Mast cell-dependent excitation of visceral-nociceptive sensory neurons in irritable bowel syndrome.

Mast Cell-Dependent Excitation of Visceral-Nociceptive Sensory Neurons in Irritable Bowel Syndrome

GIOVANNI BARBARA,* BINGXIAN WANG,‡ VINCENZO STANGHELLINI,* ROBERTO DE GIORGIO,* CESARE CREMON,* GIOVANNI DI NARDO,* MARCELLO TREVISANI,§ BARBARA CAMPI,§ PIERANGELO GEPPETTI,§ MARCELLO TONINI,‖ NIGEL W. BUNNETT,¶ DAVID GRUNDY,# and ROBERTO CORINALDESI*

*Department of Internal Medicine and Gastroenterology and CRBA, University of Bologna, Italy; ‡Department of General Surgery, University of Tuebingen, Germany; §Department of Clinical and Experimental Medicine, University of Ferrara, Italy; ‖Department of Physiological and Pharmacological Sciences, University of Pavia, Italy; ¶Departments of Surgery and Physiology, University of California, San Francisco, California; #Department of Biomedical Science, The University of Sheffield, England

Background & Aims: **Intestinal mast cell infiltration may participate to abdominal pain in irritable bowel syndrome (IBS) patients. However, the underlying mechanisms remain unknown. We assessed the effect of mast cell mediators released from the colonic mucosa of IBS patients on the activation of rat sensory neurons in vitro.** ***Methods:*** **Colonic mast cell infiltration and mediator release were assessed with quantitative immunoflorescence and immunoenzymatic assays. The effect of mucosal mediators was tested on mesenteric sensory nerve firing and Ca^{2+} mobilization in dorsal root ganglia in rats.** ***Results:*** **Mediators from IBS patients, but not controls, markedly enhanced the firing of mesenteric nerves (14.7 ± 3.2 imp/sec vs 2.8 ± 1.5 imp/sec; $P < .05$) and stimulated mobilization of Ca^{2+} in dorsal root ganglia neurons (29% ± 4% vs 11% ± 4%; $P < .05$). On average, 64% of dorsal root ganglia responsive to mediators were capsaicin-sensitive, known to mediate nociception. Histamine and tryptase were mainly localized to mucosal mast cells. IBS-dependent nerve firing and Ca^{2+} mobilization were correlated with the area of the colonic *lamina propria* occupied by mast cells ($r = 0.74$; $P < .01$, and $r = 0.78$; $P < .01$, respectively). IBS-dependent excitation of dorsal root ganglia was inhibited by histamine H_1 receptor blockade and serine protease inactivation (inhibition of 51.7%; $P < .05$ and 74.5%; $P < .05$; respectively).** ***Conclusions:*** **Mucosal mast cell mediators from IBS patients excite rat nociceptive visceral sensory nerves. These results provide new insights into the mechanism underlying visceral hypersensitivity in IBS.**

Irritable bowel syndrome (IBS) is a chronic functional bowel disorder characterized by abdominal pain and disturbed bowel habits that are not accompanied by underlying structural or biochemical changes detectable with diagnostic techniques.[1] IBS is common in western societies including the United Kingdom and the United States, where prevalence approaches 20%,[2,3] and IBS accounts for ~10% of family physician visits and 30% of gastroenterology practice.[4,5] Thus, IBS is an economic burden to society for direct (eg, diagnosis, therapy) and indirect (eg, work absenteeism) costs.[5] The pathophysiology of IBS is poorly defined, which explains the limited efficacy of current treatments.

Abdominal pain, an essential symptom of IBS,[1] correlates with the severity of the disease[6] and contributes to the patient's poor quality of life.[7] Both central (ie, central nervous system) and peripheral mechanisms (ie, gastrointestinal tract) are thought to contribute to the origin of abdominal pain in IBS.[5] Peripheral mechanisms of abdominal pain may involve an increased sensitivity of the intestinal wall to local stimuli (ie, visceral hypersensitivity),[8] which results in increased activation of splanchnic afferent nerves[9] and erroneous activation of nociceptive brain regions.[10] However, the causes of visceral hypersensitivity are unknown.

Mediators from intestinal mast cells may play a crucial role in visceral hypersensitivity of IBS patients.[11,12] Mast cells are sentinels of the immune system that are strategically located at the host–environment interface[13] in close proximity to sensory nerves.[14] Multiple factors (eg, bacteria, parasites, viruses, toxins, complements, cytokines, endogenous peptides) can activate mast cells to promote degranulation and immediate (minutes) release of preformed mediators from cytoplasmic granules (eg, histamine, tryptase, proteoglycans) or the de novo synthesis of mediators [eg, leukotriene C_4, platelet activating factor, prostaglandin (PG)D_2].[15,16] These mediators can activate sensory nerves, including those innervating the gastrointestinal tract, leading to visceral hyperalgesia/allodynia.[11,12,17,18] We have recently demonstrated a marked increase in colonic mucosal area occupied by mast cells in IBS patients compared to healthy subjects. Mast cells in IBS patients were frequently degranulated, and there was increased spontaneous release of histamine

Abbreviations used in this paper: 5-HT, 5-hydroxytryptamine; ANOVA, analysis of variance; DRG, dorsal root ganglia; IBS, irritable bowel syndrome; NSE, neuronal specific enolase.

0016-5085/07/$32.00
doi:10.1053/j.gastro.2006.11.039

and tryptase. The proximity of activated mast cells to mucosal nerve fibers correlated with the frequency and severity of abdominal pain.[19] Nonetheless, mechanisms by which mast cells could induce visceral hypersensitivity in IBS patients are completely unknown.

We investigated the mechanisms by which mast cells from IBS patients may activate visceral sensory nerves. To do so, we obtained mediators spontaneously released from the colonic mucosa of patients with IBS and healthy controls and tested their effects on mesenteric sensory nerve fibers supplying the gut and Ca^{2+} mobilization in isolated dorsal root ganglion cells (DRG) in rats in vitro.

Materials and Methods

Patients

Twenty-nine consecutive patients with IBS (aged 19–70 years; mean 39.0 ± 10.4 years; 18 females) as well as 15 controls (aged 22–30 years; mean 25.7 ± 3.8 years; 9 females) participated in the study. IBS patients were all seen in the Department of Internal Medicine and Gastroenterology of the University of Bologna and diagnosed according to the Rome II criteria.[1] Controls were recruited by public advertisement and included in the study after thorough exclusion of gastrointestinal symptoms. Exclusion criteria included also the use of nonsteroidal anti-inflammatory drugs, corticosteroids, and mast cell stabilizers, major abdominal surgery, celiac disease (excluded by detection of antitransglutaminase and antiendomysial antibodies), allergic diseases, including asthma (family and personal history and specific anti-IgE antibodies) and other organic or severe psychiatric disorders as assessed by history taking and appropriate consultations and laboratory tests. Patients and controls gave written informed consent and the study protocol was approved by the local Ethic Committee and conducted in accordance with the Declaration of Helsinki.

Each patient completed an Italian[19] modified version of the Bowel Disease Questionnaire to evaluate symptoms.[20] Patients were asked to score frequency and severity of their symptoms over the last 2 weeks before interview, as previously described.[19]

All participants underwent left colonoscopy after cleansing of the distal colon with a 500-mL water enema performed the evening before and the morning of the procedure. In all cases we obtained 6 mucosal biopsies from the proximal descending colon. Two biopsies were used for routine H&E histology and immunohistochemistry. Four biopsies were used for histamine, tryptase, and PGE_2 assays and for mucosal mediators release experiments (see below).

