The bradykinin BK2 receptor mediates angiotensin II receptor type 2 stimulated rat duodenal mucosal alkaline secretion

Sara Ewert*, Berndt Johansson, Mathias Holm, Herbert F Helander and Lars Fandriks

Address: Department of Gastrointestinal Research, Sahlgrenska Academy, Gothenburg University, Gothenburg, Sweden

Email: Sara Ewert* - sara.ewert@gastro.gu.se; Berndt Johansson - berndt.johansson@gastro.gu.se; Mathias Holm - mathias.holm@gastro.gu.se; Herbert F Helander - herberthelander@hotmail.com; Lars Fandriks - lars.fandriks@gastro.gu.se

* Corresponding author

Abstract

Background: This study investigates bradykinin and nitric oxide as potential mediators of AT2-receptor-stimulated duodenal mucosal alkaline secretion. Duodenal mucosal alkaline secretion was measured in methohexital- and α-chloralose-anaesthetised rats by means of in situ pH-stat titration. Immunohistochemistry and Western blot were used to identify the BK2 receptors.

Results: The AT2 receptor agonist CGP42112A (0.1 µg kg⁻¹ min⁻¹) administered intravenously increased the duodenal mucosal alkaline secretion by ~50%. This increase was sensitive to the selective BK2 receptor blocker HOE140 (100 ng/kg iv), but not to luminal administration of the NOS blocker L-NAME (0.3 mM). Mean arterial pressure did not differ between groups during the procedures. Immunohistochemistry showed a distinct staining of the crypt epithelium and a moderate staining of basal cytoplasm in villus enterocytes.

Conclusion: The results suggest that the AT2-receptor-stimulated alkaline secretion is mediated via BK2 receptors located in the duodenal cryptal mucosal epithelium.

Background

Alkaline secretion by the duodenal mucosa is considered to be of great physiological importance for the mucosal defense against gastric acid. This secretion has been shown to be regulated by hormones, neuronal and paracrine mechanisms [1]. We have previously demonstrated that the renin-angiotensin system (RAS) and its key mediator angiotensin II (Ang II) influence rat duodenal mucosal alkaline secretion in vivo. Two principle angiotensin II receptor subtypes have been identified in the rat duodenal mucosa: the angiotensin II subtype 1 receptor (AT1 receptor) that mediates a secretory inhibition in concert with the sympathoadrenergic system [2], and the angiotensin II subtype 2 receptor (AT2 receptor) that stimulates the duodenal mucosal alkaline secretion [3]. The AT2 receptor is involved in the regulation of fluid and electrolyte transport in the jejunum via a nitric oxide- and cGMP-dependent pathway [4,5]. A similar arrangement exists in the kidney where involvement of bradykinin has been demonstrated [6,7]. Interestingly, both bradykinin (via bradykinin receptor type 2 (BK2 receptor)) and mucosal NO formation have been shown to regulate duodenal mucosal alkaline secretion [8,9]. The present study was undertaken to elucidate the potential involvement of these regulatory factors in AT2-receptor-stimulated duodenal mucosal alkaline secretion. Based on pharmacological interference it is here reported that bradykinin, but not epithelial NO formation, is a mediator of AT2-induced duodenal mucosal alkaline secretion. Therefore, an
additional aim was to locate bradykinin receptors in the duodenal wall.

Results

Alkaline secretion and mean arterial pressure

Duodenal mucosal alkaline secretion did not differ significantly between groups at baseline. Infusion of CGP42112A at a rate of 0.1 µg kg⁻¹ min⁻¹ (n = 7) increased (p < 0.05) mucosal alkaline secretion by about 50% as compared to controls (n = 6). With L-NAME present in the luminal perfusate at a concentration of 0.3 mM, and intravenous administration of CGP42112A (n = 6), the alkaline secretion increased (p < 0.05) similarly as the group treated only with the AT2 agonist. The group treated with HOE140 as a bolus injection (n = 6) 5 min prior to CGP42112A infusion did not exhibit any increase of the duodenal mucosal alkaline secretion, neither did controls (Figure 1). It follows that net change differed significantly from controls in the groups treated with CGP42112A alone, and in the presence of L-NAME in the perfusate, which was not the case in the HOE140-treated group. Mean arterial pressure was 130(4) mmHg (n = 24) and was not significantly different between groups at baseline or change significantly in any group during the protocol.

