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# Angiotensin II type 2 receptor-mediated duodenal mucosal alkaline secretion in the rat

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Departments of <sup>1</sup>Physiology, <sup>2</sup>Surgery, and <sup>3</sup>Clinical Pharmacology,  
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**Johansson, Berndt, Mathias Holm, Sara Ewert, Anna Casselbrant, Anders Pettersson, and Lars Fändriks.** Angiotensin II type 2 receptor-mediated duodenal mucosal alkaline secretion in the rat. *Am J Physiol Gastrointest Liver Physiol* 280: G1254–G1260, 2001.—The aims of this study were to elucidate the distribution of angiotensin receptors (AT<sub>1</sub> and AT<sub>2</sub>) in the duodenal wall and to investigate whether AT<sub>2</sub> receptors are involved in the regulation of duodenal mucosal alkaline secretion, which is of importance for the mucosal defense against gastric acid. Immunohistochemistry was used to locate AT<sub>1</sub> and AT<sub>2</sub> receptors in chloralose-anesthetized rats. Duodenal mucosal alkaline output was measured by use of in situ pH-stat titration. Immunohistochemistry demonstrated a distinct staining for both AT<sub>1</sub> and AT<sub>2</sub> receptors in the lamina propria of the villi and also for AT<sub>1</sub> receptors in the muscularis interna. When angiotensin II was infused in the presence of the AT<sub>1</sub> receptor antagonist losartan, mucosal alkaline secretion increased by ~50%. This response was inhibited by the AT<sub>2</sub> receptor antagonist PD-123319. The AT<sub>2</sub> receptor agonist CGP-42112A increased mucosal alkaline secretion by ~50%. This increase was absent in the presence of PD-123319 but not in the presence of losartan or the local anesthetic lidocaine. We conclude that angiotensin II stimulates duodenal mucosal alkaline secretion by activation of AT<sub>2</sub> receptors located in the duodenal mucosa/submucosa.

duodenum; immunohistochemistry

THE ANGIOTENSINS, particularly ANG II, have well-known vasoconstrictive actions as well as influences on the renal regulation of body fluid and electrolyte homeostasis. The latter effects are mediated either directly on the tubular electrolyte transport or indirectly by the liberation of aldosterone (17). We showed previously (14, 15) that ANG II acts in concert with sympathoadrenergic regulation of duodenal mucosal alkaline secretion subsequent to hypovolemia. This alkaline secretion by the duodenal mucosa is considered to be of importance for the mucosal defense against gastric acid (7).

Two main types of ANG II receptors have been sequenced and cloned, AT<sub>1</sub> and AT<sub>2</sub> receptors (20, 21). Most effects of ANG II, including the sympathoa-

drenergic inhibition of duodenal mucosal alkaline secretion, are mediated by activation of AT<sub>1</sub> receptors, which are present in most organs including the intestine (4–6, 22).

During recent years, efforts have been made to correlate functional properties with the findings of AT<sub>2</sub> receptors, which are predominant in the ovaries, adrenal medulla, and pancreas. It has been shown that the AT<sub>2</sub> receptor is involved, for instance, in growth, differentiation, and inhibition of cell proliferation (1, 4). AT<sub>2</sub> receptors have also been demonstrated in the intestine (23), and jejunal sodium and water absorption has been shown to be influenced by activation of this receptor subtype (12).

The present study was conducted to elucidate the distribution of AT<sub>1</sub> and AT<sub>2</sub> receptors in the duodenal mucosa and to investigate whether the AT<sub>2</sub> receptors are involved in the regulation of duodenal mucosal alkaline secretion. The experiments were performed on chloralose-anesthetized rats.

## METHODS

**General.** The experiments were approved by the Animal Ethics Committee of Göteborg University and performed on nonfasted male Sprague-Dawley rats (Mölgård Breeding Center, Ejby, Denmark). For induction of anesthesia, methohexital, a barbiturate with a short duration of action, was injected intraperitoneally (75 mg/kg body wt). General anesthesia was maintained with  $\alpha$ -chloralose administered intravenously as a bolus (50 mg/kg body wt) followed by continuous infusion (25 mg·kg<sup>-1</sup>·h<sup>-1</sup>). A catheter was inserted into the trachea to ensure free airways. A femoral artery and one or two veins were catheterized for subsequent blood pressure measurements and drug infusions, respectively. The body temperature was maintained at 38°C with a heating pad and lamp, both controlled thermostatically. Blood pressure was measured by a Statham P23Dc transducer (Statham, Hato Rey, PR) connected to a PE-50 catheter in the right femoral artery. Pressure data were integrated by a microcomputer to mean arterial pressure (MAP) over 5 min. To avoid acidosis and dehydration due to surgical trauma and the long period of general anesthesia, a 1.7% glucose solution containing 0.03 M NaHCO<sub>3</sub>, made isotonic with saline, was given intravenously throughout the experiments (1 ml/h).

