Immediate-Early Gene Expression in the Inferior Mesenteric Ganglion and Colonic Myenteric Plexus of the Guinea Pig

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Activation of neurons in the inferior mesenteric ganglion (IMG) was assessed using c-fos, JunB, and c-Jun expression in the guinea pig IMG and colonic myenteric plexus during mechanosensory stimulation and acute colitis in normal and capsaicin-treated animals. Intracolonic saline or 2% acetic acid was administered, and mechanosensory stimulation was performed by passage of a small (0.5 cm) balloon either 4 or 24 hr later. Lower doses of capsaicin or vehicle were used to activate primary afferent fibers during balloon passage. c-Jun did not respond to any of the stimuli in the study. c-fos and JunB were absent from the IMG and myenteric plexus of untreated and saline-treated animals. Acetic acid induced acute colitis by 4 hr, which persisted for 24 hr, but c-fos was found only in enteric glia in the myenteric plexus and was absent from the IMG.

Balloon passage induced c-fos and JunB in only a small subset of IMG neurons and no myenteric neurons. However, balloon passage induced c-fos and JunB in IMG neurons (notably those containing somatostatin) and the myenteric plexus of acetic acid-treated animals. After capsaicin treatment, c-fos and JunB induction by balloon passage was inhibited in the IMG, but there was enhanced c-fos expression in the myenteric plexus. c-fos and JunB induction by balloon stimulation was also mimicked by acute activation of capsaicin-sensitive nerves. These data suggest that colitis enhances reflex activity of the IMG by a mechanism that involves activation of both primary afferent fibers and the myenteric plexus.

Key words: neuropeptide Y; somatostatin; prevertebral ganglia; capsaicin; immediate-early genes; enteric glia

Neurons in the inferior mesenteric ganglion (IMG) provide the sympathetic innervation of the distal colon (Szurszewski and King, 1991; Elfvin et al., 1993). Virtually all IMG neurons are noradrenergic and receive inputs from the thoracolumbar spinal cord. Some IMG neurons also contain neuropeptide Y immunoreactivity (NPY neurons; ~20% of IMG neurons), and other subsets include those containing somatostatin (SOM neurons; ~75%), both NPY and SOM (NPY/SOM neurons; 4–7%), or neither NPY nor SOM (–/– neurons; 1–5%) (Elfvin et al., 1993; Sann et al., 1995; Parr and Sharkey, 1996). Most or all NPY neurons project to the vasculature (Furness et al., 1983), whereas SOM and –/– neurons project to the myenteric and submucosal plexuses of the enteric nervous system (ENS) in the wall of the colon (Keast et al., 1993; Parr and Sharkey, 1996).

A small population of myenteric neurons in the colon have “intestinofugal” projections directly to the prevertebral ganglia, where they may reflexly regulate sympathetic outflow to the gut (Crowcroft et al., 1971; Dalsgaard and Elfvin, 1982; Furness et al., 1990; Keef and Kreulen, 1990; Szurszewski and King, 1991; Bywater, 1993; Messenger and Furness, 1993; Luckensmeyer and Keast, 1995; Miller and Szurszewski, 1997; Sharkey et al., 1998).

These neurons are cholinergic and contain a variety of neuropeptides, such as vasoactive intestinal polypeptide (VIP) (Schultzberg, 1983; Dalsgaard et al., 1983a; Mann et al., 1995; Luckensmeyer and Keast, 1996). The peripheral inputs to the IMG form dense varicose networks of fibers that are most closely associated with SOM and –/– neurons, although it is not clear whether any IMG neuronal subsets are preferentially affected by colonic stimulation (Dalsgaard et al., 1983a; Lindh et al., 1988; Parr and Sharkey, 1996).

Primary afferent fibers that accompany sympathetic nerves to the viscera also give rise to axon collaterals in the IMG and have projections throughout the ENS (Dalsgaard et al., 1983b; Matthews and Cuello, 1984; Peters and Kreulen, 1984, 1986). Like other visceral afferent fibers whose cell bodies are in the dorsal root ganglia, these fibers can also be acutely activated and chronically inhibited or killed by the neurotoxin capsaicin (Holzer, 1991). The neuronal circuitry of the distal colon and IMG are shown in Figure 1.

Mechanosensory stimulation of isolated colonic segments has been shown to increase activity in neurons of the attached decentralized IMG *in vitro* (Crowcroft et al., 1971; Szurszewski and King, 1991; Bywater, 1993). The activity of most IMG neurons is thought to reflect the integration of inputs from the CNS, neighboring prevertebral ganglia, intestinofugal neurons, and primary afferent fibers (Szurszewski and King, 1991). These nerves play an indispensable role in normal gut functions, and they may also have important roles in pathophysiological processes. Little is known about the activity of the IMG in intestinal pathology, in part because it has not been possible to record electrophysiological responses of IMG neurons to colonic stimulation in intact animals.