Distribution of BK2 receptors

Western blot showed a distinct band at the protein size 42 kDa representing specific immunostaining for the BK2 receptor in rat duodenal tissue (Figure 2). Immunohistochemical staining revealed the presence of the BK2
receptor in the mucosa of the duodenum (n = 8). Moderate staining revealed the presence of the BK2 receptor in the cytoplasm of the enterocytes along the entire villus length (Figure 3a,3b). The crypt epithelium was more strongly immunoreactive (Figure 3a,3c). Immunostaining was also detected in the Meissner and Auerbach nerve plexa (Figure 3d). Sections serving as negative controls (n = 8) were all unstained (Figure 3e).

Discussion

The present study confirms that intravenous administration of the peptidergic compound CGP42112A stimulates duodenal mucosal alkaline secretion suggesting that this secretion is under the control of the RAS [3]. The CGP42112A-mediated secretory effect was sensitive to the compound HOE140 indicating involvement of the BK2 receptor.

It has been reported from several organ systems, in particular the cardiovascular system and the kidney [13–17], that the AT2 receptor via bradykinin activates the L-arg/NO pathway. In the gut AT2-receptor activation increases fluid and sodium absorption and this process is dependent on NO and subsequent cGMP formation [4]. We have previously shown that duodenal mucosal alkaline secretion elicited by infusion of CGP 42112A at the dose 0.1 µg kg⁻¹ min⁻¹ can be blocked by PD 123319 [3] and is therefore considered to be an AT2 receptor mediated process. We have also demonstrated that mucosal acid exposure is dependent on villus epithelial NO production and can be blocked by intraluminal administration of L-NAME [9,18,19]. It should be noted that systemic administration of L-NAME elicits an upward shift of the basal duodenal mucosal alkaline secretion in the rat [18,20] reflecting other points of action for the compound and making it difficult to interpret additional effects on the secretion by other interferences. Intravenously administered L-NAME is therefore unsuitable, in our model, as a tool to elucidate the current discrete mechanisms influencing epithelial duodenal mucosal alkaline secretion, since the basal secretory levels will nearly overcome the upper limit for measurement of alkaline secretion. In the present study we found that intraluminally administered L-NAME, at a dose that blocks acid-induced alkaline secretion [18], did not inhibit CGP42112A induced secretion. It may be that the AT2-receptor-stimulated duodenal alkaline secretory response has a different pathway of mediation than the NO-dependent secretion that is induced by luminal acid. It has been suggested that the bicarbonate secreted from the villi and the crypts, respectively, are regulated via separate mechanisms [1].

Figure 2

Western blot BK2 receptor. Typical appearance of a Western blot showing a distinct band at the protein size 42 kDa representing specific immunostaining for the BK2 receptor in rat pituitary cell lysate, serving as a positive control, and in rat duodenal tissue.

It has been suggested that the bicarbonate secreted from the villi and the crypts, respectively, are regulated via separate mechanisms [1]. Apparently, the AT2 receptor does not act via the villus tip site for NO formation. Supporting this assumption is the fact that the AT2 receptors are located to the basal part of the villus core and not at the villus tip epithelium [3]. However, the possibility that NO formation at other sites within the intestinal wall is activated by AT2-receptor cannot be ruled out.