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**Secretion.** Duodenal mucosal alkaline (HCO<sub>3</sub><sup>-</sup>) secretion was measured by a pH-stat titration technique. This technique was described previously (8), but a brief summary is given here. After midline laparotomy, a segment of the duodenum (with its proximal end ~1 cm distal to the pylorus) with intact vascular supply was isolated between two glass tubes connected to a reservoir containing isotonic saline maintained at 38°C by a water jacket. Saline was recirculated through the segment by means of a gas lift (pure air). The common bile duct was catheterized ~5 mm proximal to the papilla of Vater to avoid contamination of the segment by bile and pancreatic juice. Alkaline secretion to the perfusate was continuously titrated to pH 7.4 with 0.02 M HCl controlled by a pH-stat device.

**Immunohistochemistry.** At the end of six 90-min time control experiments, the midportion of the duodenal segment under study was removed and fixed in 4% (wt/vol) paraformaldehyde in PBS (pH 7.4) overnight. The tissues were then

embedded in paraffin, and sections (3 μm) were cut and mounted on slides. After deparaffinization in xylene and ethanol, sections were boiled in citrate buffer (pH 6.0) for 20 min for antigen retrieval. Endogenous peroxidase was quenched by incubation in 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 5 min, and nonspecific binding was blocked by goat (AT<sub>1</sub>) and donkey (AT<sub>2</sub>) serum for 20 min. Subsequently, sections were incubated with primary antibody for 2 h at room temperature. The antibodies were polyclonals against the AT<sub>1</sub> or AT<sub>2</sub> receptors (Santa Cruz Biotechnology, Santa Cruz, CA) and were raised in rabbit or goat and used at 1:800 or 1:400, respectively. Slides were then incubated with secondary biotinylated goat anti-rabbit and donkey anti-goat IgG for 30 min. Horseradish peroxidase (HRP)-streptavidin complex was used to detect specific polyclonal AT<sub>1</sub> and AT<sub>2</sub> binding. Before the slides were mounted and covered with glass slips, they were counterstained with eosin and dehydrated in ethanol and xylene. Controls consisted of preimmune serum

