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ENDOGENOUS NEUROPEPTIDE Y DEPRESSES THE AFFERENT SIGNALING OF GASTRIC ACID CHALLENGE TO THE MOUSE BRAINSTEM VIA NEUROPEPTIDE Y TYPE Y2 AND Y4 RECEPTORS

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Abstract—Vagal afferents signal gastric acid challenge to the nucleus tractus solitarii of the rat brainstem. This study investigated whether nucleus tractus solitarii neurons in the mouse also respond to gastric acid challenge and whether this chemonociceptive input is modified by neuropeptide Y acting via neuropeptide Y receptors of type Y2 or Y4. The gastric mucosa of female mice was exposed to different concentrations of HCl or saline, excitation of neurons in the nucleus tractus solitarii visualized by c-Fos immunohistochemistry, gastric emptying deduced from the gastric volume recovery, and gastric lesion formation evaluated by planimetry. Relative to saline, intragastric HCl (0.15–0.35 M) increased the number of c-Fos-expressing cells in the nucleus tractus solitarii in a concentration-dependent manner, inhibited gastric emptying but failed to cause significant hemorrhagic injury in the stomach. Mice in which the Y2 or Y4 receptor gene had been deleted responded to gastric acid challenge with a significantly higher expression of c-Fos in the nucleus tractus solitarii, the increases amounting to 39 and 31%, respectively. The HCl-induced inhibition of gastric emptying was not altered by deletion of the Y2 or Y4 receptor gene. BIIE0246 ((*S*)-*N*^{2}-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6*H*)-oxodibenz[b,e] azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl] acetyl]-*N*-[2-[1,2-dihydro-3,5 (4*H*)-dioxo-1,2-diphenyl-3*H*-1,2,4-triazol-4-yl]ethyl]-argininamide; 0.03 mmol/kg s.c.), a Y2 receptor antagonist which does not cross the blood–brain barrier, did not modify the c-Fos response to gastric acid challenge. The Y2 receptor agonist peptide YY-(3-36) (0.1 mg/kg intraperitoneally) likewise failed to alter the gastric HCl-evoked expression of c-Fos in the nucleus tractus solitarii. BIIE0246, however, prevented the effect of peptide YY-(3-36) to inhibit gastric acid secretion as deduced from measurement of intragastric pH. The current data indicate that gastric challenge with acid concentrations that do not induce overt injury but inhibit gastric emptying is signaled to the mouse nucleus tractus solitarii. Endogenous neuropeptide Y acting via Y2 and Y4 receptors depresses the afferent input to the nucleus tractus solitarii by a presumably central site of action. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: nucleus of the solitary tract, neuropeptide Y Y2 receptor gene knockout, Y4 receptor gene knockout, Y2 receptor antagonist BIIE0246, c-Fos expression, gastric emptying.

Hydrochloric acid (HCl)-induced nociception in the rat stomach is mediated by vagal afferent neurons, since the visceromotor pain response to intragastric (IG) HCl administration is abolished by vagotomy (Lamb et al., 2003). This finding is consistent with the observation that gastric HCl challenge is signaled to the nucleus tractus solitarii (NTS) of the rat brainstem as shown by expression of the immediate early gene c-fos (Schuligoi et al., 1998; Danzer et al., 2004a). For the treatment of gastric pain it is hence relevant to know which endogenous factors control vagal afferent input to the NTS. It has been found that glutamate and tachykinins participate in the transmission between gastric vagal afferents and NTS neurons (Jocic et al., 2001) and morphine dampens gastric HCl-evoked c-fos transcription in the NTS (Schuligoi et al., 1998). Neuropeptide Y (NPY) may also modify vagal afferent signaling, because this neuropeptide (McLean et al., 1997; Lawrence et al., 1998; Thiele et al., 2000) and NPY receptors of the Y1, Y2 and Y4 type (Ergene et al., 1993; Gustafson et al., 1997; Larsen and Kristensen, 1997; McLean et al., 1997; Zhang et al., 1997; Dumont et al., 1998; Parker and Herzog, 1999; Glass et al., 2002; Kopp et al., 2002; Koda et al., 2005) are expressed both by certain vagal afferents and NTS neurons.