Histology, Immufluorescence and Electron Microscopy

Biopsies were fixed in buffered 10% formalin and processed for either H&E histology or immunofluorescence. For the latter, paraffin-embedded specimens were cut and processed for immunofluorescence as previously described.[19] Mast cells were identified using mouse monoclonal antibodies directed against tryptase (1:2000 dilution; Dakopatts, Glostrup, Denmark). Histamine was detected using a polyclonal antibody (1:100 dilution; Acris Antibodies, Herford, Germany), and nerves were visualized using a rabbit polyclonal NSE (1:500 dilution; Dakopatts) antibody. All histologic sections were evaluated by an expert pathologist who was unaware of the diagnosis, for exclusion of overt mucosal inflammation or microscopic colitis. Quantification of inflammatory cells was performed using a previously validated method.[19] The quantification of mast cells in close vicinity (<10 μm) of nerve fibers was carried out on sections immunolabeled for tryptase (mast cell marker) and neuronal specific enolase (NSE; general neuronal marker) adapting a previously published method.[19] Briefly, microscopic fields were digitized and randomly sampled with the aid of a grid (0.5 mm^2) located below the slide. A stereologic grid containing cross-shaped points was overlaid on the digitized sampled fields by the computer software and used to determine the area occupied by tryptase+ mast cells within 10 μm of NSE+ nerve fibers over that occupied by *lamina propria* (ie, number of points hitting the cells divided by the total number of cross-shaped points in the *lamina propria*). For the double-labeling technique, additional specificity studies were performed by incubating the primary antibodies (used at the concentration previously reported) with the supernatants at 4°C overnight before incubation on tissue slides. In addition, further appropriate controls for the double-labeling technique were performed to determine that the supernatants did not cross-react when mixed together with secondary antibodies and that the secondary antibodies recognized the appropriate antigen–antibody complexes. For electron microscopy studies, biopsies were processed as described in detail elsewhere.[19]

Collection and Assay of Mucosal Mediators

Spontaneous release of mucosal mediators from colonic biopsies was obtained using a previously described method.[19] Briefly, upon removal, biopsies were rapidly immersed in hard plastic tubes containing 1 mL of Hank's solution (Sigma, St. Louis, MO), continuously oxygenated (95% O_2/5% CO_2) at 37°C. After a 25-minute incubation, the bathing solution was removed. All samples were centrifuged at 200g for 10 minutes, and 150 μL of supernatant aliquoted and stored at –70°C until the assay. At the end of the release experiment, biopsies were blotted and weighed.

Histamine and tryptase were measured using previously described methods.[19] PGE_2 was measured from duplicate aliquots (50 μL) of the cleared supernatants using a competitive enzyme immunoassay (Cayman Chemical, Ann Arbor, MI). This assay is based on the competition between PGE_2 and a PGE_2-acetylcholinester-

ase conjugate for a limited amount of PGE_2 monoclonal antibody. Histamine and PGE_2 were measured using a microtiter plate reader (absorbance 405 nm; Tecan's Sunrise, Phoenix Research Products Candler, NC) and normalized to the weight of the biopsies.

Animals

Male Sprague-Dawley rats (250–350 g) were used in all experiments. Rats were fed regular laboratory chow with free access to water and housed under controlled conditions with a 12:12-hour light/dark cycle. All procedures were approved by the institutional Animal Care Committees.

Mesenteric Afferent Nerve Recording

The mesenteric afferent nerve recording of isolated rat terminal jejunum was performed in vitro as described previously.[21]Activity was continuously monitored in real time as spike discharge frequency (impulse/sec) and both the raw signal and spike discharge frequency were spooled to a computer running Spike-2 software (Cambridge Electronic Design Limited, Cambridge, UK). Experiments were conducted on preparations in which baseline afferent discharge was maintained for at least 10 minutes. In control experiments (n = 5), the arterial perfusion was interrupted for 2 minutes and replaced with Krebs solution, biopsy supernatants from controls, and then the sample from IBS patients, all infused at a rate of 150 μL/min (300 μL vol) in a sequential manner separated by an interval of 15 minutes. In 1 further experiment only Krebs solution and the IBS sample were administered. The reproducibility of nerve responses to the IBS samples was tested in ancillary experiments by the application of the same IBS sample twice on the same gut preparations (n = 3). The second application of same IBS sample was infused at least 10 minutes after the nerve response to the first application recovered to the baseline. Three groups of treatment were performed (n = 5 for each) by adding drug in the perfusion Krebs solution after the increased nerve discharge evoked by IBS sample had returned to the baseline and 10 minutes before the second application of the same IBS sample. The effects of the serotonin (5-HT) type 3 receptor antagonist granisetron (1 μmol/L), and the histamine H_1 receptor antagonist pyrilamine (10 μmol/L; Sigma) were tested in individual experiments.

Measurement of Intracellular [Ca^{2+}] in DRG Neurons

Rat DRG (T1-L6) were removed and placed in cold Dulbecco's modified eagle medium solution, containing (in mg/mL): 0.5 trypsin, 1 collagenase type IA, and 0.1 DNAse type IV (all from Sigma).[22] Enrichment of the fraction of nociceptive neurons was obtained following the methods reported previously.[23] Briefly, ganglia were rinsed, dissociated, and centrifuged (200*g* for 5 minutes). Cells were than plated on poly-[scap]l[r]-lysine (8.3 μmol/L, Sigma) and laminin (5 μmol/L, Sigma) coated 25-mm glass coverslips and kept for 5 to 8 days at 37°C. After 2 days, plated neurons were loaded with Fura-2-AM-ester (3 μmol/L, Sigma) for 40 minutes at 37°C and transferred to a chamber on the stage of Nikon eclipse TE300 microscope. The dye was excited at 340 nmol/L and 380 nmol/L to indicate relative $[Ca^{2+}]_i$ changes by the F_{340}/F_{380} ratio recorded with a dynamic image analysis system (Laboratory Automation 2.0, RCS, Florence, Italy).

Biopsy supernatants (200 μL) obtained from controls and IBS patients were added to the experimental chamber to obtain a final volume of 500 μL (2.5 dilution coefficient). Some experiments were performed in the presence of the histamine H_1 receptor antagonist, pyrilamine (0.1 μmol/L, Sigma), and the nonselective EP_1 and EP_2 receptor antagonist AH 6809 (5 μmol/L, Cayman Chemical, Ann Arbor, MI) injected 10 minutes prior to the stimuli.[21] The serine protease inhibitor FUT-175 (50 μg/mL; Calbiochem, Darmstadt, Germany) was added to the supernatants 10 minutes before their application to the DRG neurons.

$[Ca^{2+}]_i$ was expressed as percentage of the maximum increase detected in the presence of the calcium ionophore ionomycin (5 μmol/L, Sigma).

The stock concentration of capsaicin (10 mmol/L) was prepared in 100% ethanol. Fura-2-AM-ester, and ionomycin were dissolved in 100% DMSO. All other drugs were dissolved in Krebs buffer solution and were administered in volumes not exceeding 1% vol/vol of the bath volume.

Data Expression and Statistical Analysis

Data are reported as mean values ± standard error of the mean (SEM). Biopsies from all the enrolled IBS patients (n = 29) and controls (n = 15) were processed for immunohistochemistry and quantitative experiments relative to mast cells and nerve–mast cell interactions. Due to limited availability of tissue, different n values apply to the other experiments carried out in the present study. For Ca^{2+} imaging studies: IBS patients, n = 19; controls, n = 15. For electrophysiologic measurement of sensory nerves: IBS patients n = 16; controls, n = 5.

All data were analyzed by means of the Mann–Whitney *U* and the Yates' corrected chi-squared tests, with the following exceptions. Data relative to $[Ca^{2+}]_i$ that were analyzed by means of the Student *t* test or analysis of variance (ANOVA) and the Dunnett's test when required. Mean nerve discharge rate was calculated off-line using Spike 2 from the instantaneous discharge rates that had been saved to disk. This was then normalized by subtracting the mean discharge for a 5-minute stationary baseline period from the mean discharge rates taken at 50 second intervals up to 450 seconds after the onset of intra-arterial injection. The nerve discharge data were analyses by 2-way ANOVA followed by Bonferroni post-tests. The

Table 1. Characteristics of the Study Patients

Total number of patients	29
Age (*y*)	39.0 ± 10.4
Sex (% females)	62.1
Abdominal pain scores	
Severity (1–4)	2.4 ± 0.2
Frequency (1–4)	2.3 ± 0.2
Bowel habit	
Constipation (%)	45.5
Diarrhea (%)	54.5
Bloating (%)	90.9

effects of antagonists on the peak nerve discharge were analyzed by 1-way ANOVA and post hoc Tukey's multiple comparison test. Correlations were analyzed using the Spearman rank correlation test. Analyses were done by running the SPSS/PC+ statistical package (SPSS, Chicago, IL) on a personal computer. Two-tailed P values $<$ .05 were considered statistically significant.

Results

Patients

Twenty-nine patients referred to the Department of Internal Medicine and Gastroenterology (tertiary referral center) for gastrointestinal complaints were diagnosed with IBS according to the Rome II criteria[1] and were included in the study. Patient's clinical characteristics are reported in Table 1. Fifteen healthy subjects served as controls.