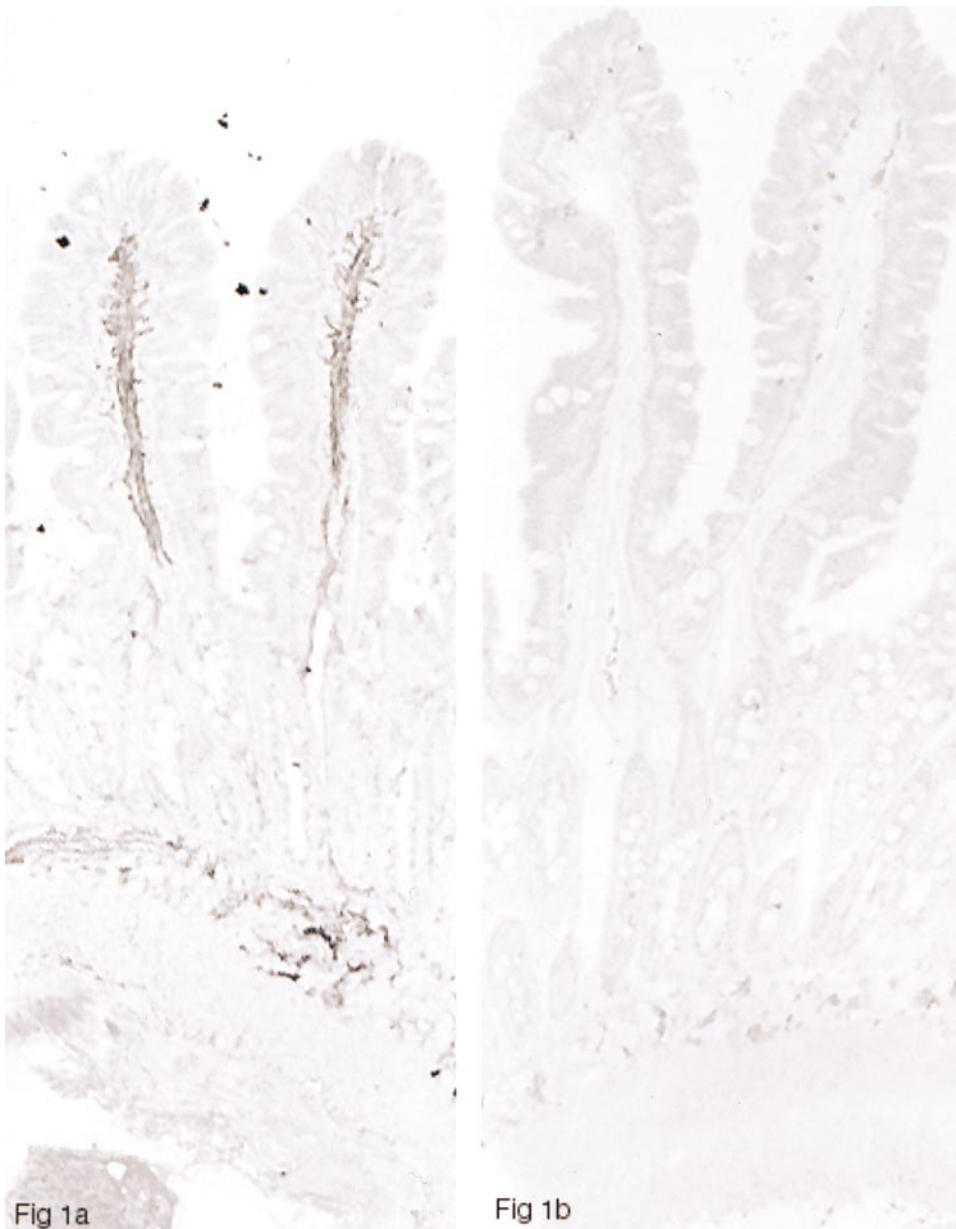


Fig. 1. Distribution of AT<sub>1</sub> receptors in a representative section of the duodenal wall in a time control animal. Distinct staining is demonstrated for AT<sub>1</sub> receptors in the lamina propria of the villi, apparently associated with vessels. In the submucosa, some staining for AT<sub>1</sub> receptors was observed but less than in the villi structures. The muscularis interna was also stained for AT<sub>1</sub> receptors (a). The negative controls were unstained in all specimens (b).

from rabbit and donkey, respectively. The Immunocruz staining system (Santa Cruz Biotechnology) was used for this immunohistochemistry protocol.

**Drugs.** For anesthesia, methohexital (Brietal, Lilly, Indianapolis, IN) and  $\alpha$ -chloralose (Sigma, St. Louis, MO) were used (for doses and administration routes see *General*). ANG II (Hypertensin, CIBA-Geigy, Basel, Switzerland), the AT<sub>1</sub> receptor antagonist losartan (Cozaar, Merck Sharp & Dohme, Whitehouse Station, NJ), the AT<sub>2</sub> receptor antagonist PD-123319 (Research Biochemicals International, Natick, MA), and the AT<sub>2</sub> receptor agonist CGP-42112A (Neosystem, Strasbourg, France) were all dissolved in saline and administered intravenously. Lidocaine HCl 1% (wt/vol)

(Xylocaine, Astra, Södertälje, Sweden) was administered topically onto the serosal side of the duodenal segment.

**Experimental protocol.** After surgery, the animals were left undisturbed for ~1 h, after which basal duodenal mucosal alkaline secretion and MAP were monitored during a 30-min control period. The animals were then treated as follows: 1) untreated controls for immunohistochemical analysis, 2) ANG II infusion alone or in the presence of either losartan or the combination of PD-123319 and losartan, and 3) CGP-42112A infusion alone or in the presence of losartan, PD-123319, or topical lidocaine (as described in Ref. 10).

