Mucosal acid challenge activates nitrergic neurons in myenteric plexus of rat stomach

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Schicho, Rudolf, Michael Schemann, Peter Holzer, and Irmgard T. Lippe. Mucosal acid challenge activates nitrergic neurons in myenteric plexus of the rat stomach. Am J Physiol Gastrointest Liver Physiol 281: G1316–G1321, 2001.—We tested the hypothesis that intrinsic neurons of the rat gastric myenteric plexus can be activated by an acid (HCl) challenge of the mucosa. Activated neurons were visualized by immunohistochemical detection of c-Fos, a marker for neuronal excitation. The neurochemical identity of the neurons activated by the HCl challenge was determined by colocalizing c-Fos with a marker for excitatory pathways, choline acetyltransferase (ChAT), and a marker for inhibitory pathways, nitric oxide synthase (NOS). Two hours after intragastric administration of HCl or saline, stomachs were removed and immunofluorescence triple labeling of myenteric neurons was carried out on whole mount preparations. Treatment with 0.35, 0.5, and 0.7 M HCl induced c-Fos in 8%, 56%, and 64%, respectively, of NOS-positive but not ChAT-positive neurons. c-Fos was also seen in glial cells of HCl-treated rats, whereas in saline-treated animals c-Fos was absent from the myenteric plexus. HCl treatment did not change the proportion of ChAT- and NOS-immunoreactive neurons in the myenteric ganglia. It is concluded that gastric acid challenge concentration-dependently stimulates a subpopulation of nitrergic, but not cholinergic, myenteric plexus neurons, which may play a role in muscle relaxation, vasodilatation, and/or secretion.

enteric nervous system; gastric mucosal acid challenge; c-Fos; triple labeling

Although the physiological functions of the enteric nervous system (ENS) in the control of gastrointestinal motility, circulation, secretion, and mucosal transport (7, 8, 11) are relatively well comprehended, the role of the ENS in pathophysiological processes of the gut, especially of the stomach, is less well studied. The stomach holds a unique position because it is located at a transition such as toxins, alcohol, and drugs. In addition, gastric acid and pepsin are major aggressive substances of endogenous origin and represent important factors in the etiology of peptic ulcer disease (21). To investigate the potential reaction of the ENS to a challenge of the gastric mucosa, we used an in vivo model in which the neuronal activation to intragastric administration of HCl was examined. Activated neurons were visualized by immunohistochemical demonstration of c-Fos. Stomachs were investigated 2 h after intragastric administration of HCl, when the c-Fos protein shows maximal expression (12).

We were particularly interested in examining the relationship between c-Fos expression and the concentration of intragastric HCl (0.35, 0.5, and 0.7 M) and in characterizing the neurochemical coding (10) of c-Fos-expressing enteric neurons. Although the coding and projection pattern of intrinsic neurons in guinea pigs have been thoroughly investigated (24, 30, 33, 37), the neurochemical coding of intrinsic neurons in the rat stomach is largely unknown. In the guinea pig stomach, nearly all neurons of the myenteric plexus can be stained for either choline acetyltransferase (ChAT) or nitric oxide synthase (NOS), which do not colocalize and are thus regarded as markers of two separate neuronal populations (33). Although ChAT-containing neurons are involved in excitatory pathways with preferentially ascending projections, NOS-containing neurons predominantly show descending projections and are involved in inhibitory pathways (24, 28, 30). Studies using NADPH diaphorase reaction and ChAT immunocytochemistry have shown that ChAT and NOS do not coexist in the rat gut either (26), although some colocalization has been reported in the rat esophagus (18). ChAT and NOS staining was therefore used to determine the principal chemical code of HCl-activated neurons in the rat stomach. The precise relationship and possible overlap between these stains was assessed by labeling of c-Fos, ChAT, and NOS. In addition, glial fibrillary acidic protein (GFAP) was double-labeled with c-Fos to examine the reaction of enteric glia to the acid insult, because glial cells have been demonstrated to express c-Fos in response to noxious stimuli (22).

METHODS

Experiments were carried out on female Sprague-Dawley rats (180–220 g) that were fasted for 20 h but had free access The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
to water. HCl (0.35, 0.5, 0.7 M) was administered intragastrically at a volume of 10 ml/kg by use of a pediatric feeding tube (Portex, Hythe, UK) as described by Schuligoi et al. (35) (n = 7). Control animals received saline (n = 6). After 2 h rats were killed by an overdose of intraperitoneal pentobarbital, and stomachs were immediately removed, washed in ice-cold oxygenated 0.1 M PBS (pH 7.4) containing 1 μM nifedipine (Sigma), and cut open along the lesser and greater curvature.