Mast Cells Infiltrate and Associate with Nerve Fibers in the Colonic Mucosa of IBS Patients

To quantify mast cells and investigate their spatial relationships with nerve fibers in the colonic *lamina propria*, we immunostained tissues for tryptase and NSE. Tryptase+ mast cells were scattered throughout the mucosal *lamina propria* of controls and IBS patients (Figure 1*A* and *D*). The mean area of mucosa occupied by tryptase+ mast cells was 152% greater in IBS patients compared with controls (7.8% ± 0.6% vs 3.0% ± 0.3%, respectively; $P < .001$) (Figure 1*D* and *K*). An increased mucosal area occupied by tryptase+ mast cells (ie, greater than mean + 2 SEM of controls: 3.6%) was present in the vast majority (72.3%) of the IBS patients studied.

In both controls and IBS patients, nerve fibers were identified in the *lamina propria* surrounding mucosal crypts (Figure 1*B* and *E*). The association of tryptase+ mast cells with nerve fibers was assessed by simultaneously localizing tryptase and NSE. The mean area of mucosa occupied by tryptase+ mast cells within 10 μm of nerve fibers in IBS patients was significantly increased by 188% compared with controls (4.9% ± 0.3% vs 1.7% ± 0.3%, respectively; $P < .01$) (Figure 1*C*, *F*, and *L*).

Mast cell activation and mast cell–nerve interactions were assessed by electron microscopy (Figure 1*G–J*). In controls, granule-filled mast cells were scattered throughout the mucosa (Figure 1*G*). Features of active degranulation, including clearing of single granules and membraneous labyrinthic arrays were particularly frequent in the mucosa of IBS patients (Figure 1*H–J*). Membrane to membrane contacts between mast cells and nerve fibers were occasionally observed, and activated mast cells with degranulation polarized toward the nerves were often found in the close proximity (0–10 μm) of nerve trunks in IBS specimens (Figure 1*H* and *I*).

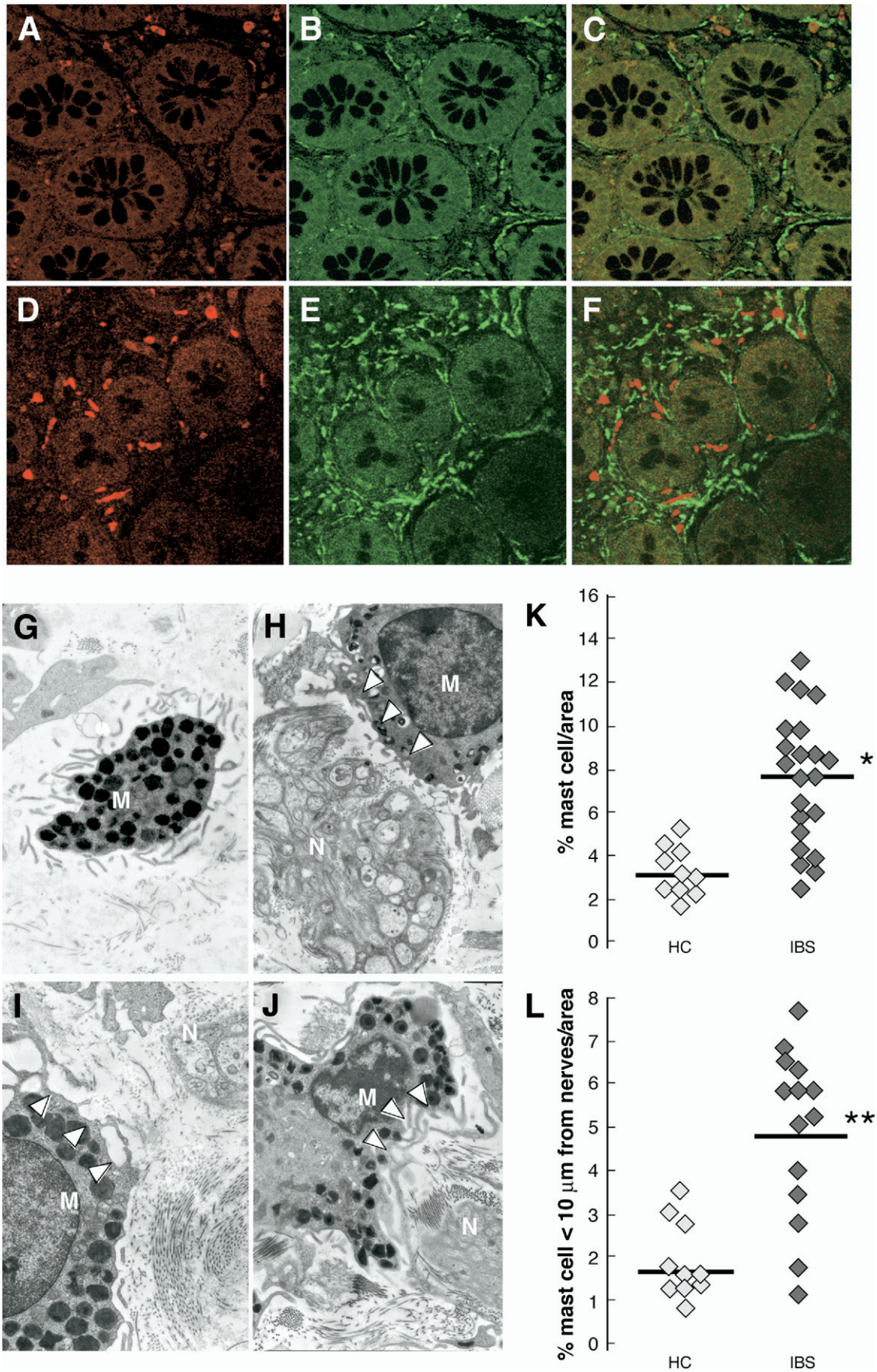
Thus, in most IBS patients there is an increased colonic mucosa area occupied by mast cells, and a closer proximity of mast cells to nerve fibers. These results provide a structural basis for enhanced mast cell–nerve interactions in the colonic mucosa of IBS patients.

Colonic Mucosa of IBS Patients Releases Increased Amounts of Mast Cell Mediators

We assessed the tissue source and the spontaneous release of mediators from the colonic mucosa of IBS patients and controls: the mast cell-specific mediator histamine and tryptase as well as the nonspecific mediator PGE_2, which derives from mast cells and other cell types. Histamine, tryptase, and PGE_2 can excite visceral sensory nerves. The tissue weight of biopsies obtained from IBS patients used for these experiments (as well as for the assessment of biopsy supernatants on the electrophysiology of mesenteric sensory nerves and DRG neurons, see below) was not different from that obtained from controls (8.4 ± 0.6 mg vs 10.3 ± 0.7 mg, respectively, $P > .05$).

The major source of histamine and tryptase in the colonic mucosa was determined in double labeling immunofluorescence experiments. As shown in Figure 2*A–C*, histamine immunoreactivity was colocalized in the majority of tryptase+ mast cells.

Mucosal biopsies were incubated in oxygenated (95% O_2/5% CO_2) buffer at 37°C, and the release of histamine, tryptase, and PGE_2 were determined. Biopsies from IBS patients released large amounts of histamine within 5 minutes (146.7 ± 28.5 ng/mL/mg), which was increased after 25 minutes (182.1 ± 27.2 ng/mL/mg). As shown in Figure 2*D* and *E*, the release of histamine and tryptase in IBS patients was significantly increased by 118% and 354%, respectively, over that in controls (241.7 ± 22.2 ng/mL/mg vs 110.8 ± 25.8 ng/mL/mg; $P < .01$ and 3.0 ± 0.6 pmol/min/mg vs 0.9 ± 0.2 pmol/min/mg; $P < .001$). The release of histamine and tryptase was significantly correlated with the number of infiltrating mast cells ($r = 0.703$; $P < .001$ and $r = 0.635$; $P < .001$, respectively). PGE_2 release was also significantly increased by 132% in IBS patients compared to controls (155.4 ± 10.8 pg/mL/mg vs 66.9 ± 23.2 pg/mL/mg, respectively; $P < .001$) (Figure 2*F*). In all subsequent experiments (ie, mediator assays, electrophysiologic testing, Ca^{2+} mobilization experiments), we assayed samples obtained after 25 minutes incubation.