**Statistics.** One-way analysis for repeated measurements and Bonferroni's post hoc test were used to evaluate signifi-

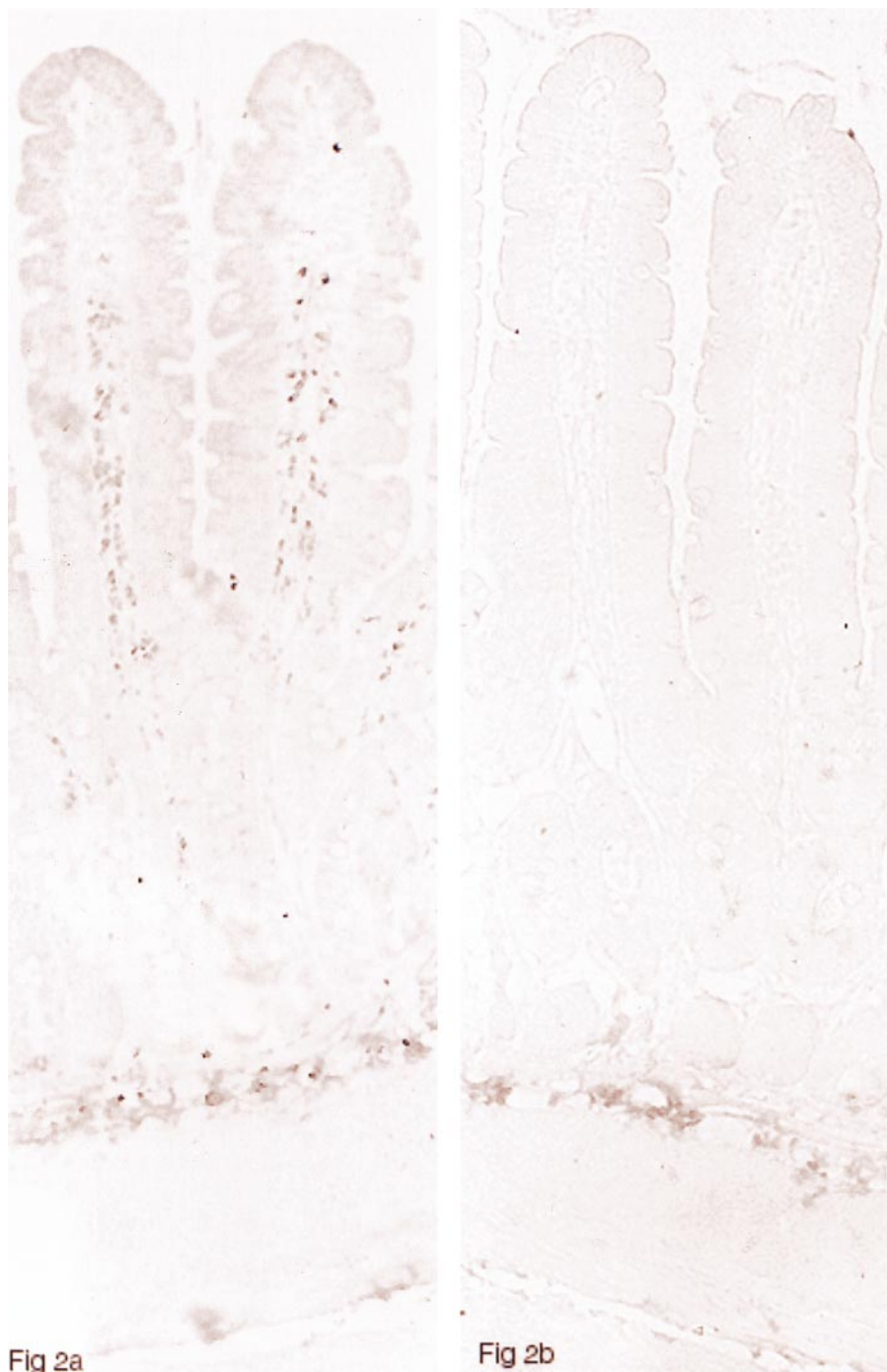


Fig. 2. Distribution of AT<sub>2</sub> receptors in a representative section of the duodenal wall in a time control animal. Staining for AT<sub>2</sub> receptors was present up to the midportion of the lamina propria of the villi. Some staining for AT<sub>2</sub> receptors in the submucosa was also observed but less than in the villi structures. The muscular layers showed almost no staining for AT<sub>2</sub> receptors (a). The negative controls were unstained in all specimens (b).