An alternative approach to this problem exploits the rapidly

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Figure 1. Schematic illustration of some of the connections of the inferior mesenteric ganglion (IMG). Intestinofugal neurons of the myenteric plexus (MP), neurons from the superior mesenteric ganglia (SMG), and spinal preganglionic neurons make excitatory connections with IMG neurons. In addition, there are axon collaterals of primary afferent fibers, whose cell bodies lie in the dorsal root ganglia (DRG) that synapse on IMG neurons. The IMG neurons themselves project to the MP, submucosal plexus (not shown), and to blood vessels (BV) of the submucosa (SM) in the distal colon.

induced expression of immediate-early genes (IEGs), such as *c-fos*, *junB*, and *c-jun*, and the detection of the gene products, Fos and Jun, by immunohistochemistry in “activated” neurons (Hughes and Dragunow, 1995). This approach has been successfully used in the ENS (Kirchgeßner et al., 1992; Parr and Sharkey, 1994b; Miampamba et al., 1997; Ritter et al., 1997), as it has in the spinal cord (Traub et al., 1992; Lantéri-Minet et al., 1993). In general, IEG expression is a feature of neurons in a high state of activity and is usually observed when intense or prolonged stimulation is applied.

This study was undertaken to examine whether induced expression of *c-fos*, *JunB*, and *c-Jun* can be used to assess activity in IMG neurons *in vivo*. Specifically, we asked whether the gene products of IEGs can be visualized in characterized neuronal subsets of the IMG and in the colonic ENS, under conditions of acute colitis with or without physiological levels of mechanosensory stimulation.

MATERIALS AND METHODS

Animals. Male albino guinea pigs (250–400 gm; Charles River, Montreal, Quebec, Canada) were used. All procedures were approved by The University of Calgary Animal Care Committee and were conducted in accordance with the guidelines established by the Canadian Council on Animal Care. Animals were fasted for 20–24 hr before use but were allowed access to water *ad libitum*. This slightly longer than usual fast was used to ensure the colon was free from fecal pellets at the time of the experiments.

Capsaicin pretreatment. One group of animals ($n = 13$) were pretreated with capsaicin dissolved in vehicle (10% ethanol, 10% Tween 80, and 80% physiological saline) 7 d before use in the studies described below, using an established method (Gamse et al., 1981). Briefly, animals were given multiple subcutaneous injections of small doses of capsaicin on day 1 until desensitized to the drug, and then larger doses were given on the second day of treatment, until a total dose of 125 mg/kg was achieved. Because guinea pigs are very susceptible to capsaicin until they are desensitized, they received prophylactic treatment with theophylline (20 mg/kg; i.p.), atropine (0.5 mg/kg; i.p.), and salbutamol by inhalation as

required. One week after treatment, they were tested for a physiological response to capsaicin by instillation of 50 μ l of 0.01% capsaicin applied to one eye. No pretreated animal responded to this application. In contrast, a naive animal will display lacrimation and vigorous wiping movements in response to capsaicin instillation.

Mechanosensory balloon stimulation. Untreated or capsaicin-treated guinea pigs were subjected to mechanosensory stimulation of the colon ($n = 49$). Mechanosensory stimulation was achieved using a small latex balloon inserted into the colon ~8 cm from the anus under halothane anesthesia (2–4% in oxygen). For these experiments, a balloon was made from the fingertip of a latex glove attached to a pediatric feeding tube. After passing the balloon into the animal, it was inflated to approximately the size of a fecal pellet with 50 μ l of saline (outside diameter of ~5 mm). The balloon was slowly withdrawn from the colon over a period of 60 sec, and this procedure was repeated six times at 5 min intervals over 30 min. At the end of the stimulation protocol, animals recovered and were returned to their cages for 2.5 hr, after which time they were deeply anesthetized and killed by exsanguination. This longer than usual survival time was chosen based on preliminary studies that showed that shorter times (90 min) produced labeling of lower intensity. Controls (sham stimulation) were treated identically, except that the balloon was inserted and withdrawn but not inflated.

Intraluminal capsaicin stimulation. In one set of experiments, animals ($n = 9$) were anesthetized as above and capsaicin (0.5 ml, 0.2% dissolved in vehicle) or vehicle alone was injected into the colonic lumen at the same site used for balloon stimulation. Animals in both groups were then subjected to balloon or sham stimulation as described above. At the end of the stimulation protocol, animals recovered and were returned to their cages for 2.5 hr, after which time they were deeply anesthetized and killed by exsanguination. At the end of the experiment, the colon was examined after removal to assess the effect of capsaicin stimulation.

