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.

METHODS

Experiments were carried out on female Sprague-Dawley rats (180–220 g) that were fasted for 20 h but had free access to water. 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 patterns of enteric neurons in the guinea pig stomach 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).

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 the entrance of the alimentary canal and therefore exposed to many harmful substances of exogenous origin 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...
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 (Sigma), and cut open along the lesser and greater curvature.

After an initial wash in 0.1 M PBS-azole (pH 7.4) tissues were preincubated in a solution of 4% donkey serum, 0.5% Triton X-100, and PBS-azole 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-azole (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-azole, preparations were mounted on poly-L-lysine-coated slides and coverslipped 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).

**Table 1. Primary antibodies used for immunohistochemistry**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Code</th>
<th>Species</th>
<th>Dilution</th>
<th>Source (Reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChAT</td>
<td>AB144P</td>
<td>Goat</td>
<td>1:100</td>
<td>Chemicon (37)</td>
</tr>
<tr>
<td>NOS</td>
<td>2F161</td>
<td>Rabbit</td>
<td>1:2,000</td>
<td>Dr. B. Mayer (1)</td>
</tr>
<tr>
<td>c-Fos</td>
<td>1G11</td>
<td>Mouse</td>
<td>1:500</td>
<td>Dr. K. Sharkey (23)</td>
</tr>
<tr>
<td>HuC/HuD</td>
<td>1G11</td>
<td>Mouse</td>
<td>20 μg/ml</td>
<td>Molecular Probes (20)</td>
</tr>
<tr>
<td>GFAP</td>
<td>Z 0334</td>
<td>Rabbit</td>
<td>1:20,000</td>
<td>DAKO (6)</td>
</tr>
</tbody>
</table>

ChAT, choline acetyltransferase; NOS, nitric oxide synthase; GFAP, glial fibrillary acidic protein.

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% noreactive 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.
Brain stem (25, 35) and because electrical stimulation challenge activates vagal afferents projecting to the sigmoid (13). Activation of NOS-positive neurons may also sensitize nerve fibers known to react to acid backdiffusion in the gastric myenteric plexus may involve mediators released from extrinsic glands (9), are absent from the guinea pig stomach (13). By agreement with results from other studies of the rat 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 4–10% of the myenteric neurons (15, 29). Such a binding site was recently proposed for the nNOS gene (31), which means that c-Fos induction in nitricergic neurons could signify enhanced transcription of transmitter-producing enzymes including NOS.

As an inhibitory transmitter of nonadrenergic, noncholinergic neurons, nitric oxide is involved in muscle relaxation (19), vasodilatation (13), acid secretion (36), and mucus secretion (5, 27). Because these processes are part of the protective mechanisms against acid-induced injury of the mucosa (13), nitricergic neurons of the gastric myenteric plexus, which express c-Fos in response to mucosal acid challenge, may form an integral part of the neural alarm and protection system in the stomach (13).

The relevance of the acid-induced expression of c-Fos in glial cells is not yet clear. It should be considered in this context that enteric glia have been shown to proliferate during inflammation (4) and are thought to play an important role in inflammatory processes of the small intestine (6).

The counts of ChAT-positive (62.5%) and NOS-positive (25%) in control animals are in fairly good agreement with results from other studies of the rat 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 neurons 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 the stomach.
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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REFERENCES