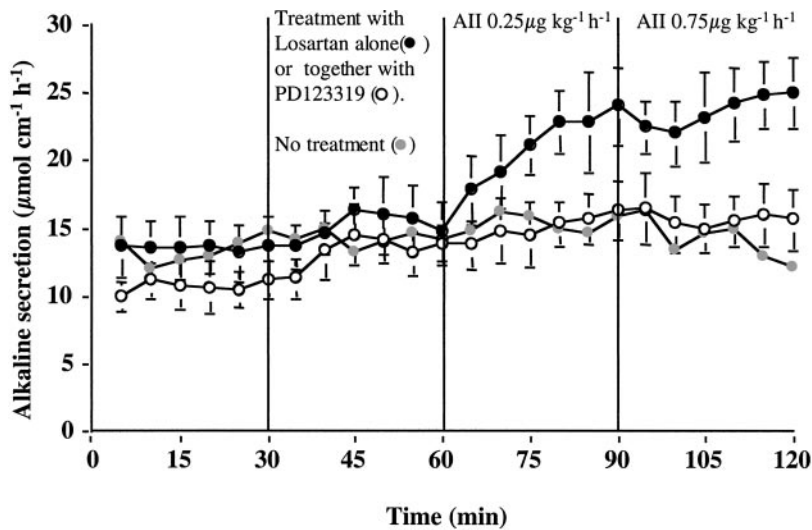


Fig. 3. Effects on duodenal mucosal alkaline secretion of ANG II infusion ( $0.25\text{--}0.75\ \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ ) alone and after treatment with the AT<sub>1</sub> receptor antagonist losartan ( $10\ \text{mg}/\text{kg}$  iv bolus) or after treatment with both the AT<sub>2</sub> receptor antagonist PD-123319 ( $0.3\ \text{mg}/\text{kg}$  iv bolus and  $0.03\ \text{mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  iv infusion) and losartan (dose as above). Data shown are means  $\pm$  SE;  $n = 5$  in the first group and 6 in the 2 latter groups.

cance of changes within groups. Data obtained during the last 15 min before administration of drug were regarded as representing basal conditions. Net change was defined as the difference between the last 15-min period during drug administration and basal conditions. Comparisons between groups were made by one-way ANOVA and a *t*-test. Values given in Figs. 1–5 are means  $\pm$  SE. A *P* value  $\leq 0.05$  was considered significant.

## RESULTS

**Distribution of AT<sub>1</sub> and AT<sub>2</sub> receptors in duodenal mucosa ( $n = 6$ ).** In time control animals, immunohistochemistry demonstrated distinct staining for both AT<sub>1</sub> and AT<sub>2</sub> receptors in the lamina propria of the villi (Figs. 1 and 2). Staining for AT<sub>1</sub> receptors was heavier than staining for AT<sub>2</sub> receptors. This speaks in favor of the AT<sub>1</sub> receptors being predominant. However, comparison between staining with different antibodies is difficult. AT<sub>2</sub> receptors were present up to the midportion of the villi, whereas AT<sub>1</sub> receptors were also present in the villi tips. No staining was present in the epithelial cells. In the submucosa, some staining for both AT<sub>1</sub> and AT<sub>2</sub> receptors was observed but less than that in the villi structures. The muscularis interna was stained for AT<sub>1</sub> receptors but showed almost no staining for AT<sub>2</sub> receptors. The negative controls were unstained in all specimens.

**Effects of ANG II infusion.** ANG II infusion alone ( $0.25\text{--}0.75\ \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ ;  $n = 5$ ) did not influence basal mucosal alkaline secretion (Fig. 3, Table 1) or MAP. Treatment with the AT<sub>1</sub> receptor antagonist losartan ( $10\ \text{mg}/\text{kg}$  bolus iv;  $n = 6$ ) did not influence mucosal alkaline secretion significantly but decreased MAP significantly by 23 mmHg. When ANG II was infused into these animals, mucosal alkaline secretion increased significantly by 49% (Fig. 3) whereas MAP was unaffected. In animals treated with both the AT<sub>2</sub> receptor antagonist PD-123319 ( $0.3\ \text{mg}/\text{kg}$  iv bolus and  $0.03\ \text{mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  iv infusion;  $n = 6$ ) and losartan (dose as above), the increase observed on ANG II infusion was absent (Fig. 3). MAP decreased equally as in animals treated

with losartan alone. PD-123319 alone had no effect on either mucosal alkaline secretion or MAP (not shown in Fig. 3).

