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. BUNNELL,† 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 immunofluorescence and immunoenzymatic assays. The effect of mucosal mediators was tested on mesenteric sensory nerve firing and Ca2+ 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 Ca2+ 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 Ca2+ 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 H1 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 disease6 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 nerves9 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 interface13 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 C4, platelet activating factor, prostaglandin (PG)D2].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.

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and trypase. The proximity of activated mast cells to mucosal nerve fibers correlated with the frequency and severity of abdominal pain. 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\textsuperscript{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. 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 modified version of the Bowel Disease Questionnaire to evaluate symptoms. Patients were asked to score frequency and severity of their symptoms over the last 2 weeks before interview, as previously described.

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\textsubscript{2} assays and for mucosal mediators release experiments (see below).

**Histology, Immunofluorescence 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. 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. 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. Briefly, microscopic fields were digitized and randomly sampled with the aid of a grid (0.5 mm\textsuperscript{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.

**Collection and Assay of Mucosal Mediators**

Spontaneous release of mucosal mediators from colonic biopsies was obtained using a previously described method. Briefly, upon removal, biopsies were rapidly immersed in hank’s solution (Sigma, St. Louis, MO), continuously oxygenated (95% O\textsubscript{2}/5% CO\textsubscript{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. PGE\textsubscript{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\textsubscript{2} and a PGE\textsubscript{2}-acetylcholinesterase.
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. 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 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 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 (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. 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$^{2+}$]$_i$, 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 Dunnnett’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 intrarterial injection. The nerve discharge data were analysed by 2-way ANOVA followed by Bonferroni post-tests. The
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 1A** 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 1D** 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 1B** 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 1C**, **F**, and **L**).

Mast cell activation and mast cell–nerve interactions were assessed by electron microscopy (**Figure 1G–J**). In controls, granule-filled mast cells were scattered throughout the mucosa (**Figure 1G**). Features of active degranulation, including clearing of single granules and membranous labyrinthic arrays were particularly frequent in the mucosa of IBS patients (**Figure 1H–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 1H 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 2A–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 2D 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 2F**). In all subsequent experiments (ie, mediator assays, electrophysiologic testing, Ca\(^{2+}\) mobilization experiments), we assayed samples obtained after 25 minutes incubation.
Mast Cells Mediate Excitation of 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$^{2+}/H1001$ ions ($[Ca^{2+}]i$) in rat DRG neurons.

Mucosal afferent discharge was characterized as a continuous pattern of ongoing 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 3A and D, the intraarterial 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

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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 (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 PGE2 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 PGE2 (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.
In sharp contrast, IBS supernatants significantly increased mesenteric afferent discharge (Figure 3B and E). Peak nerve discharge occurred at ~150 seconds and was approximately 5-fold higher than 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 3D–F). The augmented afferent discharge returned to baseline after approximately 5–6 minutes (Figure 3E). 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 (i.e., 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 H1 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 3G). However, granisetron (1 μmol/L), which blocks (5-HT3) 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 3G).