The nonapeptide bradykinin is the product of plasma kallikrein proteolytic action on kininogen in plasma. There are two main receptors for the peptide, BK1 and BK2. Most known effects of bradykinin are due to actions on the constitutively expressed BK2 receptor. The BK1 receptor expression is induced during inflammation and the receptors are mostly involved in prolonged inflammatory states. Bradykinin and kallikrein are both inactivated by kininase II (angiotensin converting enzyme). Pharmacological actions of bradykinin in the gastrointestinal tract include vasodilation (due to release of NO and PGI2), increased vascular permeability, and stimulation of epithelial ion and fluid transport [21,22] Bradykinin has been shown to be involved in longitudinal duodenal motor activity [23] and to elicit relaxant response in cultured duodenal smooth muscle cells [24]. Bradykinin induces duodenal mucosal alkaline secretion and the receptor has been proposed to be the BK2 receptor, since such secretory stimulation was blocked by intravenous administration of HOE140 [8]. The present finding that HOE140 blocked duodenal mucosal alkaline secretion elicited by CGP42112A suggests the formation and release of bradykinin in the duodenal wall following AT2-receptor activation. The BK2 receptor has previously been shown to be present in the epithelial membranes of rat jejunum and

receptor in the mucosa of the duodenum (n = 8).
Figure 3
**Immunohistochemistry BK2 receptor.** Section of rat duodenal wall showing immunoreactivity for the BK2 receptor (a). Staining is more marked in the crypt epithelium than in the villus epithelium. At higher magnification of the villus (b) immunoreactivity can be localised to the basal part of the cytoplasm in the absorptive cells. In the crypts of Lieberkühn (c) immunostaining is somewhat stronger and present in most epithelial cells. Immunoreactivity is also seen in the Auerbach nerve plexus (arrow) of the muscle layers. Negative control (d). Bar = 100 µm.
descending colon[25] and also to be located basally in the epithelium of the guinea pig ileum [26]. The present study demonstrates a general immunoreactivity to the BK2 receptor in the absorptive cells representing cytoplasmic staining. On the other hand, a stronger immunoreactivity was apparent in the crypt epithelium. The latter could be of functional significance for the duodenal mucosal alkaline secretion regarding the cryptal secretion of bicarbonate [1]. Intestinal location of AT2 receptors has previously been demonstrated [27,28]. In a recent study Johansson et al. [3] showed AT2-receptor immunoreactivity in the lamina propria at the villus base. Taken together, these findings suggest a spatial relation between the AT2 and BK2 receptors allowing for paracrine signalling. The cell types that express the AT2 receptors in the villus lamina propria are not known, nor to what extent these cells are the liberators of bradykinin in turn binding to the BK2 receptors of the crypt epithelial cells. However, in addition to the possibility of paracrine cell-to-cell signalling the vascular organisation also allows for compounds liberated in the subepithelial compartment of the villus to be transported downstream by the capillary blood flow towards the cryptal region [29]. In conclusion, although hypothetical so far, the topographical organisation of messenger receptors and the vascular arrangement support our functional findings as depicted in Figure 4. This design also explains the inability of a luminaly administered NOS inhibitor to block the duodenal mucosal alkaline secretion in response to an AT2-receptor agonist. The physiological significance of angiotensin II mediated effects on duodenal mucosal alkaline secretion remains to be elucidated. The AT2 receptor has been ascribed a counterregulatory function modulating actions by the AT1 receptor. As shown by Johansson et al [3], activation of the AT1 receptor prolongs the neural sympathoadrenergic inhibition of mucosal alkaline secretion, probably as part of a general restriction of volume and buffer excretion during stress on the circulatory system, e.g. blood loss. Restrictions of epithelial alkalisation in the upper gut increase the risk of mucosal injuries by gastric acid present in the luminal compartment. It may be speculated that the AT2 receptor, via a bradykinin dependent pathway, stimulates mucosa-protective alkaline secretion in order to offset this increased risk for autodigestive injury. The present results suggest that the AT2-receptor-stimulated alkaline secretion is mediated via BK2 receptors probably situated in the duodenal crypt epithelium.

Conclusions
The present results suggest that the AT2-receptor-stimulated alkaline secretion is mediated via BK2 receptors probably situated in the duodenal crypt mucosal epithelium.