**Effects of AT<sub>2</sub> receptor agonist CGP-42112A.** CGP-42112A infused at a rate of  $0.1\ \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  ( $n = 5$ ), which has been shown to be an optimal dose for AT<sub>2</sub> receptor agonism in the rat intestine (jejunal fluid absorption), increased mucosal alkaline secretion significantly by 54%, whereas MAP was unaffected (Fig. 4, Table 2). The increase in mucosal alkaline secretion in response to CGP-42112A was blocked in the presence of PD-123319 ( $0.3\ \text{mg}/\text{kg}$  iv bolus and  $0.03\ \text{mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  iv infusion;  $n = 6$ ), whereas losartan ( $10\ \text{mg}/\text{kg}$  iv bolus;  $n = 6$ ) was without effect (Fig. 4). Treatment with losartan decreased MAP significantly by 25 mmHg.

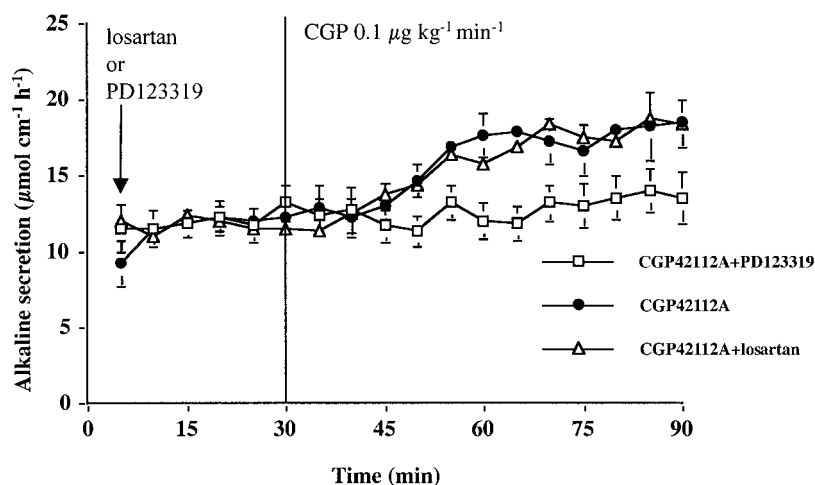
**Effects of AT<sub>2</sub> receptor agonist CGP-42112A after treatment with lidocaine on serosal side of duodenal segment.** The local anesthetic lidocaine (1% wt/vol) was applied topically onto the serosal side of the duodenal segment ( $n = 5$ ). This serosal administration of lidocaine decreased alkaline secretion significantly by 42%, whereas MAP was unaffected. When CGP-42112A was infused ( $0.1\ \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ;  $n = 5$ ) intravenously into these animals, the net increase in mucosal alkaline secretion was of the same order of magnitude as when it was infused in untreated animals (Fig. 5, Table 2).

Table 1. Effect of losartan and PD-123319 on mean arterial pressure

	<i>n</i>	After 30-min Basal Conditions	After 30-min Treatment/No Treatment	After 30-min ANG II Infusion
No treatment	5	116 $\pm$ 10.1	110 $\pm$ 12.8	111 $\pm$ 13.5
Losartan	6	109 $\pm$ 7.3	86 $\pm$ 13.3	81 $\pm$ 11.7
PD-123319 and losartan	6	107 $\pm$ 6.1	79 $\pm$ 3.5	79 $\pm$ 3.6

Values (in mmHg) are means  $\pm$  SE; *n*, no. of rats. Losartan was administered as a  $10\ \text{mg}/\text{kg}$  iv bolus; PD-123319 was administered as a  $0.03\ \text{mg}/\text{kg}$  iv bolus followed by  $0.03\ \text{mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  iv infusion.

Fig. 4. Effects on duodenal mucosal alkaline secretion of the AT<sub>2</sub> receptor agonist CGP-42112A alone (0.1  $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) and in the presence of the AT<sub>2</sub> receptor antagonist PD-123319 (0.3 mg/kg iv bolus and 0.03 mg $\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  iv infusion) or the AT<sub>1</sub> receptor antagonist losartan (10 mg/kg iv bolus) Data shown are means  $\pm$  SE;  $n = 5$  in the first group and 6 in the 2 latter groups.