A
B
C
D
E
F
G
M
H
M
N
I
N
M
J
M
N
K
% mast cell/area
16
14
12
10
8
6
4
2
0
*
HC
IBS
L
% mast cell < 10 μm from nerves/area
8
7
6
5
4
3
2
1
0
**
HC
IBS

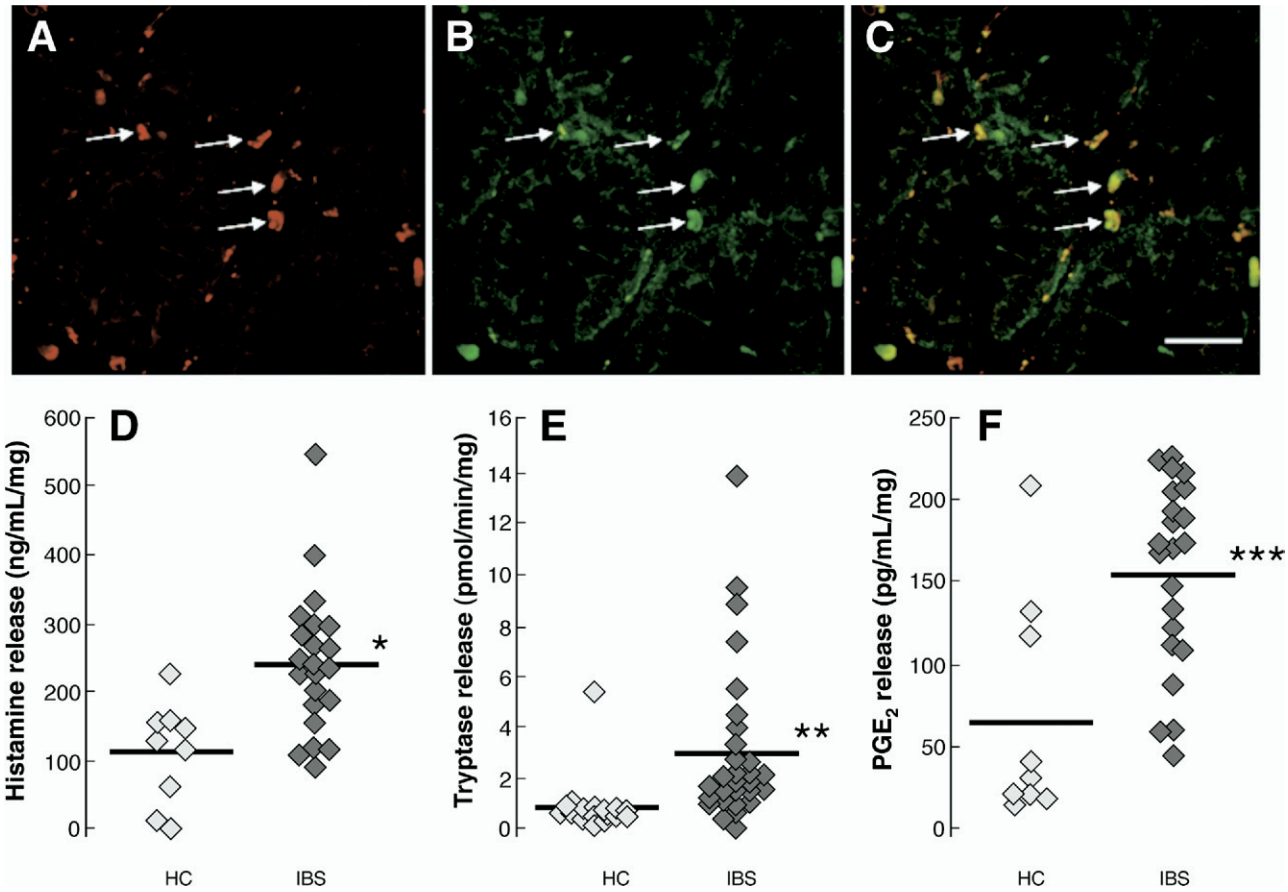


Figure 2. Localization and release of mediators from the colonic mucosa of IBS patients. The source of histamine and tryptase in the colonic mucosa was determined using immunofluorescence for tryptase (*A*) and histamine (*B*), respectively. Note that the majority of tryptase+ mast cells also expressed histamine immunolabeling (*arrows*) (*C*). Mucosal biopsies were incubated at 37°C in oxygenated culture buffer. The mast cell mediator histamine, tryptase, and PGE_2 were assayed in the cleared mucosal supernatants obtained after 25 minutes of incubation from controls (HC) and IBS patients. Note the significant increased release of histamine (*D*), tryptase (*E*), and PGE_2 (*F*) in IBS versus HC. $^*P < .01$, $^{**}P < .001$ and $^{***}P < .001$. Results are expressed as mean ± SEM, n = 9-15 for HC and n = 22-29 for IBS.

Mast Cells Mediators Excite Visceral Sensory Neurons

We used 2 methods to assess the potential of mucosal supernatants from controls and IBS patients to activate sensory nerves. In the first, we made multiunit recordings of the spontaneous activity in paravascular mesenteric afferent nerve bundles supplying the isolated rat jejunum. The second approach employed imaging of the intracellular concentration of Ca^{2+} ions ($[Ca^{2+}]_i$) in rat DRG neurons.

Mesenteric afferent discharge was characterized as a continuous pattern of on-going discharge in the absence of any intentional stimulation. Biopsy supernatants (300 μL) or buffer were injected into the mesenteric artery (0.15 mL/min) supplying an isolated segment of rat jejunum. Infusion of control buffer did not affect the nerve discharge rate. As shown in Figure 3*A* and *D*, the intra-arterial injection of supernatants from controls did not significantly increase the nerve discharge rate over that obtained with the control buffer (2.8 ± 1.5 imp/sec vs 0.0

Figure 1. Increased mast cell infiltration in close proximity to nerve fibers in the colonic mucosa of IBS patients. Identification of mast cells and nerve fibers was obtained using immunofluorescence for tryptase and NSE, respectively, in controls (HC) (*A–C*) and IBS patients (*D–F*). The area of *lamina propria* occupied by mast cells was significantly increased in IBS patients (*D*, *K*) compared with controls (*A*, *K*). Nerve fibers were identified running in the *lamina propria* surrounding mucosal crypts of both controls (*B*) and IBS patients (*E*). Increased mast cell–nerve contacts were identified in the colonic mucosa of IBS patients (*F*, *L*) compared with controls (*C*, *L*). In electron microscopy studies, mast cells (*K*) were identified as granule-filled cells in the *lamina propria* of the mucosa (*G*). A close vicinity between mast cells and nerve fibers (*L*) was frequently observed in IBS patients (*H–J*). (*H-J*) Detail of mast cells with features of active degranulation (membranous labyrinthic arrays and emptying of single granules; *arrowheads*) in proximity to nerve fibers in IBS patients. Mast cell degranulation often appeared polarized in the direction of the nerve fiber (*H*, *I*). $^*P < .001$, $^{**}P < .01$. Results are expressed as mean ± SEM, n = 10 for HC and 22 for IBS. Scale bars: *A–F*, 100 μm; *G*, 5 μm; *H–J*, 2 μm.

