Slow synaptic transmission in myenteric AH neurons from the inflamed guinea pig ileum

Kulmira Nurgali,1 Trung V. Nguyen,2 Michelle Thacker,2 Louise Pontell,2 and John B. Furness2

Departments of 1Physiology and 2Anatomy and Cell Biology, University of Melbourne, Parkville, Victoria, Australia

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Nurgali K, Nguyen TV, Thacker M, Pontell L, Furness JB. Slow synaptic transmission in myenteric AH neurons from the inflamed guinea pig ileum. Am J Physiol Gastrointest Liver Physiol 297: G582–G593, 2009.—We investigated the effect of inflammation on slow synaptic transmission in myenteric neurons in the guinea pig ileum. Inflammation was induced by the intraluminal injection of trinitrobenzene sulfonate, and tissues were taken for in vitro investigation 6–7 days later. Brief tetanic stimulation of synaptic inputs (20 Hz, 1 s) induced slow excitatory postsynaptic potentials (EPSPs) in 49% and maintained postsynaptic excitation that lasted from 27 min to 3 h in 13% of neurons from the inflamed ileum. These neurons were classified electrophysiologically as AH neurons; 10 were morphological type II neurons, and one was type I. Such long-term hyperexcitability after a brief stimulus is not encountered in enteric neurons of normal intestine. Electrophysiological properties of neurons with maintained postsynaptic excitation were similar to those of neurons with slow EPSPs. Another form of prolonged excitation, sustained slow postsynaptic excitation (SSPE), induced by 1-Hz, 4-min stimulation, in type II neurons from the inflamed ileum reached its peak earlier but had lower amplitude than that in control. Unlike slow EPSPs and similar to SSPEs, maintained excitation was not inhibited by neurokinin-1 or neurokinin-3 receptor antagonists. Maintained postsynaptic excitation was not influenced by PKC inhibitors, but the PKA inhibitor, H-89, caused further increase in neuronal excitability. In conclusion, maintained excitation, observed only in neurons from the inflamed ileum, may contribute to the dysmotility, pain, and discomfort associated with intestinal inflammation.

Enteric neurons of another class, S neurons, have prominent fast postsynaptic excitatory potentials (EPSPs) and do not have late AHPs following their action potentials in normal conditions. These neurons have single axons and lamellar or filamentous dendrites (Dogiel type I or filamentous shapes). Functionally, these neurons are motor and interneurons (11). In the inflamed guinea pig ileum, a subpopulation of Dogiel type I neurons significantly alter their electrophysiological properties and start to behave in a similar way to AH neurons, exhibiting prominent late AHPs and a TTX-resistant component of their action potential (33). Fast EPSPs in S neurons are substantially augmented in the guinea pig jejunum and colon after inflammation induced by Trichinella spiralis and 2,4,6-trinitrobenzenesulfonic acid (TNBS) (16, 20, 34).

The mechanisms of the changes in fast synaptic transmission after inflammation in the intestine have been investigated in more detail in the guinea pig distal colon myenteric and submucosal neurons (16, 20). In the submucosal plexus, recruitment of additional neurotransmitters is associated with inflammation-induced facilitation of fast EPSPs (20), whereas in the myenteric neurons inflammation-induced augmentation of fast EPSPs was not accompanied by alteration in pharmacological profiles of EPSPs or in synaptic density but involved a presynaptic increase in PKA activity and an increase in the readily releasable pool of synaptic vesicles (16).

Three forms of slow postsynaptic excitation have been described in enteric neurons, slow EPSPs, induced by brief tetanic stimulation (10–20 Hz for 1 s) of synaptic inputs; sustained slow postsynaptic excitation (SSPE) in AH neurons, induced by low frequency (1 Hz) stimulation of presynaptic inputs for 4 min or more; and a tachykinin-mediated slow excitatory potential in S neurons (inhibitory motor neurons), evoked by low frequency stimulation (1, 11). A single stimulus pulse can also evoke slow EPSPs in secretomotor and vasodilator neurons in the guinea pig submucosal plexus (26). Slow EPSPs that last for 1–4 min can be induced in both AH and S neurons (28); SSPEs that last from 20 min to 4 h were observed only in AH neurons in normal intestine (1, 10, 32). Slow EPSPs in S, but not AH, neurons in the guinea pig colon are affected by inflammation (19, 20). The proportion of myenteric S neurons receiving slow EPSPs was increased following TNBS-induced inflammation in the guinea pig distal colon (19), and the amplitudes of stimulus-evoked slow EPSPs recorded in submucosal S neurons were significantly increased during inflammation in the guinea pig distal colon (20). Until the present investigation, the effect of inflammation on the SSPE has not been studied in any region.