Acetic acid-induced colitis. In some animals ($n = 38$), mechanosensory stimulation was performed after the induction of colitis using a standard method (MacPherson and Pfeiffer, 1978). Briefly, animals were anesthetized as above, and acetic acid (0.5 ml, 2% dissolved in physiological saline) or vehicle (saline) alone was injected into the colonic lumen at the same site used for balloon stimulation. Animals were allowed to recover for either 4 or 24 hr, after which animals in both groups were then subjected to balloon or sham stimulation as described above. Animals kept 24 hr after injection of acetic acid were fed immediately after injection and allowed access to food for an additional 8 hr, after which they were fasted for ~12 hr before balloon stimulation. In all cases, an examination was made of the colon after removal to assess the extent of inflammation. Quantitative assessment of colonic damage was made using a standard method (McCafferty et al., 1994). Briefly, the method of macroscopic damage scoring takes into account the presence and severity of adhesions (score 0–2), the maximum bowel wall thickness (in millimeters), and the presence or absence of diarrhea (0–1), in addition to assigning a score based on the extent of mucosal damage from normal (score of 0) to a score of 10 depending on the presence and extent of ulceration and the extent of hyperemia. At the end of the balloon stimulation protocol, animals recovered and were returned to their cages for 2.5 hr, after which time they were deeply anesthetized and killed by exsanguination.

Tissue preparation. The IMG and colon were removed from all animals and fixed by overnight immersion in 4% paraformaldehyde and Zamboni's fixative, respectively, at 4°C. It was found that the colon was free from fecal pellets in virtually all animals. The colon was first immersed in PBS, pH 7.4, containing nifedipine (1 μ M) to allow it to be stretched flat before fixation. After fixation, tissues were washed in PBS. The IMG was cryoprotected in PBS containing 20% sucrose, and sagittal frozen sections (12–14 μ m) were serially mounted onto slides, each of which contained representative sections, spaced by at least 120 μ m, of the whole ganglion. The colon was dissected to produce whole-mount preparations as described previously (Sharkey et al., 1990; Parr and Sharkey, 1994a).

Immunohistochemistry. Sections and whole-mount preparations were processed for immunohistochemistry as described previously (Sharkey et al., 1990; Parr and Sharkey, 1994a, 1996). Briefly, after washing in PBS containing 0.1% Triton X-100, they were incubated for 18–48 hr in primary antibodies (4°C). They were then washed in PBS (three times for 10 min) and incubated for 1 hr at room temperature in secondary antibodies that were conjugated to a variety of fluorochromes. After this, they were washed again and mounted in bicarbonate-buffered glycerol. Primary antibodies used were rabbit and mouse anti-*c-fos* [TF3 (rabbit) and TF161 (mouse) provided by Dr. K. Riabowol, University of Calgary,

Calgary, Alberta, Canada] (Riabowol et al., 1988), rabbit anti-JunB (ab 725; provided by Dr. R. Bravo, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ), rabbit anti-c-Jun (provided by Dr. K. Riabowol), mouse anti-somatostatin (soma 10; provided by the Medical Research Council Regulatory Peptide Group, University of British Columbia, Vancouver, British Columbia, Canada), rat anti-NPY (NT115; Eugene Tech, Ridgefield Park, NJ), rabbit anti-calcitonin gene-related peptide (CGRP) (IHC 6006; Peninsula Laboratories, Belmont, CA), rabbit anti-VIP (7913; provided by Dr. J. H. Walsh, Center for Ulcer Research and Education: Digestive Diseases Research Center, University of California at Los Angeles, Los Angeles, CA), and rabbit anti-Fos4 (SC52; Santa Cruz Biotechnology, Santa Cruz, CA) that labels all neuronal nuclei in the guinea pig (Parr and Sharkey, 1994a, 1996). Specificity controls for these have been described previously (Parr and Sharkey, 1994a; Parr and Sharkey, 1996). Secondary antibodies used were goat anti-rabbit IgG conjugated to FITC or CY3 (Sigma, St. Louis, MO), donkey anti-mouse IgG conjugated to aminomethylcoumarin or CY3 (Jackson ImmunoResearch, West Grove, PA), and sheep anti-rat IgG conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch). For double or triple labeling, the primary and secondary antibodies were mixed before use. Immunofluorescence was viewed using a Zeiss (Oberkochen, Germany) Axioplan microscope with appropriate filter sets. Photographs were taken with Kodak (Eastman Kodak, Rochester, NY) TMax 400 ASA film.

Drugs. Capsaicin, atropine, nifedipin, and theophylline were obtained from Sigma. Salbutamol was obtained from NovoPharm Ltd. (Novo-Salmol; Toronto, Ontario, Canada). All other reagents were from BDH Chemicals (Edmonton, Alberta, Canada).

