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

RUDOLF SCHICHO,1 MICHAEL SCHEMANN,2 PETER HOLZER,1 AND IRMGARD T. LIPPE1

1Department of Experimental and Clinical Pharmacology, University of Graz, A-8010 Graz, Austria; and 2Department of Physiology, School of Veterinary Medicine, D-30173 Hannover, Germany

Received 29 March 2001; accepted in final form 28 June 2001

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 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 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).

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 solution containing the same substances. Before immunohistochemistry, whole mounts of the gastric corpus were prepared by removing the mucosa and the circular muscle layer under a dissecting microscope to expose the myenteric plexus.

**Immunohistochemistry.** 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 coverslipped with PBS-glycerol. Negative controls were performed by leaving out the primary antibodies during the staining procedure. Blocking of the ChAT antisera 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. 1, B–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). Double 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-

---

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

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Code</th>
<th>Host</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></td>
<td>Chemicon (37)</td>
</tr>
<tr>
<td>NOS</td>
<td></td>
<td>Rabbit</td>
<td>1:2,000</td>
<td></td>
<td>Dr. B. Mayer (1)</td>
</tr>
<tr>
<td>c-Fos</td>
<td>TF161</td>
<td>Mouse</td>
<td>1:500</td>
<td></td>
<td>Dr. K. Sharkey (23)</td>
</tr>
<tr>
<td>HuC/HuD</td>
<td>16A11</td>
<td>Mouse</td>
<td>20 μg/ml</td>
<td></td>
<td>Molecular Probes (20)</td>
</tr>
<tr>
<td>GFAP</td>
<td>Z 0334</td>
<td>Rabbit</td>
<td>1:20,000</td>
<td></td>
<td>DAKO (6)</td>
</tr>
</tbody>
</table>

ChAT, choline acetyltransferase; NOS, nitric oxide synthase; GFAP, glial fibrillary acidic protein.
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 of the vagal nerve (13). Activation of NOS-positive neurons may also be observed in sensory nerve fibers known to react to acid backdiffusion (15). Although acid can excite intrinsic sensory neurons of the guinea pig intestine (3), electrophysiological studies have shown that AH type II neurons, which are unlikely to be sensory neurons, are involved in nonadrenergic, noncholinergic neural mechanisms that include 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), nitrergic 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).

Figure 3 compares the acid concentration-dependent expression of c-Fos in the myenteric plexus, determined 2 h after HCl challenge, with the extent of epithelial ablation determined 45 min after HCl in a previous study (35). Although the time of measurement is different, this comparison is valid because the acid-induced mucosal damage appears to be maximal 45 min after HCl and has significantly diminished by 2 h (P. Holzer, E. Bock, M. A. Pabst, unpublished data). As can be seen, there is a dissociation of c-Fos induction from formation of epithelial injury, which is most conspicuous at the concentration of 0.5 M HCl. With this treatment, c-Fos expression appears to be stimulated to a submaximal degree while the extent of epithelial ablation is only ~10%. It therefore seems that the acid challenge itself rather than the acid-induced injury determines the extent of myenteric c-Fos expression. A similar conclusion was previously reached for the gastric acid-evoked transcription of c-fos in the rat brain stem (14).

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%) cells 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 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 corpus (34). If this also reflects the situation in the rat gastric corpus, the c-Fos-positive nuclei seen here are unlikely to be sensory neurons.

Further pathways whereby a mucosal acid challenge induces c-Fos in neurons of the gastric myenteric plexus may involve mediators released from extrinsic sensory nerve fibers known to react to acid backdiffusion (13). Activation of NOS-positive neurons may also derive from a vagal reflex because gastric mucosal challenge activates vagal afferents projecting to the brain stem (25, 35) and because electrical stimulation of vagal afferents leads to a preferential activation of NADPH diaphorase-containing neurons in the gastric myenteric plexus (2).

Fos regulates the production of transmitter-synthesizing enzymes such as ChAT by acting at the AP1-binding site in the promoter region of the target gene (15, 29). Such a binding site was recently proposed for the nNOS gene (31), which means that c-Fos induction in nitrergic 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), nitrergic 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).
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.

Antibodies to c-Fos and NOS were kindly provided by Dr. K. Sharkey and Dr. B. Mayer, respectively. We thank Evelyn Painsipp and Birgit Brodlacz for excellent technical assistance.

This work was supported by the Jubilee Foundation of the Austrian National Bank (Grant 7843) and the Austrian Science Fund (FWF Grant P14295-MED).

REFERENCES