Effects of inflammation on neuronal excitability are confined to specific morphologically defined neurons in the guinea pig ileum (33), but effects on slow transmission have not been reported in this region. Thus in the present work we have
investigated whether slow postsynaptic events in myenteric neurons of the small intestine are altered by inflammation.

MATERIALS AND METHODS

All experiments were performed on guinea pigs (150–275 g) of either sex from the inbred Hartley strain colony of the Department of Anatomy and Cell Biology at the University of Melbourne. All procedures were conducted according to the Code of Practice of the National Health and Medical Research Council of Australia and were approved by the University of Melbourne Animal Experimentation Ethics Committee. All animals were maintained in a controlled environment at 21°C on a 12-h:12-h light-dark cycle with free access to food and water. At the time of taking tissue, animals were stunned by a blow to the head and killed by having the carotid arteries cut and the spinal cord severed.

Induction of inflammation. Guinea pigs were anesthetized with a mixture of xylazine (20 mg/kg) and ketamine hydrochloride (100 mg/kg; Troy Laboratories, Smithfield, NSW, Australia) given intramuscularly. The abdomen was opened by a 1.5-cm incision in the midline, and the distal part of the ileum was exteriorized. TNBS (Wako Industries, Nagoya, Japan), 30 mg/kg in 1 ml of 30% ethanol, was injected into the lumen of the ileum ~8 cm proximal to the ileocecal junction through a 30-gauge needle over the course of 1 min. The intestine was temporarily occluded just distal to the injection site during the injection and for a further minute following injection. A fine silk ligature (5.0) was tied loosely around a nearby blood vessel to mark the injection site for later location. The intestine was then returned to the abdominal cavity, the abdominal wall and peritoneum were closed with sutures, and the skin was closed with stainless steel staples. The guinea pigs were housed individually and monitored during recovery from anesthesia (2–3 h) and then housed together with free access to food and water. The guinea pigs showed no signs of stress, exhibited apparently normal exploratory behavior, and ate soon after they awoke from anesthesia. Animals were taken at 6 and 7 days after TNBS injection. Segments of inflamed ileum were removed for electrophysiological studies and for histological assessment of the level of inflammation. Animals were weighed before administration of TNBS and daily following surgery. Inflammation was assessed as previously described (33).

Control data were from guinea pigs of the same colony that were in the same age range but were not subjected to surgery.

Tissue preparation for electrophysiology. Segments of inflamed ileum, 2–3 cm long, were taken from the region of inflammation in TNBS-treated animals and from control (untreated) animals, 8–10 cm proximal to the ileocecal junction. The oral end was marked with a fine pin passed through the tissue. The segments were placed in physiological saline (composition in mM: 118 NaCl, 4.8 KCl, 25 NaHCO3, 1.0 Na2HPO4, 1.2 MgSO4, 11.1 glucose, and 2.5 CaCl2; equilibrated with 95% O2:5% CO2) and initially kept at room temperature. The solution contained 3 µM nicardipine and 1 µM hyoscine (both from Sigma-Aldrich, Sydney, Australia) to inhibit muscle movement. The mucosa, submucosa, and circular smooth muscle were carefully removed to expose the myenteric plexus. The preparation was pinned to the silgard elastomer base of a recording dish (volume 1 ml), which was placed on the stage of an inverted microscope and continuously superfused (4 ml/min) with physiological saline that had been preheated to yield a bath temperature of 34–35°C. The tissue was equilibrated with perfusate for 1–2 h before recording commenced.

Electrophysiological recordings. Neurons were impaled with conventional borosilicate glass microelectrodes filled with 1% biocytin (Sigma-Aldrich) in 1 M KCl. Electrode resistances were 100–170 MΩ. Recordings were made using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA). Signals were digitized at 1–10 KHz with the use of a Digidata 1322A interface (Axon Instruments) and stored using PC-based data acquisition software (Axoscope 8.2, Axon Instruments). Measurements of electrophysiological properties were made after allowing the impalements to stabilize for at least 15 min without applying intracellular holding current. At this time, the ability of the cell to fire an action potential was assessed. Action potentials were evoked both by extracellular stimulation to induce antidromically activated action potentials and by injection of brief intracellular current pulses. Only cells that were able to fire an action potential, had resting membrane potentials (RMPs) more negative than ~40 mV, and were adequately filled with dye to reveal their morphology were included in the electrophysiological analysis. The properties of the action potentials and late AHs in all groups were recorded at ~60 mV, which was achieved by injection of holding current when necessary.

Small intracellular hyperpolarizing current pulses (duration 500 ms, intensity 30 pA, yielding voltage shifts of 5–10 mV) were used to determine input resistance (Rm) and cell capacitance (Cm). Excitability was assessed by injecting 0.5–2-s depolarizing current pulses, at an intensity of 20–300 pA at 20-s intervals, through the recording electrode while a holding current was used to maintain the RMP at ~60 mV.