Statistical analysis. Quantitative data were obtained by counting immunoreactive neurons in representative sections of the IMG from all animals. Approximately 12 triple-labeled sections were counted from each animal, spaced through a ganglion such that double counting of a single cell was avoided. Neurons were defined as c-fos-immunoreactive when clear nuclear labeling was evident. Binuclear neurons were counted as a single cell. The nonspecific, faint nuclear labeling of IMG neurons by the NPY antibody noted previously (Parr and Sharkey, 1996) was used as a convenient way to identify those neurons that lacked either NPY or SOM (–/– neurons). The average (mean $\pm$ SEM) proportion of c-fos-immunoreactive cells also containing SOM, NPY, or neither was determined for each group. Multiple groups were compared by ANOVA and a Tukey–Kramer *post hoc* test. Two groups were compared with a Student's *t* test. Probabilities of $<5\%$ ($p < 0.05$) were considered significant.

RESULTS

c-fos, c-Jun, and JunB

In sections of the IMG from untreated animals or animals treated with an intraluminal injection of saline, c-fos and JunB antisera were found to label some nerve fibers and to faintly label the cytoplasm of neurons, but nuclear labeling was not found. The c-Jun antiserum also lightly labeled the cytoplasm of IMG neurons, and nuclear c-Jun-IR was found in IMG neurons of smaller animals (<250 gm; $n = 8$ animals) but was less evident or undetectable in larger animals (>300 gm; $n = 6$ animals). The smaller chromaffin cells sometimes contained nuclear c-Jun-, c-fos-, and JunB-IR as well (data not shown). In sections triple-labeled for SOM, NPY, and c-Jun, large subsets of SOM neurons and NPY neurons and smaller groups of NPY/SOM neurons and –/– neurons were visible (Fig. 2*A,B*). When present, nuclear c-Jun-IR in double- and triple-labeled sections was found in some neurons of all of these subsets but was most evident in SOM and –/– neurons. However, no clear association was found between any of the experimental procedures (see below) and c-Jun expression, and so this was not characterized further. In all cases, c-fos and JunB gave identical results, and so for most studies only c-fos was used for quantification purposes.

c-fos-IR and JunB were absent from the colonic myenteric plexus of untreated animals, and c-Jun was found in a small fraction of neuronal nuclei (data not shown).

Induction of c-fos and JunB by balloon stimulation

In an attempt to mimic a low-threshold physiological, mechanical stimulus to the colon, a saline-filled small intraluminal balloon was used to activate colonic nerves by imitating the passage of fecal pellets. In saline-treated animals that received balloon stimulation, an insignificant number of total IMG neurons contained c-fos or JunB, but this was exclusively localized in the –/– neurons (Fig. 3). In triple-labeled sections (SOM, NPY, and c-fos), c-fos expression was found in $40 \pm 18\%$ ($n = 4$ animals) of these neurons treated with balloon stimulation 4 hr after saline administration and in $65 \pm 20\%$ ($n = 4$) 24 hr after saline administration. The –/– neurons account for ~ 1 –3% of total IMG neurons (Parr and Sharkey, 1996). Thus, only ~ 0.5 –2% of total IMG neurons were apparently activated by physiological levels of balloon stimulation and only those in this specific subclass.

In the colonic myenteric plexus, 50% of animals examined (two of four animals) had c-fos expression 4 hr after saline treatment and balloon stimulation (Fig. 4). The nuclei were relatively small, lacked a visible nucleolus, and were not immunoreactive for a selective neuronal nuclear antigen (Parr and Sharkey, 1994a). On this basis, we have classified these cells as enteric glia. Twenty-four hours after saline administration and then balloon stimulation, all animals ($n = 4$) had glial c-fos expression but no neuronal c-fos. JunB-IR was not observed in the myenteric plexus.

Colonic damage induced by saline or acetic acid

Intracolonic administration of saline produced no macroscopically observable colonic damage, but slight hyperemia was evident 4 hr after treatment. By 24 hr, the saline-treated animals were indistinguishable from untreated guinea pigs. In contrast, the colons of animals treated with 2% acetic acid consistently exhibited mild colitis, with hyperemia and mucosal hemorrhaging within 4 hr. Twenty-four hours after receiving acetic acid, colonic damage consisted of hyperemia, hemorrhage, and regions of ulceration. In some animals, muscle thickening was also apparent. Nevertheless, animals showed no other obvious signs of distress and would eat when allowed. Quantitative assessment of colonic damage is given in Table 1.

Induction of c-fos and JunB by acetic acid-induced colitis

Surprisingly, colitis caused very little enhanced expression of c-fos or JunB in the IMG. Four hours after treatment, c-fos expression was observed in $1 \pm 1\%$ of SOM neurons, $3 \pm 2\%$ of –/– neurons, and none of the NPY neurons ($n = 4$ animals). At 24 hr after treatment, c-fos expression was not observed in any class of IMG neuron ($n = 4$). In the treated segments of distal colon, c-fos labeling was not observed in the myenteric plexus either 4 ($n = 4$ animals) or 24 ($n = 4$) hr after treatment.