Figure 4
Hypothetical depiction of mechanisms involved in CGP42112A-stimulated alkaline secretion. Hypothetical depiction of mechanisms involved in CGP42112A-stimulated alkaline secretion. Black arrows indicate alkaline secretions from crypts and the villi (†1-Flemstrom [1]). Red and blue arrows indicate precapillary and postcapillary blood flow, respectively (†2 – Jodal et al [29]). The location of AT2 receptors is indicated by dotted area (†4 - Johansson et al [3]). Orange areas indicate the location of BK2 receptors (†5 – Ewert et al, present study).

Methods
The study was approved by the Ethics Committee of Experiments on Animals, Goteborg University. Animals were housed in thermostatically controlled humidified rooms with a daylight-darkness cycle of 12 h and fed standard rat chow and water ad libitum.

Anaesthesia and surgical procedure
Experiments were performed on non-fasted male Sprague-Dawley rats weighing 300–350 g. Anaesthesia was induced by methohexital (60 mg/kg i.p.) and maintained by α-chloralose as a bolus injection (50 mg kg⁻¹) followed by a continuous infusion (25 mg kg⁻¹ h⁻¹). Lack of response to interdigital reflex stimuli confirmed an adequate anaesthetic condition. A thermostatically controlled heating pad and a lamp kept the body temperature at 38°C. Free airways were ensured by a catheter inserted into the trachea. The right femoral vein and artery were catheterised for drug infusions and measurement of blood
pressure (using a Statham P23Dc pressure transducer and a computer to obtain averages over 5-min periods), respectively. To avoid acidosis and compensate for basal needs and fluid losses due to the surgical trauma an isotonic buffered glucose (2.5%) solution was infused intrave-
nously (1 ml h⁻¹) throughout the experiments. A mid-
line laparotomy was performed. The common bile duct was catheterised 5 mm proximal to the papilla of Vateri and secretions of bile and pancreatic juice were collected outside the animal to avoid contamination of the duode-
nal perfusate. A duodenal segment (length 1.5 cm and proximal end 0.5 cm distal to the pylorus) was isolated between two glass tubes connected to a reservoir enclosed by a water jacket for maintenance of 38°C. Saline solution (150 mMNaCl) was perfused and recirculated through the reservoir and duodenal segment by means of a gas lift (air, 150 ml min⁻¹). Alkaline secretion into the luminal per-
 fusate was titrated to pH 7.40 by automatic infusion of isotonic HCl using a pH-stat equipment [10].

Immunohistochemistry
After the completed protocol, the midportion of the duode-
nal segment under study was removed and fixed in 4%
formaldehyde in phosphate buffered saline (pH 7.4) over
night. The specimens were subsequently dehydrated in ethanol, cleared in xylene and embedded in paraffin. Sections (4 µm) were cut and mounted on glass slides. The sections were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol and then boiled for 15 min in citrate buffer (pH 6.0) for antigen retrieval. The Immunocruz™ Staining System (Santa Cruz Biotechnolo-
gy Inc, CA, USA) was used for the immunohistochemistry
protocol. Endogenous peroxidase was quenched by 5 min
incubation in peroxidase blocking solution. Endogenous
biotin in the tissue was blocked using Biotin Blocking Sys-
tem (Dakopatts AB, Alvsjo, Sweden). Non-specific
binding was blocked by incubation in 5% non-fat dry
milk in PBS pH 7.5 and incubation in normal goat serum.
Sections were incubated over night at 4°C with mouse
monoclonal primary antibody specific for the BK2 recep-
tor (Transduction Laboratories, Lexington, USA) at a con-
centration of 5 µg/ml prior to incubation with secondary
antibody (anti-mouse IgG of goat origin) for 30 min at
room temperature. Sections serving as negative controls
were instead incubated with normal mouse IgG. Immu-
noreactivity was detected by means of horseradish-perox-
idase-streptavidin complex using diaminobenzidine as a
marker. Subsequently, the sections were dehydrated in
ethanol and xylene and finally mounted and covered by
glass slips.

