BMC Physiology

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Research article

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

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Published: 22 February 2003

BMC Physiology 2003, **3**:1

Received: 27 September 2002
Accepted: 22 February 2003

This article is available from: http://www.biomedcentral.com/1472-6793/3/1

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

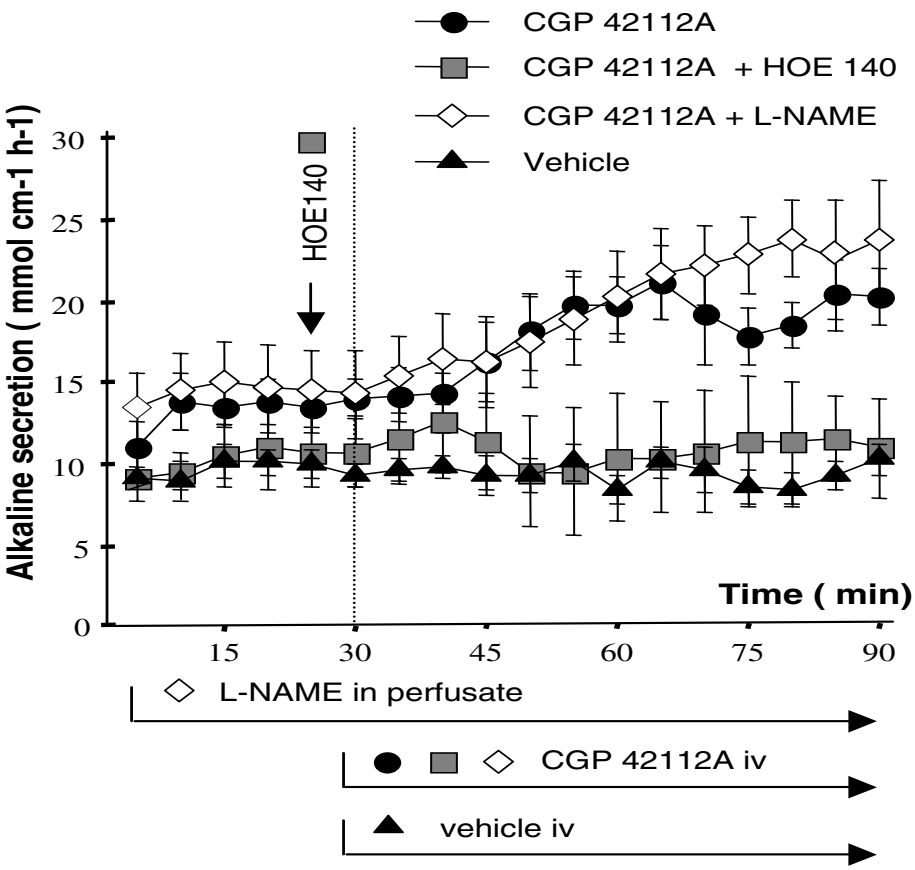

Figure 1

Alkaline secretion. Effects of intravenous administration of the AT2-receptor agonist CGP42112A (0.1 μg kg^{-1} min^{-1}) alone (n = 7), or combined with L-NAME (0.3 mM) in the luminal perfusate (n = 6) or intravenous injection of the BK2-receptor blocker HOE140 (100 ng/kg) (n = 5). Displayed also are untreated time controls receiving only vehicle (NaCl 150 mM) (n = 6). Data shown are means(SEM).

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

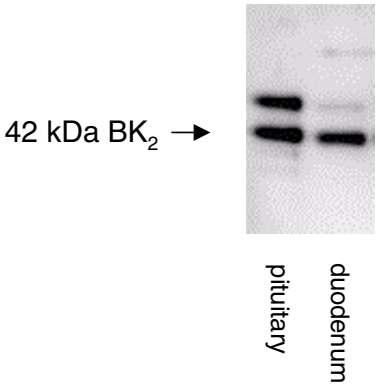

Figure 2
Western blot BK2 receptor. Typical appearance of a Western blot showing a distinct band at the protein size 42 kDa representing specific immunostaining for the BK2 receptor in rat pituitary cell lysate, serving as a positive control, and in rat duodenal tissue.

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].