Induction of c-fos and JunB by acetic acid-induced colitis and balloon stimulation

Costimulation of animals with balloon stimulation after the induction of colitis (4 and 24 hr) resulted in extensive expression of c-fos and JunB in some IMG neurons (Figs. 2, 3). NPY neurons did not express c-fos or JunB at any time. SOM and –/– neurons had elevated expression compared with either balloon stimulation alone or acetic acid alone (Figs. 3, 5). Between 9–12% of SOM neurons expressed c-fos under these conditions, as did 65–80% of –/– neurons. Because these cells represent 75% of the total IMG neurons (Parr and Sharkey, 1996), these data suggest that ~ 8 –

Figure 2. Fluorescence micrographs of c-Jun (*A*) and JunB (*C*) in the IMG from animals treated with acetic acid (*A, B*) or acetic acid and balloon stimulation (*C, D*) before perfusion. Preparations were double-labeled with anti-somatostatin (*SOM, B, D*). c-Jun-immunoreactive nuclei were found in all classes of neurons, of which two are shown by the *arrowheads* (non-SOM) and the *double arrowheads* (SOM neurons). c-Jun-immunoreactive nuclei were also found in control animals, and no differences in the distribution of c-Jun were associated with any of the stimuli used in this study. JunB-immunoreactive nuclei were absent from control animals and after acetic acid treatment alone. After balloon stimulation, in acetic acid-treated animals, JunB was primarily found in SOM neurons (*arrowheads*). Scale bar, 50 μ m.

10% of the total population of IMG neurons were activated by this stimulus.

In the colon, there was a similar enhancement of c-fos expression. c-fos was observed in glia in virtually all animals examined (five of five at 4 hr; two of three at 24 hr) after colitis and was also seen in myenteric neurons 4 hr after treatment (Fig. 4).

Induction of c-fos and JunB by acetic acid-induced colitis and balloon stimulation in capsaicin-treated animals

Capsaicin treatment of adult animals was performed 1 week before experimentation. Its effectiveness was assessed behaviorally (see Materials and Methods) and by immunohistochemistry. All animals were behaviorally insensitive to capsaicin. Sections of IMG or colonic submucosal blood vessels from control animals had an extensive CGRP innervation. After capsaicin treatment, CGRP-IR was virtually absent from these animals (data not shown). In contrast, VIP-IR was not different between untreated and capsaicin-treated guinea pigs in either location.

Colitis alone in capsaicin-treated animals gave rise to no c-fos or JunB expression in the IMG either 4 or 24 hr after treatment. However, in the colonic myenteric plexus, c-fos expression was present in glia (4 hr, one of three animals) and neurons (4 hr, one of three animals; 24 hr, two of three animals) in capsaicin-treated

animals (Fig. 6), in contrast to untreated animals with colitis (see above).

Balloon stimulation in the presence of colitis in capsaicin-treated guinea pigs was far less effective in stimulating c-fos or JunB expression in the IMG compared with the effects of balloon stimulation in vehicle-treated animals (Figs. 3, 5). In vehicle-treated guinea pigs, 9–12% of SOM and ~75% of $-/-$ neurons express c-fos. Only 2–7% of SOM and 14–37% of $-/-$ neurons were activated in capsaicin-treated animals. In the colonic myenteric plexus, c-fos expression was present in glia (4 hr, four of four animals) and neurons (4 hr, four of four animals; 24 hr, two of three animals) in capsaicin-treated guinea pigs (Fig. 6).

Induction of c-fos by intraluminal capsaicin treatment

To determine whether activation of capsaicin-sensitive nerves might have a role in the responses of IMG neurons, 0.2% intracolonic capsaicin or vehicle was given to anesthetized animals to acutely stimulate primary afferent fibers in combination with balloon or sham stimulation. Despite obvious acute physiological effects of capsaicin application, including transiently elevated respiration, heart rate, and faint traces of blood on the mucosal surface, animals showed no obvious signs of distress after recovery from anesthesia 0.5 hr later. Intraluminal capsaicin failed to

Figure 3. Fluorescence micrographs of c-fos (*A, D, G, J*), NPY (*B, E, H, K*), and SOM (*C, F, I, L*) in the IMG from normal animals treated with saline 24 hr before balloon stimulation (*A–C*), with acetic acid 24 hr before balloon stimulation (*D–F*), a capsaicin-treated animal given acetic acid 24 hr before balloon stimulation (*G–I*), and an animal given intraluminal capsaicin treatment (*J–L*). Preparations were triple-labeled. After balloon stimulation in the normal colon, few cells were activated, and these were restricted to the non-NPY/non-SOM population (*A–C*, double arrowheads). Note that the apparent nuclear labeling in the NPY cells (*B*) is an artifact (see Materials and Methods). Balloon stimulation of inflamed colon resulted in more extensive c-fos induction, predominantly in SOM neurons (*D–F*, arrowheads). After capsaicin treatment, very few c-fos cells were visualized, but when present, these were primarily SOM or non-SOM/non-NPY cells (*G–I*, arrowheads). Intraluminal capsaicin treatment activated similar neurons to those seen after inflammation and balloon stimulation (*J–L*); primarily SOM neurons (arrowheads). Scale bar, 50 μ m.