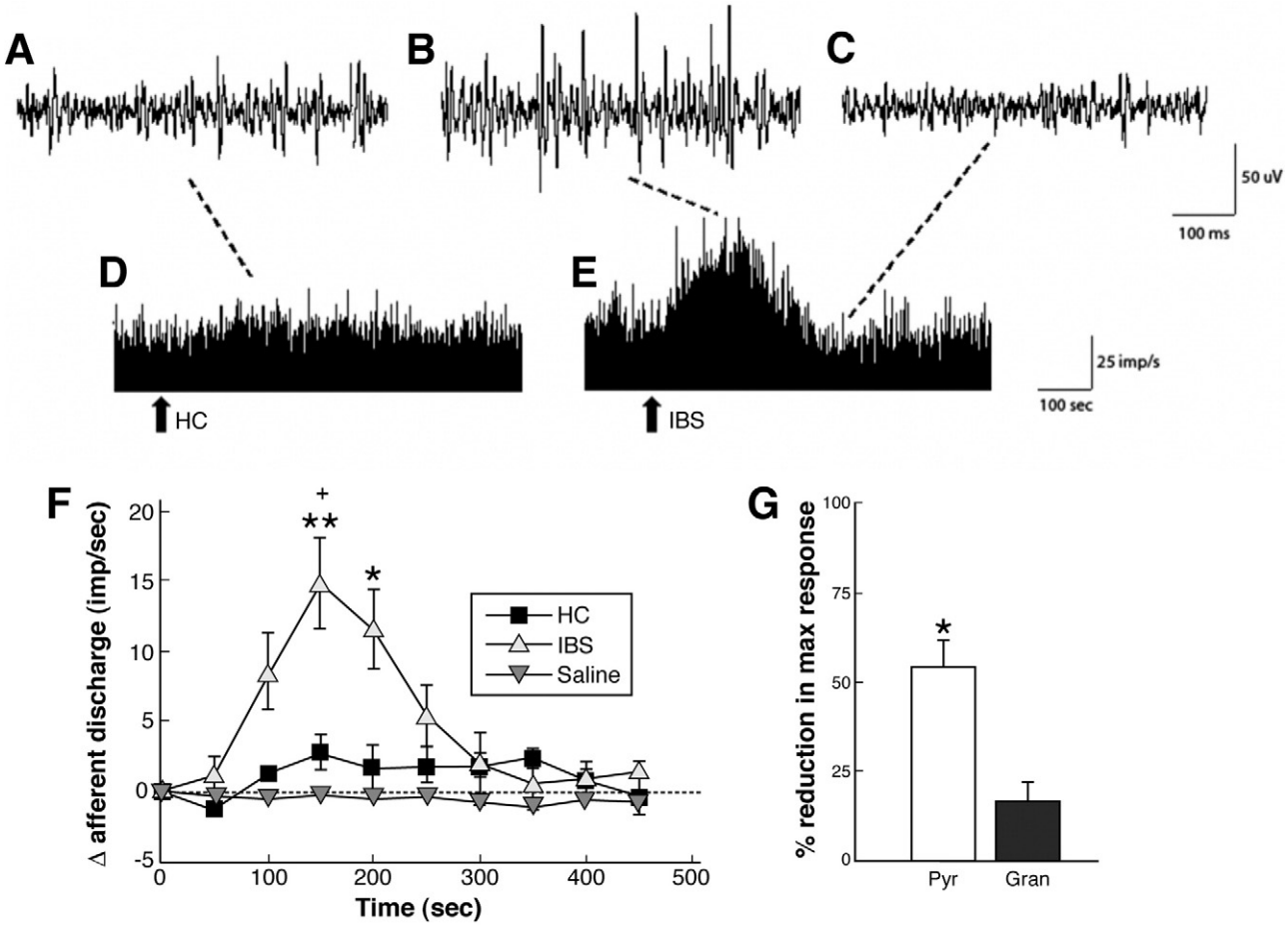


Figure 3. Mucosal supernatants obtained from colonic mucosal biopsies of IBS patients increase nerve discharge of rat mesenteric sensory nerves. Representative tracings (*A–E*) and quantitative data (*F*) showing the increase in afferent nerve discharge above baseline in response to healthy control (HC; n = 5) or IBS (n = 6) mucosal supernatants in comparison to saline buffer (n = 5). Note that although the majority of HC supernatants had no significant effect on the afferent response (*A, D*), IBS supernatants induced a significant marked increase in nerve discharge (*B, E*). On some occasions the augmented firing in response to IBS samples was followed by a transient period of reduced activity (*C*). The data in *F* were analyses by 2-way ANOVA followed by Bonferroni post-tests. * and ** represent statistically significant differences from buffer ($P < .05$ and 0.01, respectively), while + shows statistical difference ($P < .05$) between IBS and HC supernatants. The histograms in *G* show the magnitude of the reduction in the afferent response to IBS supernatants following treatment with pyrilamine (10 μmol/L) and granisetron (1 μmol/L), normalized prior to treatment. Data were analyzed by 1-way ANOVA and post hoc Tukey's multiple comparison test. * shows $P < .05$ for pyrilamine versus granisetron (n = 5 for each).

± 0.6; $P > .05$). In sharp contrast, IBS supernatants significantly increased mesenteric afferent discharge (Figure 3*B* and *E*). Peak nerve discharge occurred at ~150 seconds and was approximately 5-fold higher that that obtained with control samples (14.7 ± 3.2 imp/sec vs 2.8 ± 1.5 imp/sec at the peak, respectively; $P < .05$) (Figure 3*D–F*). The augmented afferent discharge returned to baseline after approximately 5–6 minutes (Figure 3*E*). The peak nerve discharge obtained with IBS supernatants was significantly correlated with both *lamina propria* area occupied by mast cells and with histamine release ($r = 0.7$, $P < .01$ and $r = 0.6$, $P < .05$, respectively). Although there was variability in the nerve responses to supernatants from different IBS patients, nerve responses (ie, response over time and peak discharge) were reproducible following repeated application of IBS supernatants obtained from the same patient at 10-minute intervals, which allowed before and after studies using treatments designed to interfere with mast cell to nerve signaling to be examined. The histamine H_1 antagonist pyrilamine (10 μmol/L) significantly decreased the peak nerve response to IBS supernatants (6.0 ± 2.2 imp/sec vs 12.4 ± 3.5 imp/sec; $P < .05$) (Figure 3*G*). However, granisetron (1 μmol/L), which blocks (5-HT_3) receptors, adds no significant effect on the peak afferent response to IBS supernatants (22.3 ± 2.9 imp/sec vs 19.4 ± 2.7 imp/sec; $P > .05$) (Figure 3*G*).

To further examine the capacity of mucosal supernatants to regulate sensory nerves, we measured their effects on $[Ca^{2+}]_i$ of DRG neurons in culture. By challenging neurons with capsaicin, a specific agonist of transient receptor potential vanilloid (TRPV)-1 that is known to play an essential role in inflammatory pain, we determined whether supernatants regulate those DRG neu-

rons that mediate neurogenic inflammation and pain transmission. Capsaicin (1 μmol/L) evoked a prompt increase in $[Ca^{2+}]_i$ in isolated DRG neurons. On average, 64% of the tested DRG neurons were capsaicin-sensitive. There was no significant difference in the proportion of capsaicin-sensitive DRG neurons used for experiments involving the application of healthy control or IBS mucosal supernatants (61% vs 67%, respectively; $P > .05$). Both healthy control and IBS supernatants stimulated a prompt increase in $[Ca^{2+}]_i$ that was maximal within 32.8 ± 5.8 seconds and declined to basal concentration within 211.1 ± 30.7 seconds (Figure 4*A*). Mucosal supernatants obtained from IBS patients evoked a detectable $[Ca^{2+}]_i$ increase in a significantly higher number of isolated DRG neurons compared with DRG exposed to healthy control supernatants (70% vs 35%; $P < .05$). The average $[Ca^{2+}]_i$ increase obtained with IBS samples was significantly higher than that obtained with samples of controls (29% ± 4% vs 11% ± 4%; $P < .05$) (Figure 4*A* and *B*). There was no difference in the proportion of capsaicin sensitive DRG neurons responsive to either IBS or to control samples (75% vs 71%; $P > .05$). The increase in $[Ca^{2+}]_i$ obtained with IBS samples was significantly correlated with the area of the *lamina propria* occupied by mast cells ($r = 0.78$, $P < .01$), but not with histamine release ($r = 0.25$, $P > .05$). In DRG neurons challenged with control supernatants, pyrilamine (0.1 μmol/L) did not affect the $[Ca^{2+}]_i$ responses (10% ± 6%) compared with its vehicle (11% ± 4%; $P > .05$) (Figure 4*C* and *D*). In contrast, in the presence of pyrilamine (0.1 μmol/L), the magnitude of the $[Ca^{2+}]_i$-increase evoked by IBS supernatants was markedly and significantly reduced as compared with its vehicle (14% ± 3% vs 29% ± 4%, respectively; $P < .05$) (Figure 4*E* and *F*). The role of proteases on $[Ca^{2+}]_i$ in DRG neurons was assessed by incubation of supernatants with the serine protease inhibitor FUT-175 (50 μg/mL). As shown in Figure 4*G* and *H*, protease inhibition significantly reduced the magnitude of the $[Ca^{2+}]_i$-increase evoked by IBS supernatants as compared to its vehicle (11% ± 3% vs 44% ± 7%, respectively; $P < .05$). The nonselective EP_1 and EP_2 receptor antagonist AH 6809 (5 μmol/L) failed to antagonize the effects of mucosal supernatants (20% ± 2% vs 21% ± 4%, respectively, $P > .05$).