## DISCUSSION

The present study demonstrates the distribution of AT<sub>1</sub> and AT<sub>2</sub> receptors in the rat duodenal wall. It has been proposed that a local renin-angiotensin system is acting in the gastrointestinal tract, based on the demonstration of ANG II binding sites in the jejunal muscular layer and angiotensin-converting enzyme (ACE) in the mucosa (6). Sechi et al. (23) demonstrated AT<sub>2</sub> binding sites in the rat jejunal and ileal mucosa and showed that they account for ~15% of total binding sites for ANG II. Functional properties were also linked to these data when it was shown that the regulation of jejunal sodium and water absorption involves AT<sub>2</sub> receptors (12).

We showed previously (14) that ANG II is involved in the regulation of duodenal mucosal alkaline secretion during hypovolemia. It was observed that ANG II prolongs the sympathoadrenergic inhibition of mucosal alkaline secretion seen after moderate hypovolemia and that ANG II in this regard has a peripheral site of action. The present immunohistochemical analysis of the presence of AT<sub>1</sub> and AT<sub>2</sub> receptors further strengthens this hypothesis because staining for AT<sub>1</sub> receptors was detected in the duodenal wall, particularly associated with the lamina propria of the villi structures and the muscularis interna. Staining for AT<sub>1</sub> receptors was heavy, but staining for AT<sub>2</sub> receptors occurred as well, which is in line with earlier

studies (23). Because the quality of staining could differ between antibodies, quantitative estimations should be made with care. It is not possible to know from the images which structures are stained. Endothelial cells and smooth muscle cells in vessel walls but also fibroblasts are well-known sites for ANG receptors (1, 4). AT<sub>2</sub> receptors were present up to the midportion of the villi, whereas AT<sub>1</sub> receptors were also present in the villi tips. No staining was present within the epithelial cells. In the submucosa, some staining for both AT<sub>1</sub> and AT<sub>2</sub> receptors was observed, although considerably less than that within villi structures. The muscularis interna was stained for AT<sub>1</sub> receptors but showed almost no staining for AT<sub>2</sub> receptors.

In this study, we also investigated whether AT<sub>2</sub> receptors have any physiological correlation to duodenal mucosal alkaline secretion. In addition to ANG II, the alkaline secretion by the duodenal epithelium can be influenced by several other hormones, drugs, and

Table 2. Effect of losartan or PD-123319 on mean arterial pressure

	<i>n</i>	After 30-min Treatment	After 30-min CGP-42112A Infusion
No treatment	5	129 $\pm$ 5.8	128 $\pm$ 5.0
Losartan	6	128 $\pm$ 5.2	103 $\pm$ 6.6
PD-123319	6	129 $\pm$ 4.5	127 $\pm$ 2.7
Xylocaine and PD-123319	5	121 $\pm$ 4.6	119 $\pm$ 4.4

Values (in mmHg) are means  $\pm$  SE; *n*, no. of rats. Losartan was administered as a 10 mg/kg iv bolus; PD-123319 was administered as a 0.03 mg/kg iv bolus followed by 0.03 mg $\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  iv infusion.

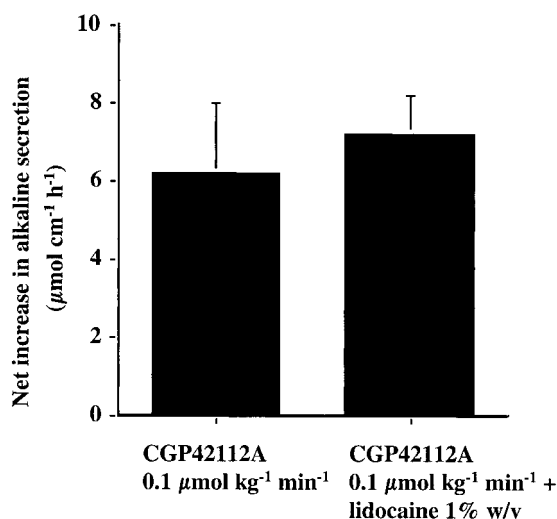


Fig. 5. Net increase in duodenal mucosal alkaline secretion after the AT<sub>2</sub> receptor agonist CGP-42112A (0.1  $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) alone and after treatment with lidocaine (1% wt/vol) on the serosal side of the duodenal segment. Data shown are means  $\pm$  SE;  $n = 5$  in both groups