**Tissue preparation.** The stomachs were divided into two parts, which were stretched and pinned flat on a petri dish with a silicon elastomer bottom, fixed overnight in Zamboni's parts, which were stretched and pinned flat on a petri dish (Sigma), and cut open along the lesser and greater curvature.

**Immunochemistry.** After an initial wash in 0.1 M PBS-azide (pH 7.4) tissues were preincubated in a solution of 4% donkey serum, 0.5% Triton X-100, and PBS-azide for 1 h to block nonspecific binding. Antibodies were diluted in a solution containing the same substances. Single, double, and triple labeling were performed by incubating tissues overnight at room temperature using antibodies against c-Fos, ChAT, neuronal NOS (nNOS), GFAP, and neuronal HuC/HuD antigen. After a wash in PBS-azide (3 × 10 min), preparations were exposed for 1.5 h to the species-specific fluorophore-conjugated secondary antibodies (Cy2- or Cy3-conjugated anti-mouse IgG, Cy3-conjugated anti-goat IgG, AMCA- or Cy2-conjugated anti-rabbit IgG; Jackson ImmunoResearch). After a final wash in 0.1 M PBS-azide, preparations were mounted on poly-L-lysine-coated slides and coveredslipped with PBS-glycerol. Negative controls were performed by leaving out the primary antibodies during the staining procedure. Blocking of the CHAT antiserum by preabsorption with ChAT rat recombinant protein (Chemicon, Temecula, CA) before the immunohistochemical procedure completely abolished immunostaining. The specificities for all other primary antibodies have been demonstrated elsewhere (see references in Table 1).

Whole mount preparations were examined under a fluorescence microscope (Olympus IX 70) equipped with four separate filter cubes (wide band cube for AMCA: DM400, excitation filter BP330–385, barrier filter BP460–490; narrow band cube for Cy2: DM505, excitation filter BP470–490, barrier filter BA515–550; Cy3 cube: DM568, excitation filter 540–560, barrier filter 575–645) and ×40 and ×60 water-immersion objectives to examine colocalization. No cross fluorescence was observed when fluorophores were examined through a filter cube unsuitable for the emitting wavelength of the fluorophore. Images were taken by a high-resolution digital camera (Olympus DP 50; 2,776 × 2,074 pixels) and processed using SIS image analysis software (Münster, Germany).

**Counting procedures and statistical analysis.** The number of immunoreactive neurons or nuclei was counted in 30–50 ganglia of the myenteric plexus in each animal. Only a clearly defined group of neurons was considered as a myenteric ganglion, whereas single cells lying in internodal strands were not counted. The total neuronal population of one ganglion was determined by staining for the neuronal HuC/HuD antigen and set at 100%. All other neuronal populations are expressed as percentages of this population. Data are expressed as means ± SD for all ganglia investigated and evaluated by ANOVA and multiple-comparison test (Student-Newman-Keuls or Dunnett’s method) using SigmaStat software. Probability P values <0.05 were regarded as significant.

**RESULTS**

**Hu neuronal protein, c-Fos, ChAT, and NOS in saline-treated rats.** Antibodies to the neuronal HuC/HuD antigen were used to determine the total number of neurons present in the gastric corpus myenteric ganglia of saline-treated rats. On average, ganglia contained 33.6 ± 11.9 neurons (Fig. 1A). ChAT was counted in 21 ± 5.7 neurons/ganglion (62.5%), whereas NOS was present in 8.4 ± 3.1 neurons/ganglion (25%). In the majority of neurons, ChAT and NOS were not colocalized, except for a small population of 3.9% somata (1.3 ± 1.1 neurons/ganglion; Fig. 1B–D). Immunostaining for NOS appeared to be weaker in cell bodies coexpressing CHAT than in CHAT-negative neurons (Fig. 1C). Administration of saline did not cause corpus myenteric neurons and glial cells to express c-Fos.

**c-Fos, ChAT, NOS, and GFAP in acid-treated rats.** Intragastric administration of 0.35, 0.5, and 0.7 M HCl induced c-Fos in 2%, 12%, and 16%, respectively, of corpus myenteric plexus neurons (0.7 ± 1.1, 4.1 ± 2, 5.4 ± 1.9 nuclei/ganglion; Figs. 2A and 3) and also in nuclei of glial cells (Fig. 2, A and E). Triple labeling with antibodies to c-Fos, NOS, and ChAT revealed that c-Fos was present in NOS-positive neurons but not in ChAT/positive or ChAT/NOS-coexpressing neurons (Fig. 2D). Of the NOS-positive neurons, 8%, 56%, and 64% showed expression of c-Fos after intragastric application of 0.35, 0.5, and 0.7 M HCl, respectively. The relative proportion of ChAT (20 ± 7.4 cells/ganglion; 59.5%), NOS (7.7 ± 3.5 cells/ganglion; 22.9%), and ChAT/NOS (1.5 ± 1.3; 4.5%)-positive neurons in HCl (0.5 M)-treated animals did not differ from that seen after saline administration.