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

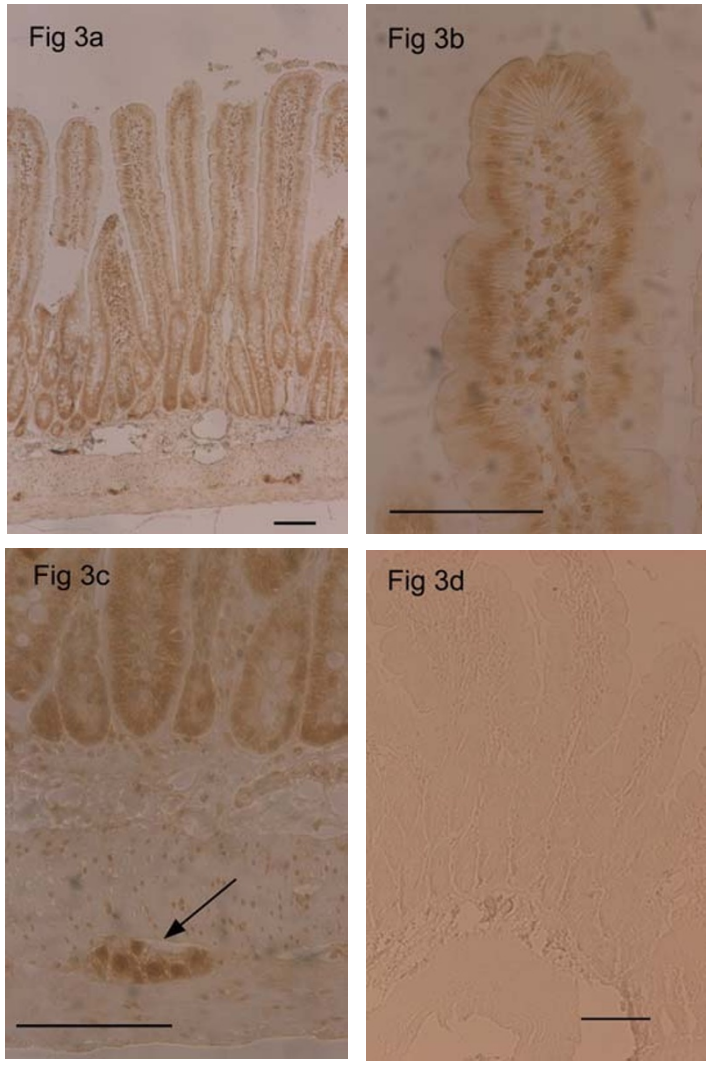

Figure 3
Immunohistochemistry BK2 receptor. Section of rat duodenal wall showing immunoreactivity for the BK2 receptor (a). Staining is more marked in the crypt epithelium than in the villus epithelium. At higher magnification of the villus (b) immunoreactivity can be localised to the basal part of the cytoplasm in the absorptive cells. In the crypts of Lieberkühn (c) immunstaining 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 cryptal 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 down-stream by the capillary bloodflow 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 luminally 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 prolong 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 alkalinisation 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 cryptal mucosal epithelium.

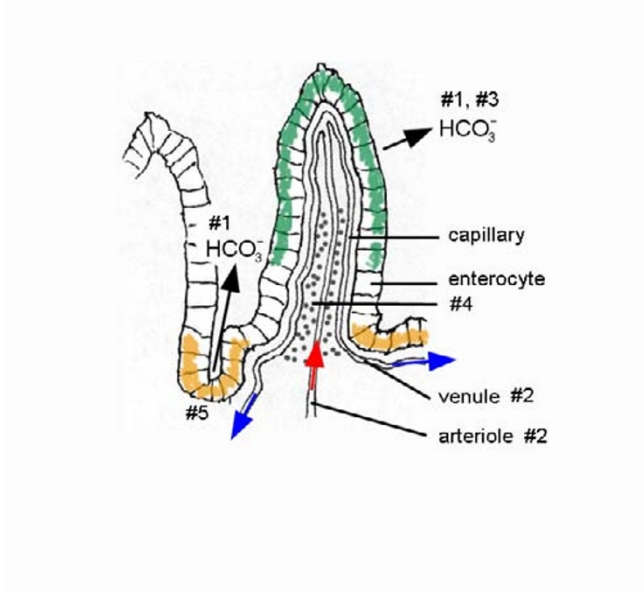

Figure 4
Hypothetical depiction of mechanisms involved in CGP42112A-stimulated alkaline secretion. Hypothetical depiction of mechanisms involved in CGP42112A-stimulated alkaline secretion. Black arrows indicate alkaline secretions from crypts and the villi (#1-Flemstrom [1]). Red and blue arrows indicate precapillary and postcapillary blood flow, respectively (#2 – Jodal et al [29]). Green area indicates the location of iNOS protein involved in acid-induced alkaline secretion by the villus epithelium (#3 -Holm et al [9,18]). 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 12h 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 intraarterially (1 ml h^{-1}) throughout the experiments. A midline 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 duodenal 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 perfusate 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 duodenal 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 Biotechnology 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 System (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 receptor (Transduction Laboratories, Lexington, USA) at a concentration 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. Immunoreactivity was detected by means of horseradish-peroxidase-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 phenylmethylsulfonyl fluoride, 2 mmol/L EDTA, 2 mmol/L EGTA, 10 mmol/L sodium orthovanadate, 10 μg/mL leupeptin, and 10 μg/mL aprotinin) [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 centrifugation 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 (Invitrogen AB) and one lane was loaded with rat pituitary cell lysate (Transduction Laboratories) serving as a positive control. After the electrophoresis the proteins were transferred to a polyvinyldifluoride membrane (Amersham, Buckinghamshire, UK) which was incubated with a specific antibody of mouse origin directed against the BK2 receptor (Transduction Laboratories). An alkaline phosphatase conjugated goat anti-mouse IgG2b (Santa Cruz) and CDP-Star (Tropix, Bedford, MA, USA) as a substrate were used to identify immunoreactive proteins by chemiluminescense. 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 immediately 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 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 tetraborate decahydrate (Merck, Darmstadt, Germany) and titrated to pH 7.40. CGP42112A (peptidergic AT2-receptor

agonist)(Neosystem, Strasbourg, France), N^G-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. BJ and MH carried out animal experiments and participated in study design. HH contributed to analysis of protein expression and production of images. LF conceived and coordinated the study and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Christina Ek for excellent laboratory assistance. This study was supported financially by the Swedish Medical Research Council (grant no. 8663), the Gothenburg Medical Society and the Knut and Alice Wallenberg foundation.

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