Western blot analysis
In a separate series of experiments duodenal specimens of
full wall thickness were collected, immediately frozen in
liquid nitrogen and stored at -70°C. The specimens were
homogenized on ice (Polytron, Kinematica AG, Switzerland) in buffer A (10% glycerol, 20 mmol/L Tris-HCl pH
7.3, 100 mmol/L sodium chloride, 2 mmol/L phenyl-
methylsulfonyl fluoride, 2 mmol/L EDTA, 2 mmol/L EG-
TA, 10 mmol/L sodium orthovanadate, 10 µg/mL
leupeptin, and 10 µg/mL aprotenin) [11].

Centrifugation was performed at 30,000 g for 30 min at
4°C. The pellet was resuspended in buffer B (1% NP-40
(Sigma Chem Inc, St Louis, MO, USA) in buffer A) and
subsequently stirred at 4°C for one hour before centri-
fugation at 30,000 g for 30 min at 4°C. The supernatant was
analysed for protein content by the method of Bradford
[12] and stored at -70°C for further analysis. Samples were
diluted in SDS buffer and heated at 70°C for 10 min
before they were loaded on a NuPage 10% Bis-Tris gel and
then electrophoresed in a MOPS buffer (Invitrogen AB,
Lidingo, Sweden). One lane of each gel was loaded with
SeeBlue™ prestained molecular weight standards (Invitro-
gen AB) and one lane was loaded with rat pituitary cell
lysate (Transduction Laboratories) serving as a positive
control. After the electrophoresis the proteins were trans-
ferred to a polyvinylidifluoride membrane (Amersham,
Buckinghamshire, UK) which was incubated with a specif-
ic antibody of mouse origin directed against the BK2 re-
ceptor (Transduction Laboratories). An alkaline
phosphatase conjugated goat anti-mouse IgG2b (Santa
Cruz) and CDP-Star (Tropix, Bedford, MA, USA) as a sub-
strate were used to identify immunoreactive proteins by
chemiluminescence. Images were captured by a LAS-100
cooled CCD-camera (Fujifilm, Tokyo, Japan).

Experimental protocol
After surgery the animals were left undisturbed for 30
min. Subsequently, duodenal mucosal alkaline secretion
and mean arterial pressure were recorded during a 30 min
baseline period. Intravenous infusion of the AT2-receptor
agonist CGP42112A (0.1 µg kg⁻¹ min⁻¹) was initiated im-
mediately after the baseline period and maintained
throughout the protocol. In animals treated with L-
NAME, the drug was present (0.3 mM) in the doudenal
perfusate during baseline recordings. Animals treated
with HOE140 received this drug as an iv bolus injection
(100 ng/kg) 5 min prior to onset of CGP42112A infusion
(0.1 µg kg⁻¹ min⁻¹). Controls received only the saline v
vehicle. Animals were monitored for 60 min after the onset
of the drug infusions and then the experiment was
terminated.

Drugs
Methohexital (Brietal™, Lilly Inc., IN, USA) was dissolved
in saline solution (150 mM NaCl). α-chloralose (Kebo
Lab, Spanga, Sweden) was dissolved in tetaborate dec-
brydrate (Merck, Darmstadt, Germany) and titrated to pH
7.40. CGP42112A (peptidergic AT2-receptor
agonist)(Neosystem, Strasbourg, France), NG-nitro-L-arginine methyl ester hydrochloride (L-NAME) (Sigma) and HOE140 (Hoechst AG, Frankfurt, Germany) were each freshly dissolved in saline solution.

Statistics
Differences in duodenal mucosal alkaline secretion and in mean arterial pressure between groups were analysed by ANOVA and Bonferroni post-hoc test. Net change was defined as the difference between an average of the last 15-min period of drug administration and basal conditions. All data presented are means(SEM). A p-value < 0.05 was considered significant.

Authors' contributions
SE carried out animal experiments, molecular genetic and protein analysis, performed the statistical analysis and drafted the manuscript. HH and MH carried out animal experiments and participated in study design. LF conceived and coordinated the study and drafted the manuscript. All authors read and approved the final manuscript.

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