To exclude the possibility that the effects evoked by mucosal mediators could be the result of activation of tissue mast cells in the recipient rat tissue, we carried out immunohistochemistry using antitryptase antibodies that showed the lack of mast cell immunolabeling in the DRG neuronal culture.

In summary, mediators released from biopsies of the colonic mucosa of IBS patients evoke increased excitation of mesenteric afferent nerves and of nociceptive neurons of DRG compared to controls. Mast cell histamine and serine proteases participated to a great extent to the sensory neuron excitation. However, the incomplete inhibition of responses obtained with the histamine H_1 receptor antagonist and the serine protease inhibitor suggests that other mediators may be involved.

Discussion

We have identified a novel mechanism by which mast cells in the mucosa of patients with IBS can release specific mediators that excite afferent neurons and may thereby cause visceral hypersensitivity. The most important findings of our study were the marked increase in the firing rate of visceral sensory nerves and the enhanced Ca^{2+} mobilization in the vast majority of capsaicin-sensitive DRG neurons following exposure to IBS mucosal supernatants compared to controls. Several observations support a prominent role of mucosal mast cells of IBS patients in the enhanced activation of sensory nerves. First, we confirmed, in a different patient population, an increased number of mast cells in close proximity to colonic neural processes. Second, we observed increased release of histamine, tryptase, and PGE_2 in IBS patients compared to controls. Third, histamine H_1 receptor blockade, and serine protease inactivation significantly reduced the effects of IBS supernatants on neuronal firing rate and Ca^{2+} mobilization. Together, these results suggest that mast cell histamine and proteases as well as likely other mediators released by the colonic mucosa of patients with IBS can excite visceral, nociceptive sensory nerves innervating the intestine, which may induce visceral hyperalgesia.

There Is an Influx of Mast Cells and Increased Release of Mediators in IBS Patients

In agreement with our previous studies,[19] in a different patient population, we confirmed that the area of the *lamina propria* occupied by mast cells was markedly increased in patients with IBS. Furthermore, the double labeling quantitative immunofluorescence experiments showed the presence of an increased infiltration of mast cells in close proximity (<10 μm) of nerve fibers in IBS. Although the increased density of mast cells in the mucosa per se could account for the increased proportion of mast cells in close vicinity to nerves, previous studies indicate that mast cell-to-nerve association in the rat intestinal mucosa unlikely occurs by chance.[24] For example, in coculture studies, sympathetic neurons form contacts with mast cells, suggesting that either mast cell mediators may influence neurite attraction or neural factors attract mast cells.[25] There are many factors involved in the nonrandom close vicinity of mast cells and nerves in different tissues in general and in the intestine in particular (for review, see ref. 12). We did not provide evidence of the extrinsic sensory nature of the neuronal fibers in contact with mast cells. However, previous studies in the intestinal mucosa of nematode-infected rats reported that the majority of nerve fibers adjacent to

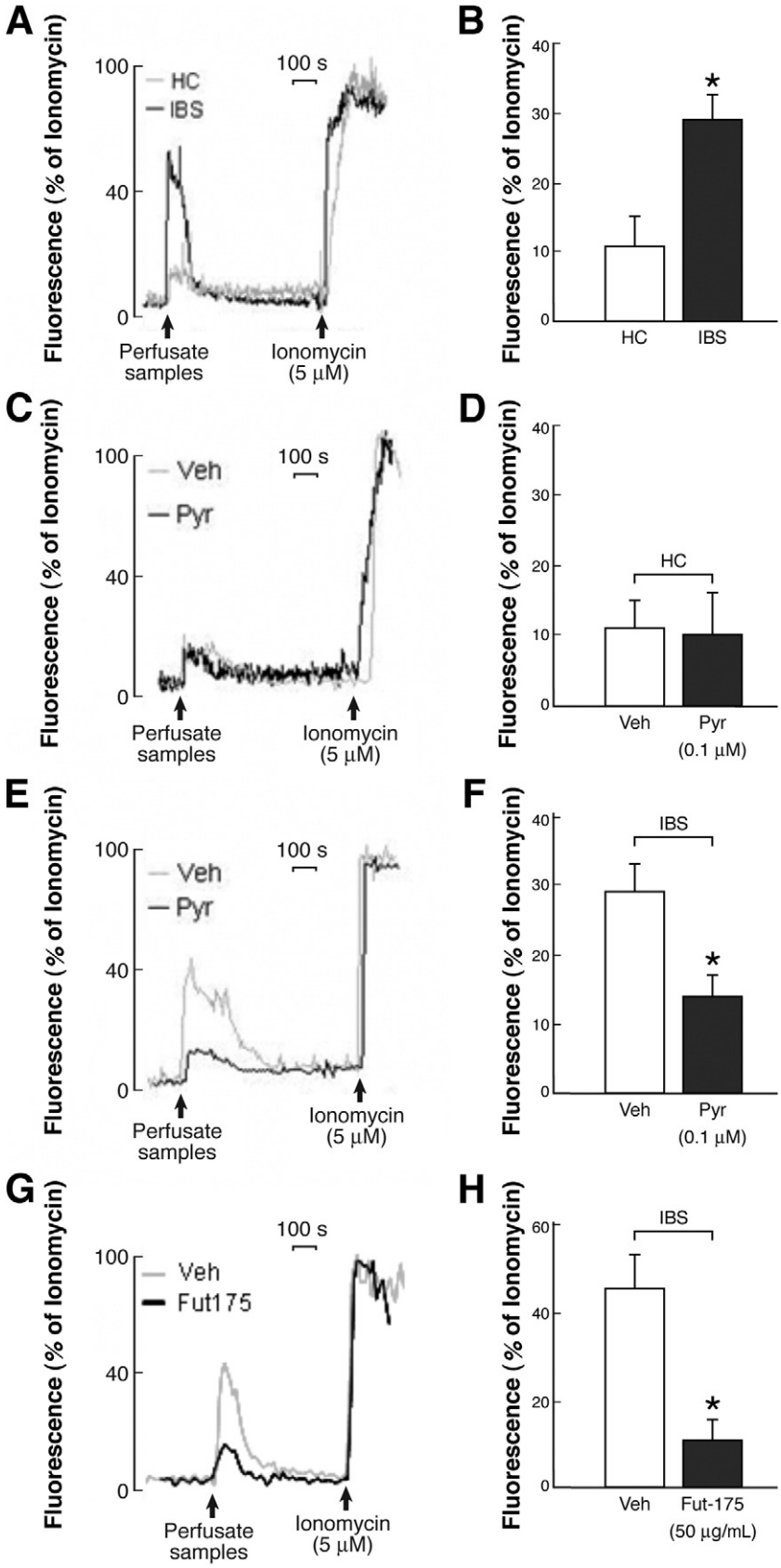


Figure 4. Mucosal supernatants obtained from colonic mucosal biopsies of IBS patients increase the intracellular calcium concentration ($[Ca^{2+}]_i$) in cultured rat DRG neurons. (*A*) Biopsy supernatants obtained from healthy control (HC) and IBS patients produced an increase in $[Ca^{2+}]_i$ in DRG neurons. (*B*) IBS supernatant-induced increase of $[Ca^{2+}]_i$ was significantly higher than that obtained with supernatants from healthy control (HC). (*C*) shows the increase in $[Ca^{2+}]_i$ induced by HC supernatants in the presence (*black lines*) or absence (*gray lines*) of the histamine H_1 receptor antagonist, pyrilamine (Pyr, 0.1 μmol/L). (*D*) Pyrilamine had no significant effect on the $[Ca^{2+}]_i$ evoked by the HC supernatants. (*E*) The increase in $[Ca^{2+}]_i$ induced by IBS supernatants in the presence (*black lines*) or absence (*gray lines*) of the histamine H_1 receptor antagonist, pyrilamine (Pyr, 0.1 μmol/L), in cultured DRG neurons. (*F*) Pyrilamine significantly decreased the enhanced $[Ca^{2+}]_i$ evoked by IBS supernatants. $^*P < .05$. (*G*) The increase in $[Ca^{2+}]_i$ induced by IBS supernatants in the presence (*black lines*) or absence (*gray lines*) of the protease inhibitor, FUT-175 (50 μg/mL). (*H*) FUT-175 significantly decreased the enhanced $[Ca^{2+}]_i$ evoked by IBS supernatants. $^*P < .05$. Results are expressed as means ± SEM of at least 5 different biopsy supernatants.

infiltrating mast cells contained substance P and calcitonin gene-related peptide,[24] and are therefore likely to be sensory in nature. Our findings of a close relationship between nerves and mast cells in the colonic mucosa of IBS patients provide a morphologic basis for facilitated functional communication between mast cells and nerves. The electron microscopy results, showing mast cells releasing the content of their granules in the space surrounding nerve endings, is in line with this concept.