To further examine the capacity of mucosal supernatants to regulate sensory nerves, we measured their effects on [Ca2+]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 \([Ca^{2+}]\), 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 \([Ca^{2+}]\), that was maximal within 32.8 ± 5.8 seconds and declined to basal concentration within 211.1 ± 30.7 seconds (Figure 4A). Mucosal supernatants obtained from IBS patients evoked a detectable \([Ca^{2+}]\), 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 \([Ca^{2+}]\), increase obtained with IBS samples was significantly higher than that obtained with samples of controls (29% ± 4% vs 11% ± 4%; \(P < .05\)) (Figure 4A 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 \([Ca^{2+}]\), 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 \([Ca^{2+}]\), responses (10% ± 6%) compared with its vehicle (11% ± 4%; \(P > .05\)) (Figure 4C and D). In contrast, in the presence of pyrilamine (0.1 μmol/L), the magnitude of the \([Ca^{2+}]\), increase evoked by IBS supernatants was markedly and significantly reduced as compared with its vehicle (14% ± 3% vs 29% ± 4%, respectively; \(P < .05\)) (Figure 4E and F). The role of proteases on \([Ca^{2+}]\), in DRG neurons was assessed by incubation of supernatants with the serine protease inhibitor FUT-175 (50 μg/mL). As shown in Figure 4G and H, protease inhibition significantly reduced the magnitude of the \([Ca^{2+}]\), 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$^{2+}$]) in cultured rat DRG neurons. (A) Biopsy supernatants obtained from healthy control (HC) and IBS patients produced an increase in [Ca$^{2+}$] in DRG neurons. (B) IBS supernatant-induced increase of [Ca$^{2+}$] was significantly higher than that obtained with supernatants from healthy control (HC). (C) Shows the increase in [Ca$^{2+}$], induced by HC supernatants in the presence (black lines) or absence (gray lines) of the histamine H$_1$ receptor antagonist, pyrilamine (Pyr, 0.1 mol/L). (D) Pyrilamine had no significant effect on the [Ca$^{2+}$], evoked by the HC supernatants. (E) The increase in [Ca$^{2+}$], induced by IBS supernatants in the presence (black lines) or absence (gray lines) of the histamine H$_1$ receptor antagonist, pyrilamine (Pyr, 0.1 mol/L), in cultured DRG neurons. (F) Pyrilamine significantly decreased the enhanced [Ca$^{2+}$] evoked by IBS supernatants. *P < .05. (G) The increase in [Ca$^{2+}$], 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$^{2+}$], evoked by IBS supernatants. *P < .05. Results are expressed as means ± SEM of at least 5 different biopsy supernatants.
controls could be identified, this suggests that not all the though substantial overlap between IBS patients and the majority of IBS patients compared to controls; all-features of mast cell activation in the colonic mucosa of in agreement with our previous data showing increased in infiltrating the mucosa or, more likely, also an increased 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. This variable state of activation of mast cells among IBS pa-tients 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. 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 demon-stration of a significant correlation between degranula-tion of mast cells in close proximity to mucosal nerves in the colonic mucosa of IBS patients. Other causes, as previously discussed, 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 con-ceivable that both populations may be influenced by mediators released from mast cells acting directly on the sensory terminals or secondary to release of other medi-ators 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 re-sponded with a more pronounced increase in [Ca^{2+}], to the supernatants of IBS patients compared to the super-natants 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 H1 receptor antago-nist, pyrilamine and by inactivation of proteases in the mucosal supernatants. Third, mast cell infiltration and histamine release were significantly correlated with mesen-teric nerve activation, and, at least in part with [Ca^{2+}], in DRG neurons. These findings are in agreement with previous reports showing that histamine directly activates sensory nerves through an H1 receptor-mediated mechanism. The importance of histamine in excitation of sensory nerves is also illustrated by the report that H1 receptor antagonists suppress the activation of mesen-teric afferent nerves following mast cell degranulation in models of intestinal anaphylaxis. On the other hand, proteases, and particularly mast cell tryptase, may also activate sensory afferents by acting on protease activated receptor-2 (PAR2) located on sensory neurons. Indeed, PAR2 agonists evoked long-lasting visceral hypersensitiv-ity in rats. Our results are in keeping with this hypo-thesis 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+}], in rat DRG neurons. Nonetheless, because pyril-amine and serine proteases reduced only in part the potentiation of mesenteric afferents discharge of [Ca^{2+}], in DRG neurons caused by IBS supernatants, it is likely that mediators other than histamine and proteases con-tributed to the increased neuronal discharge. Although prostanoids are known to activate sensory neurons in-cluding those innerving the gastrointestinal tract, the contribution of PGE2 in our experiments was negligible because the EP1–EP2 antagonist had no effect on the
activation of $[\text{Ca}^{2+}]_{\text{i}}$ in DRG neurons exposed to IBS supernatants. Similarly, 5-HT had a limited effect, because 5-HT$_3$ receptor 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 (e.g., 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. 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. 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. 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 [e.g., psychologic factors, neurohormonal factors (e.g., neuropeptides, corticotropin 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 (e.g., mast cell stabilizers, anti-IgE antibodies, inhibitors of the intracellular tyrosine kinase, Syk), or antagonism of the effects of mast cell mediators (e.g., histamine and proteases receptor antagonists). These still open issues could represent the objective of future ad hoc-designed studies.

References


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

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