Figure 4. Fluorescence micrographs of the myenteric plexus from animals treated with vehicle 4 hr before balloon stimulation (*A, B*), acetic acid 4 hr before sham stimulation (*C, D*), and acetic acid 4 hr before balloon stimulation (*E, F*). Preparations were double-labeled with an anti-neuronal nuclear antibody (*A, C, E*) and c-fos (*B, D, F*). After balloon stimulation in the normal colon (*A, B*), c-fos was confined primarily to enteric glia (arrowheads) and not neurons (double arrowheads). Acetic acid-induced colitis did not cause c-fos induction (*C, D*), but after inflammation, balloon stimulation caused glial (arrowheads) and neuronal (double arrowheads) c-fos expression. Scale bars, 50 μ m.

induce c-fos in most IMG neurons from sham-stimulated animals ($n = 3$ animals) (Table 2). In animals treated with vehicle and balloon stimulation, c-fos was again absent from most neurons but was consistently found in ~40% of the $-/-$ neurons as before ($n = 3$). In animals treated with both capsaicin and the balloon stimulation ($n = 3$), nuclear c-fos-IR was significantly increased in SOM neurons but was still primarily absent from NPY or NPY/SOM neurons (Fig. 3).

In the treated segments of colon from these animals, c-fos was not present in the myenteric plexus from vehicle-treated–balloon-stimulated animals but was again evident in primarily glial nuclei from animals in both capsaicin-treated groups.

DISCUSSION

Stimulation of the colon *in vivo* leads to IEG expression in both the IMG and myenteric plexus of the guinea pig. c-fos and JunB

Table 1. Quantitative assessment of colonic damage [mean $\pm$ SEM (*n*)]

Treatment group	Colonic damage	
	4 hr	24 hr
Saline and balloon stimulation	0.8 $\pm$ 0.5 (4)*	0.5 $\pm$ 0.3 (4)*
Acetic acid	3.6 $\pm$ 2.1 (4)	5.7 $\pm$ 1.3 (5)
Acetic acid and balloon stimulation	2.1 $\pm$ 0.8 (5)	6.6 $\pm$ 2.0 (4)
Capsaicin pretreatment and acetic acid	5.0 $\pm$ 0 (3)	6.3 $\pm$ 0.2 (3)
Capsaicin pretreatment, acetic acid, and balloon stimulation	4.4 $\pm$ 0.5 (4)	8.5 $\pm$ 0.8 (3)

**p* < 0.05 compared with all acetic acid groups.

Figure 5. Quantitative assessment of the effects of balloon stimulation 4 and 24 hr after acetic acid treatment in normal and capsaicin-treated animals (*n* = 4 per group). c-fos was counted in triple-labeled sections in either non-NPY/non-SOM or SOM neurons and is expressed as a percentage of the total population of these two classes of neurons, which make up ~5 and 75% of total IMG neurons, respectively. **p* < 0.05 compared with control.

are expressed in neurons of the IMG and myenteric plexus, and c-fos is also expressed in enteric glia, in response to a combination of inflammation and mechanical stimulation of the gut. c-Jun was present under basal conditions in neurons in both the myenteric plexus and IMG, but none of the stimuli used in this study

altered c-Jun expression. There appeared to be less c-Jun in neurons of older animals than younger ones, for reasons that are unclear; however, this type of phenomenon has been described in the CNS (Lloyd et al., 1994). Considerable ongoing activity of the myenteric inputs to the IMG has been found in acutely isolated preparations of colon with attached IMG (Crowcroft et al., 1971; Szurszewski and King, 1991). We found that IEG expression was absent from the IMG of untreated or saline-treated animals, and we found no evidence that ongoing activity *in vivo* could act synergistically with intraluminal capsaicin or colitis to induce IEG expression in the IMG. It is possible that any ongoing activity was below the threshold for IEG induction, although it is also possible that the levels of activity observed *in vitro* have been introduced by excision of the colon-IMG preparation through loss of spinal inputs.