**DISCUSSION**

This study has shown that exposure of the rat gastric mucosa to HCl activates a subpopulation of NOS-positive myenteric plexus neurons, as determined by immunohistochemical localization of c-Fos. Depending on the applied HCl concentration (0.35, 0.5, and 0.7 M), 8–64% of NOS-positive neurons displayed c-Fos-immunoreactive nuclei, whereas ChAT-positive and ChAT/NOS-coexpressing neurons did not respond to the mu-
Fig. 1. Neuronal Hu antigen (A) and choline acetyltransferase (ChAT) and nitric oxide synthase (NOS) (B–D) immunofluorescence in the myenteric plexus of the gastric corpus taken from saline-treated rats. A: individual ganglia in the myenteric plexus stained for the neuronal marker Hu using Cy3 as fluorochrome. Calibration bar = 200 μm. B–D: matching fields of the same ganglion double-labeled for ChAT (B, red) and NOS (C, green) using Cy3- and Cy2-conjugated secondary antibodies. Arrows in B and C denote a neuron coexpressing ChAT and NOS, which displays weaker immunoreactivity for NOS than the other NOS-positive cells. D: B and C were superimposed to show that ChAT-positive (red) and NOS-positive (green) neurons are not colocalized except for a single neuron (arrow). Calibration bar = 50 μm.

Fig. 2. c-Fos, ChAT, NOS, and glial fibrillary acidic protein (GFAP) immunofluorescence in the myenteric plexus of the gastric corpus after intragastric administration of 0.5 M HCl. A–D show matching fields of the same myenteric ganglion after triple labeling for c-Fos, ChAT, and NOS. A: acid-induced c-Fos expression in neurons (arrows) and in glial cells (arrowheads; Cy2 label). B: ChAT-positive neurons in the same ganglion (Cy3 label) C: NOS-positive neurons of the same ganglion (AMCA label). D: A–C were superimposed to show that c-Fos is exclusively expressed in NOS-positive neurons. Calibration bar = 50 μm. E: detail of a myenteric ganglion after double labeling for GFAP (Cy2 label, green) and c-Fos (Cy3 label, red) showing c-Fos immunoreactivity in nuclei of glial cells (arrows). Calibration bar = 50 μm.
The expression of c-Fos in the rat stomach and intestine has previously been used to investigate enteric neuron responses to various in vivo stimuli such as electrical stimulation of the vagal nerve (39), application of cholecystokinin (32), injection of formalin into the colonic wall (22), and intestinal anaphylaxis (23). In the present study, we have used three concentrations of HCl that are increasingly capable of breaking the mucosal barrier (35). The more the gastric mucosal epithelium is ablated (Fig. 3; Ref. 35), the more HCl is likely to diffuse into the lamina propria, where it may come in contact with proton-sensitive receptors on enteric nerve terminals. It remains to be investigated whether acid-induced c-Fos expression in gastric myenteric neurons involves paracrine mediators like serotonin, which is released by acid challenge of the rat gastric mucosa (38) and induces c-Fos in presumably intrinsic sensory neurons of the guinea pig intestine (17). Although acid can excite intrinsic sensory neurons of the guinea pig small intestine (3), electrophysiological studies have shown that AH type II neurons, thought to be intrinsic sensory afferents in the guinea pig intestine (9), are absent from the guinea pig stomach and guinea pig stomach (16, 26, 33), although species differences between the rat and the guinea pig are noticeable. Whereas in the gastric corpus of the guinea pig NOS- and ChAT-positive somata represent two completely separate populations of neurons (33), the two enzymes are colocalized in ~4% of the myenteric neurons in the rat stomach. Furthermore, the total number of NOS- and ChAT-positive somata in the rat stomach was less than the total number of somata stained for the panneuronal marker HuC/HuD, whereas in the guinea pig stomach NOS and ChAT neurons account for the total population of neurons in glandular mucosa. Values represent means; n = 6–8 animals. *P < 0.01, 0.5 M vs. 0.35 M HCl; **P < 0.01, 0.7 M vs. 0.5 M HCl.
each ganglion (33). This indicates the presence of a population of non-NOS/non-ChAT neurons in the rat stomach.

In summary, we have demonstrated that nitrergic, but not cholinergic, myenteric plexus neurons in the rat gastric corpus respond to mucosal acid challenge with a concentration-dependent expression of c-Fos. These activated neurons may represent inhibitory motor neurons, vasodilator neurons, and/or secretomotor neurons, and may thus belong to a neuronal circuit that participates in mechanisms of gastric mucosal protection.

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