Among a putative wide range of mediators released by mucosal biopsies, we elected to focus on histamine, tryptase, and PGE_2 because they are prototype substances that are released when mast cells degranulate, and because they can excite and sensitize sensory nerves by activating specific receptors (for review, see ref. 26). Our results showed a higher mucosal release of the mast cell-specific mediator histamine and tryptase as well as of PGE_2, although we cannot rule out that the increased production of PGE_2 originates, at least in part, from other cell types. The increased release of these mediators could either reflect the higher number of mast cells infiltrating the mucosa or, more likely, also an increased state of activation of mast cells. The latter hypothesis is in agreement with our previous data showing increased features of mast cell activation in the colonic mucosa of the majority of IBS patients compared to controls; although substantial overlap between IBS patients and controls could be identified, this suggests that not all the patients showed increased mast cell activation.[19] This variable state of activation of mast cells among IBS patients is in keeping with our findings showing that some IBS samples did not elicit a neuronal activation higher than that found with control samples. The causes of the increased mast cell infiltration/activation are presently unknown. Previous studies suggested that mast cell degranulation, membrane ruffling, and increased cellular content of histamine can be evoked by the release of sensory neuropeptides (eg, substance P) by adjacent nerves.[27–29] This is also in agreement with our finding that mast cell degranulation was frequently polarized in the direction of nerve fibers and with our previous demonstration of a significant correlation between degranulation of mast cells in close proximity to mucosal nerves in the colonic mucosa of IBS patients.[19] Other causes, as previously discussed,[12] including stress, unrecognized food allergies, and changes in intestinal microflora may also participate in the increased activation of mast cells in IBS. However, further work has to be done to confirm these hypotheses.

Mast Cell Mediators from IBS Patients Excite Nociceptive Neurons

Visceral hypersensitivity, which can be detected in up to 60% of patients with IBS, plays an essential role in abdominal nociception. Although the underlying mechanisms remain unclear, both peripheral and central mechanisms are considered to contribute. Our data are in keeping with a key role for peripheral sensitization and support the concept that mediators released in the mucosal milieu contribute to visceral hypersensitivity. In this study, exposure of mesenteric sensory nerves to colonic mucosal supernatants obtained from patients with IBS evoked a rapid and marked increase in nerve discharge compared with controls. Mesenteric nerve bundles contain both vagal and spinal afferent fibers, and it is conceivable that both populations may be influenced by mediators released from mast cells acting directly on the sensory terminals or secondary to release of other mediators from various cell types in the gut wall. That a direct spinal mechanism is likely to be involved stems from observation that these mediators have a direct effect on capsaicin sensitive DRG neurons. DRG neurons responded with a more pronounced increase in $[Ca^{2+}]_i$ to the supernatants of IBS patients compared to the supernatants of controls. Several observations suggest that histamine and proteases from mucosal mast cells of IBS patients are of crucial importance in exciting nociceptive neurons. First, IBS mucosal supernatants contained markedly increased amounts of histamine and tryptase. Second, enhanced responses evoked by IBS samples on mesenteric afferents and DRG neurons were significantly reduced by the selective histamine H_1 receptor antagonist, pyrilamine and by inactivation of proteases in the mucosal supernatants. Third, mast cell infiltration and histamine release were significantly correlated with mesenteric nerve activation, and, at least in part with $[Ca^{2+}]_i$ in DRG neurons. These findings are in agreement with previous reports showing that histamine directly activates sensory nerves through an H_1 receptor-mediated mechanism.[30] The importance of histamine in excitation of sensory nerves is also illustrated by the report that H_1 receptor antagonists suppress the activation of mesenteric afferent nerves following mast cell degranulation in models of intestinal anaphylaxis.[31] On the other hand, proteases, and particularly mast cell tryptase, may also activate sensory afferents by acting on protease activated receptor-2 (PAR_2) located on sensory neurons.[32] Indeed, PAR_2 agonists evoked long-lasting visceral hypersensitivity in rats.[33] Our results are in keeping with this hypothesis because we demonstrated that colonic biopsies of IBS patients contained large amounts of tryptase and the serine–protease inhibitor FUT-175 markedly reduced $[Ca^{2+}]_i$ in rat DRG neurons. Nonetheless, because pyrilamine and serine proteases reduced only in part the potentiation of mesenteric afferents discharge of $[Ca^{2+}]_i$ in DRG neurons caused by IBS supernatants, it is likely that mediators other than histamine and proteases contributed to the increased neuronal discharge. Although prostanoids are known to activate sensory neurons including those innervating the gastrointestinal tract,[34] the contribution of PGE_2 in our experiments was negligible because the EP_1–EP_2 antagonist had no effect on the

activation of $[Ca^{2+}]_i$ in DRG neurons exposed to IBS supernatants. Similarly, 5-HT had a limited effect, because 5-HT_3 receptors' antagonism did not affect the magnitude of the afferent response to the IBS samples.

Our data indicate that mast cells in the biopsies are the major source of the mediators (eg, histamine, tryptase) involved in sensory afferent activation, which is in keeping with evidence demonstrating that mast cells are the largest storage site of histamine in the human body.[35] However, other mediators or bacterial products could potentially cause afferent sensitivity indirectly following release of these mediators from tissue resident mast cells in the rat mucosa. Certainly mast cell degranulation has been shown to excite these afferents.[31] However, rodent mast cells contain 5-HT and the afferent response during intestinal anaphylaxis in the rat is attenuated by 5-HT_3 receptor blockade. In contrast, the afferent response to patient mucosal supernatants was attenuated by H_1, but not 5-HT_3 blockers, suggesting a direct effect on the afferents, which have been shown to express both receptors.[30] The DRG neuronal cultures do not contain mast cells as demonstrated by our immunohistochemistry so an indirect action via mast cells on these neurons is unlikely.

In conclusion, our data support the concept that colonic mediators derived from mucosal mast cells of IBS patients contribute to the excitation of sensory afferent pathways. Thus, our data pave the way for considering the release of these mediators as a potential mechanism contributing to IBS pathophysiology. However, our study has some limitations, and a number of issues remain to be addressed, including the assessment of: (1) mediators (other than histamine and tryptase) of mast cell and nonmast cell origin, which are likely involved in the activation of sensory neurons; (2) participation of mechanical stimuli on the sensitization of sensory fibers; (3) causes underlying the increased release of mucosal mediators involved in sensory neuron activation [eg, psychologic factors, neurohormonal factors (eg, neuropeptides, corticotrophin releasing factor) food allergens, intestinal microflora, intestinal permeability]; (4) potential clinical correlates between sensory neural pathways activation evoked by mucosal mediators and abdominal pain perceived by patients; (5) clinical usefulness of inhibition of mast cell activation (eg, mast cell stabilizers,[36,37] anti-IgE antibodies,[38] inhibitors of the intracellular protein tyrosine kinase, Syk[39]), or antagonism of the effects of mast cell mediators (eg, histamine and proteases receptor antagonists). These still open issues could represent the objective of future ad hoc-designed studies.