Mechanosensory stimulation in animals treated with saline 4 or 24 hr before balloon stimulation was sufficient to cause IEG expression in a small fraction of IMG neurons, restricted to the class of noradrenergic neurons that lacked SOM and NPY (–/– neurons). It is notable that the relatively rare –/– neurons were activated by balloon passage alone, perhaps indicating unique inputs, responses, and/or thresholds of these neurons compared with other subsets. In this respect, it may be valuable in future experiments to examine the more abundant –/– neurons in the celiac/superior mesenteric ganglion (Macrae et al., 1986).

Only when mechanosensory stimulation was combined with either colitis or intraluminal capsaicin was there more extensive activation of IMG neurons involving both –/– neurons and SOM neurons. At no time did we observe IEG expression in either class of NPY neurons (NPY or NPY/SOM neurons). It is interesting that NPY neurons appear to have a much reduced or nonexistent innervation from intestinofugal neurons (Dalsgaard et al., 1983a; Lindh et al., 1988; Parr and Sharkey, 1996), suggesting that intestinofugal inputs are required for IEG expression in IMG neurons.

In the myenteric plexus, intraluminal capsaicin was found to cause c-fos expression in enteric glia but in few, if any, neurons. We and others have observed previously glial c-fos expression after neural stimulation in the guinea pig and colitis in the rat (Kirchgessner et al., 1992; Parr and Sharkey, 1994b; Miampamba and Sharkey, 1998). Only 4 hr after acetic acid treatment, when also stimulated by mild balloon distension, were neurons found to express c-fos. Curiously, after animals were pretreated with capsaicin, acetic acid-induced colitis alone was a sufficient stimulus to cause neuronal (and glial) activation, suggesting that some feature of the primary afferent innervation of colonic myenteric neurons normally suppresses their activity. In the rat colon, stretch-induced enteric reflexes are mediated by activation of

Figure 6. Fluorescence micrographs of the myenteric plexus from capsaicin-treated animals treated with acetic acid 24 hr before sham stimulation (*A, B*) and acetic acid 24 hr before balloon stimulation (*C, D*). Preparations were double-labeled with an anti-neuronal nuclear antibody (*A, C*) and c-fos (*B, D*). After acetic acid or acetic acid and balloon stimulation, c-fos was present in enteric glia and neurons. In contrast, in untreated animals, acetic acid-induced colitis did not cause c-fos induction (see Fig. 4*C, D*). Scale bar, 50 μ m.

Table 2. The percentage of each of the IMG neuronal subsets with c-Fos in animals treated with colonic balloon stimulation, intraluminal capsaicin, or both (mean $\pm$ SEM; $n = 3$ animals per group)

Treatment group	SOM neurons	–/– Neurons	NPY neurons	NPY/SOM neurons
Balloon stimulation	0	38.2 $\pm$ 12.4*	0	0
Intraluminal capsaicin	0.33 $\pm$ 0.33	0	0	0
Capsaicin and balloon stimulation	37.6 $\pm$ 16.1**	47.9 $\pm$ 11.3†	2.9 $\pm$ 2.9	1.7 $\pm$ 1.7

* $p < 0.05$ compared with SOM, NPY, and NPY/SOM neurons within the balloon-stimulated group and compared with –/– neurons in the capsaicin-treated group. ** $p < 0.05$ compared with NPY and NPY/SOM neurons within the capsaicin-treated–balloon-stimulated group and compared with SOM neurons of both of the other groups. † $p < 0.01$ compared with NPY and NPY/SOM neurons within the capsaicin-treated–balloon-stimulated group and compared with –/– neurons of the capsaicin-treated group.

capsaicin-sensitive nerves, whereas mucosal stimuli activate intrinsic primary afferent neurons (Grider and Jin, 1994; Grider et al., 1996). If such an arrangement exists in the guinea pig colon, then it may be that stretch-activated enteric reflex pathways have a net inhibitory effect on colonic myenteric neurons stimulated by mucosal damage caused by colitis.

In the IMG, however, capsaicin pretreatment reduced the extent of IEG expression such that 24 hr after the induction of colitis c-fos expression in both SOM and –/– neurons was virtually abolished. It may be that the loss of stretch-activated reflexes also reduced the activity in the second-order intestinofugal neurons to such an extent that very little in the way of a stimulus reached the IMG. A peripheral model of IMG activation is supported by an electrophysiological and anatomical study by

King and Szurszewski (1984), who showed that stretch mechanoreceptor information from the colon is primarily referred to the IMG, with relatively little involvement of the spinal cord. However, distal colitis in rats has been shown to alter viscerosomatic reflexes and proximal colonic transit (Morteau et al., 1994), suggesting that some spinal involvement occurs, at least under pathophysiological conditions.

