase activities. Furthermore, they suggested that a lower $[Ca^{2+}]_i$ enhances a PGE_2-evoked sustained increase in I_{sc}. Therefore, one possible explanation for the ANG II-enhanced PGE_2 response is that ANG II may continually affect $[Ca^{2+}]_i$ and enhance cAMP metabolism in the PGE_2 response.

There are two major isoforms of the ANG II receptor: AT_1R and AT_2R. Sechi et al. (41) showed that AT_1R and AT_2R subtypes are present in the mucosa and the muscularis mucosa of rat jejunum, ileum, and colon and that the predominant ANG II receptor subtype is AT_1R, but a small proportion of AT_2R is also present. Jin et al. (26) reported that a low dose of ANG II given in an intravenous infusion stimulates net Na^+ absorption through AT_2R, whereas a high dose of ANG II inhibits Na^+ absorption by PGE_2 synthesis through AT_1R on rat small intestine. The report has suggested that, in small and large intestines, AT_1R and AT_2R regulate the ANG II-evoked ion transport. In the present study the AT_1R antagonist FR-130739 (10^{-5} M) completely blocked an ANG II (10^{-6} M)-evoked increase in $I_{sc,}$ whereas the AT_2R antagonist PD-123319 had no effect (Fig. 6). We have also shown that AT_1R-immunoreactive neurons are located in the submucosal plexus (Fig. 7*A*) and the ANG II-evoked increase in I_{sc} was partly blocked by pretreatment with TTX. Moreover, the moderate immunoreactivity of AT_1Rs was also found in surface epithelial cells (Fig. 7*C*). It has been shown that Cl^- secretion is restricted to the crypts (19, 20). However, Kockerling and Fromm (28) showed that in rat distal colon the cAMP-dependent Cl^- secretion is not confined to crypts but is also performed by surface epithelial cells. Thus the results of the present study suggest that AT_1R regulates ANG II-evoked Cl^- secretion and that AT_1R in the submucosal plexus and surface epithelial cells most likely contributes to the secretory effect. In the present study the ANG II response was reduced to 16% of control by pretreatment with piroxicam. Therefore, ANG II-evoked Cl^- secretion is partially linked with prostaglandin synthesis. Many subepithelial cells, including mast cells, fibroblasts, and also crypt epithelial cells are able to produce prostaglandins (2, 5, 36). However, we did not observe AT_1R immunoreactivity in these cells. Thus it is unlikely that ANG II directly induces prostaglandin synthesis through AT_1R. Therefore, further study is needed to identify an ANG II-induced prostaglandin synthesis mechanism in guinea pig distal colonic mucosa.

We have also shown that AT_1R-immunoreactive nerves are located in the colonic myenteric plexus (Fig. 7*B*). Leung et al. (32) showed that ANG II induces smooth muscle contractions in guinea pig small and large intestines and that these responses are inhibited by TTX and AT_1R antagonist but are not affected by AT_2R antagonist. These results suggest that ANG II regulates colonic motility as well as electrolyte transport through AT_1R in the enteric nervous system.

In the present study we have used a concentration of ANG II much higher than the concentration of circulatory ANG II. Hatch et al. (22) reported that the calculated extracellular tissue space concentration of ANG II was 2.7×10^{-6} M when the bath concentration was 1×10^{-4} M and suggested that the active concentration of ANG II at the tissue level might be much lower than the concentration in the bath solution. Using the rate of ANG II tissue hydrolysis reported, we have estimated the tissue concentration of ANG II in guinea pig distal colon when the ANG II concentration of the bathing solution was 10^{-6} M. The estimated tissue concentration of ANG II was two orders of magnitude less than the bath concentration. These results indicate that relatively high bath concentrations of ANG II are necessary to evoke Cl^- secretion under experimental conditions.

Some reports have shown the possibility that ANG II exists as the tissue renin-angiotensin system in the gastrointestinal tract. The hypothesis is supported by the following observations: *1*) angiotensinogen mRNA has been detected in rat mesentery and large intestine (3, 37); *2*) kallikrein was observed in rat colonic goblet cells (40); and *3*) ANG II receptors and ACE have been shown to be present in rat colon by in vitro autoradiography (15, 41). These findings suggest that ANG II may be produced and may regulate local intestinal functions in the gastrointestinal tract.

In conclusion, the results provide evidence for the involvement of ANG II in the local regulation of Cl^- secretion in guinea pig distal colon. ANG II-evoked Cl^- secretion may involve submucosal cholinergic and tachykinergic neurons and prostanoid synthesis pathways. These responses may be regulated by AT_1R on the submucosal plexus and surface epithelial cells. Furthermore, ANG II and PGE_2 synergistically evoke ion transport. Thus the present study indicates that ANG II functions as a local mediator in colonic mucosa in physiological and pathophysiological states.

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