References

1. Thompson WG, Longstreth GF, Drossman DA, Heaton KW, Irvine EJ, Muller-Lissner SA. Functional bowel disorders and functional abdominal pain. Gut 1999;45(suppl 2):II43–II47.
2. Jones R, Lydeard S. Irritable bowel syndrome in the general population. BMJ 1992;304:87–90.
3. Talley NJ, Zinsmeister AR, Van Dyke C, Melton LJ III. Epidemiology of colonic symptoms and the irritable bowel syndrome. Gastroenterology 1991;101:927–934.
4. Harvey RF, Salih SY, Read AE. Organic and functional disorders in 2000 gastroenterology outpatients. Lancet 1983;1:632–634.
5. Drossman DA, Camilleri M, Mayer EA, Whitehead WE. AGA technical review on irritable bowel syndrome. Gastroenterology 2002; 123:2108–2131.
6. Sandler RS, Drossman DA, Nathan HP, McKee DC. Symptom complaints and health care seeking behavior in subjects with bowel dysfunction. Gastroenterology 1984;87:314–318.
7. Spiegel BM, Gralnek IM, Bolus R, Chang L, Dulai GS, Mayer EA, Naliboff B. Clinical determinants of health-related quality of life in patients with irritable bowel syndrome. Arch Intern Med 2004; 164:1773–1780.
8. Delvaux M. Role of visceral sensitivity in the pathophysiology of irritable bowel syndrome. Gut 2002;51(suppl 1):i67–i71.
9. Lembo T, Munakata J, Mertz H, Niazi N, Kodner A, Nikas V, Mayer EA. Evidence for the hypersensitivity of lumbar splanchnic afferents in irritable bowel syndrome. Gastroenterology 1994;107: 1686–1696.
10. Silverman DH, Munakata JA, Ennes H, Mandelkern MA, Hoh CK, Mayer EA. Regional cerebral activity in normal and pathological perception of visceral pain. Gastroenterology 1997;112:64–72.
11. Barbara G, De Giorgio R, Stanghellini V, Cremon C, Corinaldesi R. A role for inflammation in irritable bowel syndrome? Gut 2002; 51(suppl 1):i41–i44.
12. Barbara G, Stanghellini V, De Giorgio R, Corinaldesi R. Functional gastrointestinal disorders and mast cells: implications for therapy. Neurogastroenterol Motil 2006;18:6–17.
13. Metcalfe DD, Baram D, Mekori YA. Mast cells. Physiol Rev 1997; 77:1033–1079.
14. Stead RH, Dixon MF, Bramwell NH, Riddell RH, Bienenstock J. Mast cells are closely apposed to nerves in the human gastrointestinal mucosa. Gastroenterology 1989;97:575–585.
15. Galli SJ, Maurer M, Lantz CS. Mast cells as sentinels of innate immunity. Curr Opin Immunol 1999;11:53–59.
16. Stevens RL, Austen KF. Recent advances in the cellular and molecular biology of mast cells. Immunol Today 1989;10:381–386.
17. Bueno L, Fioramonti J, Delvaux M, Frexinos J. Mediators and pharmacology of visceral sensitivity: from basic to clinical investigations. Gastroenterology 1997;112:1714–1743.
18. Vergnolle N, Ferazzini M, D'Andrea MR, Buddenkotte J, Steinhoff M. Proteinase-activated receptors: novel signals for peripheral nerves. Trends Neurosci 2003;26:496–500.
19. Barbara G, Stanghellini V, De Giorgio R, Cremon C, Cottrell GS, Santini D, Pasquinelli G, Morselli-Labate AM, Grady EF, Bunnett NW, Collins SM, Corinaldesi R. Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. Gastroenterology 2004;126:693–702.
20. Talley NJ, Phillips SF, Melton J, III, Wiltgen C, Zinsmeister AR. A patient questionnaire to identify bowel disease. Ann Intern Med 1989;111:671–674.
21. Wang B, Glatzle J, Mueller MH, Kreis M, Enck P, Grundy D. Lipopolysaccharide-induced changes in mesenteric afferent sensitivity of rat jejunum in vitro: role of prostaglandins. Am J Physiol Gastrointest Liver Physiol 2005;289:G254–G260.
22. Rigoni M, Trevisani M, Gazzieri D, Nadaletto R, Tognetto M, Creminon C, Davis JB, Campi B, Amadesi S, Geppetti P, Harrison S. Neurogenic responses mediated by vanilloid receptor-1 (TRPV1) are blocked by the high affinity antagonist, iodo-resiniferatoxin. Br J Pharmacol 2003;138:977–985.
23. Gilabert R, McNaughton P. Enrichment of the fraction of nociceptive neurones in cultures of primary sensory neurones. J Neurosci Methods 1997;71:191–198.

24. Stead RH, Tomioka M, Quinonez G, Simon GT, Felten SY, Bienenstock J. Intestinal mucosal mast cells in normal and nematode-infected rat intestines are in intimate contact with peptidergic nerves. Proc Natl Acad Sci U S A 1987;84:2975–2979.
25. Blennerhassett MG, Tomioka M, Bienenstock J. Formation of contacts between mast cells and sympathetic neurons in vitro. Cell Tissue Res 1991;265:121–128.
26. Grundy D. What activates visceral afferents? Gut 2004;53(suppl 2):ii5–ii8.
27. Shanahan F, Denburg JA, Fox J, Bienenstock J, Befus D. Mast cell heterogeneity: effects of neuroenteric peptides on histamine release. J Immunol 1985;135:1331–1337.
28. Mori N, Suzuki R, Furuno T, McKay DM, Wada M, Teshima R, Bienenstock J, Nakanishi M. Nerve-mast cell (RBL) interaction: RBL membrane ruffling occurs at the contact site with an activated neurite. Am J Physiol Cell Physiol 2002;283:C1738–C1744.
29. Janiszewski J, Bienenstock J, Blennerhassett MG. Picomolar doses of substance P trigger electrical responses in mast cells without degranulation. Am J Physiol 1994;267:C138–C145.
30. Kreis ME, Haupt W, Kirkup AJ, Grundy D. Histamine sensitivity of mesenteric afferent nerves in the rat jejunum. Am J Physiol 1998;275:G675–G680.
31. Jiang W, Kreis ME, Eastwood C, Kirkup AJ, Humphrey PP, Grundy D. 5-HT(3) and histamine H(1) receptors mediate afferent nerve sensitivity to intestinal anaphylaxis in rats. Gastroenterology 2000;119:1267–1275.
32. Steinhoff M, Vergnolle N, Young SH, Tognetto M, Amadesi S, Ennes HS, Trevisani M, Hollenberg MD, Wallace JL, Caughey GH, Mitchell SE, Williams LM, Geppetti P, Mayer EA, Bunnett NW. Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. Nat Med 2000;6:151–158.
33. Kirkup AJ, Jiang W, Bunnett NW, Grundy D. Stimulation of proteinase-activated receptor 2 excites jejunal afferent nerves in anaesthetised rats. J Physiol 2003;552:589–601.
34. Haupt W, Jiang W, Kreis ME, Grundy D. Prostaglandin EP receptor subtypes have distinctive effects on jejunal afferent sensitivity in the rat. Gastroenterology 2000;119:1580–1589.
35. Pearce FL, Befus AD, Gauldie J, Bienenstock J. Mucosal mast cells. II. Effects of anti-allergic compounds on histamine secretion by isolated intestinal mast cells. J Immunol 1982;128:2481–2486.
36. Lunardi C, Bambara LM, Biasi D, Cortina P, Peroli P, Nicolis F, Favari F, Pacor ML. Double-blind cross-over trial of oral sodium cromoglycate in patients with irritable bowel syndrome due to food intolerance. Clin Exp Allergy 1991;21:569–572.
37. Stefanini GF, Saggioro A, Alvisi V, Angelini G, Capurso L, di Lorenzo G, Dobrilla G, Dodero M, Galimberti M, Gasbarrini G, . Oral cromolyn sodium in comparison with elimination diet in the irritable bowel syndrome, diarrheic type. Multicenter study of 428 patients. Scand J Gastroenterol 1995;30:535–541.
38. Milgrom H, Fick RB, Jr., Su JQ, Reimann JD, Bush RK, Watrous ML, Metzger WJ. Treatment of allergic asthma with monoclonal anti-IgE antibody. rhuMAb-E25 Study Group. N Engl J Med 1999;341:1966–1973.
39. Wong BR, Grossbard EB, Payan DG, Masuda ES. Targeting Syk as a treatment for allergic and autoimmune disorders. Expert Opin Investig Drugs 2004;13:743–762.

Received October 21, 2005; Accepted October 12, 2006.

Address requests for reprints to: Giovanni Barbara, MD, St. Orsola Hospital, Building No. 5, Via Massarenti, 9, I-40138, Bologna, Italy. e-mail: gbarbara@med.unibo.it; fax: (39) 051 345-864.

Supported by the Italian Ministry of Education, University and Research (No. 2002052573, to G.B., V.S., R.De.G., and R.C.), from the University of Bologna (to G.B. and R.C.), from the National Institute of Health (No. DK57480, DK43207, DK39957, to N.W.B.), and Alexander von Humboldt Foundation Grant, Bonn, Germany (to B.W.).