There is evidence that NPY modulates visceral afferent input to the NTS via activation of presynaptic NPY receptors of type Y2 (Y2 receptors) (Ergene et al., 1993) and stimulation of NPY receptors in the rat brainstem influences gastric motility, acid secretion and mucosal integrity (Humphreys et al., 1992; Chen et al., 1997; Yang et al., 1998, 1999; Yang, 2002). Since the NPY receptors prevailing in the NTS are of the Y2 and Y4 type (Gustafson et al., 1997; Larsen and Kristensen, 1997; Dumont et al., 1998; Parker and Herzog, 1999), we hypothesized that endogenous NPY acting via Y2 or NPY receptors of type Y4 (Y4 receptors) controls the gastric HCl-evoked input to the NTS. As this question was addressed with Y2 and Y4 receptor knockout mice, the first specific aim was to establish and characterize the experimental model of NTS

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Abbreviations: AEC, 3-amino-9-ethylcarbazole; ANOVA, analysis of variance; BIIE0246, (*S*)-*N*^{2}-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6*H*)-oxodibenz[b,e] azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl] acetyl]-*N*-[2-[1,2-dihydro-3,5 (4*H*)-dioxo-1,2-diphenyl-3*H*-1,2,4-triazol-4-yl]ethyl]-argininamide; DAB, 3,3-diaminobenzidine; EAAC1, excitatory amino acid transporter-1; HCl, hydrochloric acid; IG, intragastric; NPY, neuropeptide Y; NTS, nucleus tractus solitarii; PBS, phosphate-buffered saline; PYY, peptide YY; Y2 receptor, neuropeptide Y receptor of type Y2; Y4 receptor, neuropeptide Y receptor of type Y4.

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activation by gastric HCl challenge in the mouse. The effect of HCl on gastric emptying and injury was also assessed, given that in the rat excess IG HCl blocks gastric emptying (Holzer et al., 2003).

The second specific aim was to compare the effect of gastric HCl challenge on c-Fos expression in the NTS, gastric emptying and gastric lesion formation in control, Y2 and Y4 receptor knockout mice. In addition, we examined whether the neurochemical code of the NTS neurons that respond to gastric HCl challenge with c-Fos expression (Danzer et al., 2004b) differs between control and Y2 receptor knockout mice in terms of the codistribution of c-Fos with NPY and the excitatory amino acid transporter-1 (EAAC1, a marker of glutamatergic neurons). Finally, we investigated whether systemic administration of the Y2 receptor agonist peptide YY-(3-36), abbreviated PYY-(3-36) (Challis et al., 2003), and the Y2 receptor antagonist (*S*)-N^2-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6*H*)-oxodibenz[b,e] azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl] acetyl]-*N*-[2-[1,2-dihydro-3,5 (4*H*)-dioxo-1,2-diphenyl-3*H*-1,2,4-triazol-4-yl]ethyl]-argininamide (BIIE0246) modify (Doods et al., 1999) the gastric HCl-evoked c-Fos response in the NTS. The activity of BIIE0246 as a Y2 receptor antagonist was tested by its ability to prevent the inhibitory effect of PYY-(3-36) on gastric acid secretion.

EXPERIMENTAL PROCEDURES

Experimental animals

This study was carried out with female mice which were housed in groups of three to four per cage under controlled temperature (21 °C) and a 12-h light/dark cycle (lights on at 6:00 AM, lights off at 6:00 PM). All experiments were approved by an ethical committee at the Federal Ministry of Education, Science and Culture of the Republic of Austria and conducted according to the Directive of the European Communities Council of 24 November 1986 (86/609/EEC). The experiments were designed in such a way that the number of animals used and their suffering was minimized.

Studies 1, 2, 3 and 7 and part of study 6 were performed with outbred mice (strain Him:OF1, Abteilung für Labortierkunde und -genetik, Medical University of Vienna, Himberg, Austria) weighing 17–23 g. For studies 4 and 5 and part of study 6, germline Y2 and Y4 receptor knockout (Y2−/− and Y4−/−) mice and non-induced conditional Y2 and Y4 receptor knockout (FY2 and FY4) mice (Department of Pharmacology, Medical University of Innsbruck, Innsbruck, Austria) weighing 16–24 g were used. The generation of Y2−/−, Y4−/−, FY2 and FY4 mice has been described previously (Sainsbury et al., 2002a,b). Germline Y2−/− and Y4−/− mice were generated from the same founders on the same mixed C57BL/6-129SvJ background as the conditional FY2 and FY4 knockout mice. Germline Y2−/− and Y4−/− knockout mice were obtained by crossing chimeric mice carrying a Y2 floxed gene ($Y2^{lox/lox}$) or a Y4 floxed gene ($Y4^{lox/lox}$), respectively, with oozyte-specific Cre recombinase-expressing C57BL/6 mice (Sainsbury et al., 2002a,b). Non-induced conditional FY2 and FY4 knockout mice were used as controls in all experiments and termed control mice throughout the paper. As demonstrated before, these non-induced conditional $Y2^{lox/lox}$ and $Y4^{lox/lox}$ mice do not differ from wild-type mice, as the level of expression of Y2 and Y4 receptors is not influenced by the introduction of the loxP sites (Sainsbury et al., 2002a,b). The deletion or presence of Y2 and Y4 receptors in the germline and non-induced conditional knockout mice was verified by receptor autoradiography using [^{125}I]PYY_{3-36} and [^{125}I]PP, respectively, and *in situ* hybridization (Tschenett et al., 2003) as well as by polymerase chain reaction using oligonucleotide primers recognizing DNA sequences adjacent to the loxP sites flanking the deleted or residing Y2 and Y4 receptor gene (Sainsbury et al., 2002a,b).