neural activities. In the present study, it was observed that ANG II infusion alone (0.25–0.75  $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ ) had no effect on basal mucosal alkaline secretion or MAP, confirming a previous report (14). This infusion rate is within the range that has been reported to give physiological plasma levels of ANG II (18). However, when ANG II was infused in the presence of the non-peptide-selective AT<sub>1</sub> receptor antagonist losartan (27), mucosal alkaline secretion increased by ~50%. The increased mucosal alkaline secretion observed on ANG II infusion in losartan-treated animals was absent in the presence of the non-peptide-selective AT<sub>2</sub> antagonist PD-123319. The ability of AT<sub>1</sub> receptor blockade to reveal increased mucosal alkaline secretion in response to ANG II infusion could be explained in terms of “receptor unmasking,” i.e., enhanced response to an exogenous agonist by one receptor subtype when another subtype is blocked (24). It follows that the increased mucosal alkaline secretion observed after ANG II infusion in losartan-treated animals could be mediated by AT<sub>2</sub> receptors.

This observation is supported by the finding that the peptidergic-selective AT<sub>2</sub> agonist CGP-42112A raises alkaline secretion by ~50%. In low concentrations, CGP-42112A is a highly selective ligand to the AT<sub>2</sub> receptor. However, CGP-42112A at high concentrations occupies both AT<sub>1</sub> and AT<sub>2</sub> receptors (19). At the dose used in this study (0.1  $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ), CGP-42112A has been shown to act as a selective AT<sub>2</sub> agonist on jejunal water absorption (12, 13). The fact that the increase in mucosal alkaline secretion was absent in animals treated with PD-123319 but not in those receiving losartan strongly indicates that the effect of CGP-42112A in this dose range is mediated by AT<sub>2</sub> receptors.

As previously mentioned, this study demonstrates AT<sub>2</sub> receptors within the duodenal mucosa/submucosa. To show that ANG II increases duodenal mucosal alkaline secretion via AT<sub>2</sub> receptors located in the duodenal mucosa/submucosa and not elsewhere, e.g., within the central nervous system or on sympathetic nerves or ganglia (28), the local anesthetic lidocaine was applied topically onto the serosal side of the duodenal segment. By this administration route lidocaine has been shown to diffuse into the submucosa, and when applied topically in this way the compound mainly blocks nerve conductance in the myenteric plexus as well as the extrinsic nerves entering the intestinal segment via the mesenterium (2, 10). In these animals, basal mucosal alkaline secretion decreased by ~40%, but when CGP-42112A was infused intravenously the net increase in mucosal alkaline secretion was of the same order of magnitude as when it was infused in control animals. These data suggest a peripheral site of action for ANG II in AT<sub>2</sub> receptor-mediated mucosal alkaline secretion, probably within the duodenal mucosa/submucosa.

The staining in the lamina propria of the villi for both AT<sub>1</sub> and AT<sub>2</sub> receptors suggests receptor localization in proximity to blood vessels. Moderate changes in local blood flow cannot be excluded but seem unlikely

to influence duodenal mucosal alkaline secretion (16). The exact mediation of the increased alkaline secretion on AT<sub>2</sub> receptor stimulation remains to be further investigated. Holm (11) recently showed that duodenal mucosal alkaline secretion is regulated by the L-arginine-nitric oxide (NO) pathway, involving NO synthase within the surface epithelium, and Chen et al. (3) demonstrated that an ACE inhibitor increased alkaline secretion by a bradykinin-dependent pathway. Interestingly, the influence of these two pathways has been postulated regarding other functions related to AT<sub>2</sub> receptor activation (9, 25). Data obtained in this study, together with the observation by Johansson et al. (14) that ANG II prolongs the inhibition of mucosal alkaline secretion during hypovolemia, support the view that ANG II has a dual action on duodenal mucosal alkaline secretion. A counterregulatory function of AT<sub>1</sub> versus AT<sub>2</sub> receptors is, however, not exclusive for duodenal mucosal alkaline secretion. It has been suggested in, for instance, jejunal water absorption and renal wrap hypertension (12, 13, 26). The receptors seem to antagonize each other's actions to achieve both homeostasis at an individual level and tissue integrity at an organ level.

In summary, we have been able to show the distribution of AT<sub>1</sub> and AT<sub>2</sub> receptors within the duodenal wall of the rat. Our data suggest that activation of AT<sub>2</sub> receptors located in the duodenal mucosa/submucosa has a potent stimulatory action on duodenal mucosal alkaline secretion.

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