Based on these findings, our data point to the need for convergent afferent signals to reach the IMG to activate the principal ganglion cells sufficiently to cause IEG expression. Convergent inputs have been suggested previously on the basis of electrophysiological studies *in vitro* (Davison et al., 1977; Keef and Kreulen, 1988, 1990; King and Szurszewski, 1989). Whether the convergence occurs in the wall of the colon, at the level of the IMG by

activation of axon collaterals of primary afferent fibers, via spinal reflexes, or by a combination of these is not clear and requires further study. Comparison of IMG with the superior mesenteric ganglion would be instructive in this regard. Our data support the concept that the functional circuitry of the IMG and distal colon relies on the convergent inputs shown in Figure 1.

Previous electrophysiological studies have found that the activity of fast cholinergic inputs to IMG neurons from the myenteric plexus are increased by intracolonic pressure (Crowcroft et al., 1971; Szurszewski and King, 1991; Bywater, 1993; Miller and Szurszewski, 1997). Noncholinergic slow excitatory potentials that have also been observed in these cells are thought to originate in part from transmitters released from primary afferent fibers (Peters and Kreulen, 1984, 1986; Kreulen and Peters, 1986). Nevertheless, either of these stimuli may be insufficient to generate action potentials in IMG neurons, and in fact the activity of these neurons is thought to reflect integration of multiple inputs as mentioned above. Our data support the idea that these peripheral inputs have synergistic effects on IMG neurons and suggest that these effects may become apparent when the inflamed colon is stimulated, for example, by fecal transit. It may also be that sensitization of afferent inputs together with ongoing mechanical activity is sufficient to cause sympathetic activation, which, through IEG activation, leads to long-term changes in the sympathetic innervation of the bowel and may underlie some functional bowel disorders.

Previous studies have demonstrated correlations between the neurochemistry, projections, major inputs, and presumably the function of neurons in the guinea pig celiac ganglion that supplies sympathetic nerves to the small bowel (Macrae et al., 1986; Keast et al., 1993; Luckensmeyer and Keast, 1995, 1996). These data also support the idea that immunohistochemically defined subsets of IMG neurons represent chemically coded groups with distinct functions. For example, noradrenergic NPY neurons project to vasculature and are likely to be vasoconstrictor neurons. The lack of IEG expression seen in NPY neurons may correlate with reduced activity in these cells, consistent with the vasodilatation that accompanies inflammation. The projections of $-/-$ neurons in the IMG have not been identified conclusively, but $-/-$ neurons in the celiac ganglion project exclusively to the myenteric plexus (Macrae et al., 1986). Similar projections of IMG neurons to the colon could conceivably provide a low threshold reflex pathway modulating motor patterns. Interestingly, acute colitis has marked effects on colonic transit, consistent with sympathetic activation (Myers et al., 1997a), but also potentially because of altered contractility of smooth muscle (Goldhill et al., 1995; Myers et al., 1997b). SOM neurons in the IMG project to both the myenteric and submucosal plexuses (Parr and Sharkey, 1996) and might therefore modulate all enteric nerve functions. Because activation of IEGs in these neurons also appears to require noxious colonic stimulation, they may represent the efferent limb of a high-threshold pathway that affects enteric nerve functions in a distinct and/or more profound manner.

The role of sympathetic nerves in colitis is not yet clear, but IEG expression observed in this study suggests that sympathetic activation is a feature of colitis. Because c-fos has been implicated as a factor in the regulation of tyrosine hydroxylase (Gizang-Ginsberg and Ziff, 1990), an enzyme required for noradrenaline synthesis, intestinal inflammation may change some aspect of noradrenaline turnover in sympathetic ganglia. Alternatively, there may be altered local transmitter or mediator release leading to peripheral changes, as seen, for example, in rat articular joints

(Basbaum and Levine, 1991). Evidence for this in the gut comes from a recent study from this laboratory in which we demonstrated a proinflammatory deleterious role for sympathetic nerves in trinitrobenzene sulfonic acid-induced colitis in the rat (McCafferty et al., 1997). In other models of intestinal inflammation, noradrenaline release has been shown to be reduced (Swain et al., 1991). Inhibition of noradrenaline release appears to involve inflammatory mediators within the myenteric plexus (Collins et al., 1992); however, our data suggest that this may be preceded by activation of the sympathetic nerve cell bodies.

In conclusion, we have demonstrated IEG expression in sympathetic prevertebral ganglion neurons of the guinea pig under physiological and pathophysiological conditions *in vivo*. Acute colitis caused the activation of sympathetic neurons when accompanied by mechanosensory stimulation. Myenteric neuronal activation is not a major feature of acute colitis, but enteric glia express c-fos in response to inflammation. Recent work suggests that enteric glia proliferate in response to inflammation (Bradley et al., 1997) and may play a central immunosuppressive role in the gastrointestinal tract (Bush et al., 1998). Based on this work, further studies are now warranted to examine the consequences of c-fos expression in the myenteric plexus and IMG and the role of enteric glia in intestinal inflammation.

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