Experimental protocols

In all studies, the mice were fasted for 20 h to ensure that the stomach was empty, but had free access to water, before HCl or physiological saline (0.15 M NaCl) was administered IG at a volume of 0.01 or 0.02 ml/g through a soft infant feeding tube (outer diameter 1.5 mm; Rüsch, Montevideo, Uruguay). After the IG treatment the animals were no longer allowed to drink until tissue collection 0.5, 1 or 2 h later. For this purpose, the mice were killed by i.p. injection of an overdose of pentobarbital (100 mg/kg).

Seven specific studies were carried out. The aim of study 1 which involved 26 Him:OF1 mice was to investigate how the c-Fos expression in the NTS after IG treatment with HCl depends on the time of examination post-treatment and on the volume of the IG HCl bolus. To this end, two groups of mice were treated IG with HCl (0.25 M; 0.02 ml/g) and the expression of c-Fos in the NTS examined 1 and 2 h later, respectively. In two other groups of Him:OF1 mice, HCl (0.25 M) was administered IG at volumes of 0.01 ml/g and 0.02 ml/g, respectively, and c-Fos in the NTS visualized 2 h post-treatment. In study 2, which was carried out with 24 Him:OF1 mice, the relationship between the IG concentration of HCl and the c-Fos expression in the NTS was investigated. For this purpose, the gastric mucosa was exposed to saline (0.15 M NaCl; 0.02 ml/g) or different concentrations of HCl (0.15, 0.25, 0.35 M; 0.02 ml/g) and the expression of c-Fos in the NTS determined 2 h post-treatment. Study 3 involved 66 Him:OF1 mice and addressed the relationship between IG concentration of HCl, duration of exposure to HCl, IG pH, gastric volume recovery (an indirect measure of gastric emptying) and gastric mucosal lesion formation. Specifically, mice were treated IG with saline (0.15 M NaCl; 0.02 ml/g) or different concentrations of HCl (0.15, 0.25, 0.35 M; 0.02 ml/g) 30, 60 or 120 min before IG pH, gastric volume recovery and gastric injury were quantified.

Study 4 was carried out with 59 mice to test whether the c-Fos expression in the NTS evoked by IG HCl treatment differs in FY2, FY4, Y2−/− and Y4−/− mice. For this purpose, saline (0.15 M NaCl; 0.02 ml/g) or HCl (0.25 M; 0.02 ml/g) was administered IG 2 h before the expression of c-Fos was determined in the NTS. In study 5, which involved 27 mice, analogous experiments were carried out to examine gastric volume recovery and gastric lesion formation in FY2, FY4, Y2−/− and Y4−/− mice 30 min after IG treatment with HCl (0.25 M; 0.02 ml/g).

Study 6 was performed with 24 Him:OF1 mice and 17 control (FY2 and FY4) mice, in which the effect of the Y2 receptor agonist PYY-(3-36) (Bachem, Basel, Switzerland) and the Y2 receptor antagonist BIIE0246 (Boehringer Ingelheim, Biberach, Germany) on the gastric HCl-evoked expression of c-Fos in the NTS was investigated. In the Him:OF1 mice BIIE0246 (0.03 mmol/kg; Chu et al., 2003) or its vehicle (30% polyethylene glycol 200 in distilled water) was injected s.c. 10 min before IG treatment with HCl (0.25 M; 0.02 ml/g). PYY-(3-36) (0.1 mg/kg; Challis et al., 2003) or its vehicle (saline) was injected intraperitoneally 5 min before IG treatment with HCl. The expression of c-Fos in the NTS was analyzed 60 min post-HCl. The control (FY2 and FY4) mice were injected s.c. with BIIE0246 (0.03 mmol/kg) or its vehicle 10 min before IG treatment with HCl (0.25 M; 0.02 ml/g), and the NTS was subjected to immunohistochemistry 2 h later. In study 7, 24 Him:OF1 mice were used to examine the effect of PYY-(3-36) on IG pH and gastric volume recovery in the absence and presence of BIIE0246. To this end, BIIE0246 (0.03 mmol/kg) or its vehicle was injected s.c. 10 min before IG treatment with saline (0.02 ml/g), while PYY-(3-36) (0.1 mg/kg) or its vehicle was injected intraperitoneally 5 min before IG treatment with saline. IG pH and gastric volume recovery were determined 30 min post-saline.