Electrical stimuli were applied to interganglionic connectives using a fine tungsten stimulating electrode (10–50-µm tip diameter) that was insulated except at the tip. The stimulating electrode was positioned at the end of the ganglion circumferential to the recording electrode so that the inputs from most processes including circumferential, ascending, and descending were stimulated. Stimuli were delivered via an ISO-Flex stimulator controlled by a Master-8 programmable pulse generator (both from AMPI, Jerusalem, Israel). Fast EPSPs were evoked by extracellular pulses of 0.1-ms duration and 0.3–0.5-mA intensity at 10-s intervals while the membrane potential was held at ~90 mV. Slow EPSPs were evoked by 20-Hz trains of 0.1-ms pulses at an intensity of 0.08–0.5 mA for 1 s. SSPEs were evoked by 1-Hz stimulation of internodal strands for 4 min. Pulses were again 0.1 ms in duration and 0.08–0.5 mA in intensity. Excitability during slow postsynaptic events was assessed by injecting 500-ms depolarizing current pulses, at an intensity of 50–100 pA at 30-s intervals, through the recording electrode. To determine input resistance during slow postsynaptic events, small hyperpolarizing current pulses (duration 100 ms, intensity 20–50 pA) were injected before the depolarizing pulse. All parameters were determined using in-house analysis routines written in Igor Pro 4.0 analysis software (Wave Metrics, Portland, OR).

Drugs. H-89 dihydrochloride (Calbiochem, La Jolla, CA), bisindolylmaleimide hydrochloride (Calbiochem), chelerythrine chloride (Sigma-Aldrich), calphostin C (Sigma-Aldrich), cesium chloride (CsCl), SB 223412 (SmithKline Beecham, Harlow, UK), and SR140333 (Sanofi-Aventis, Montpellier, France) were used. Compounds were applied by addition to the superfusion solution. Evoked responses were measured before drug application and after at least 15 min in the presence of drug in the extracellular solution.

Neuron identification. Biocytin was passed from the recording electrodes into the neurons during impalement. Once a neuron in a ganglion had been injected with biocytin, a diagram of the positions of the ganglion and of the impaled neuron was prepared so that the neuron could be later identified under the microscope. If further recordings were taken, the electrode was moved to a fresh ganglion to avoid ambiguity of cell identity. At the end of each experiment, the tissue was fixed overnight in 2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate-buffer (pH 7.0), cleared in three changes of 10 min each of DMSO, and washed in PBS (3 × 10 min). Fixed tissue was stored at 4°C in PBS containing sodium azide (0.1%).

Preparations were incubated overnight at 4°C. The tissues were then washed (3 × 10 min) in PBS before incubation with streptavidin coupled to Texas red (Amersham Biosciences, Piscataway, NJ), 1:400, for 2 h at room temperature. Preparations were then washed (3 × 10 min) in PBS and mounted on glass slides using buffered glycerol (pH 8.4).
To analyze the morphologies and projections of the impaled neurons, preparations in which impaled nerve cells had been identified were removed from the slides and washed in PBS before conversion of the streptavidin and bound to biocytin to a permanent deposit (9). This was achieved using goat anti-streptavidin antiserum coupled to biotin (Vector Laboratories, Burlingame, CA) diluted at 1:50 and incubated overnight at 4°C. The biotin was in turn localized using an avidin-biotin-horseradish peroxidase kit (Vectastain, Vector Laboratories). The horseradish peroxidase was reacted with diaminobenzidine and hydrogen peroxide to yield a permanent deposit. Cell shapes, positions, and projections were evaluated on an Olympus BH microscope under positive low-phase contrast optics and drawn with the aid of a camera lucida drawing tube at 185/400 or 185/1,000 magnification.

Statistics. Electrophysiological data are presented as means ± SE. Statistical differences were determined by Student’s t-test (paired and unpaired) and one-way ANOVA with Tukey-Kramer post hoc test for multiple group comparisons. Differences were considered statistically significant at \( P < 0.05 \).

RESULTS

TNBS caused inflammation of a 5–8-cm length of the ileum as has been previously reported (33). Electrophysiology experiments utilized myenteric plexus-longitudinal muscle preparations from 94 guinea pigs with TNBS-induced ileitis and from 51 control (untreated) animals. All neurons were characterized electrophysiologically, labeled by intracellular injection of biocytin during recording, and were later analyzed morphologically. Only neurons that had both electrophysiological characterization and morphological identification were used for analysis. AH neurons, which were the main focus of this study, were characterized by the presence of a late AHP following one or more action potentials and an inflection on the repolarizing phase of the action potential. In total, 133 neurons with AH electrophysiological properties from inflamed tissues and 52 AH neurons from control guinea pigs were included in the analysis.

Morphologically, AH neurons were classified into one of two categories, multiaxonal Dogiel type II neurons (n = 124 in the inflamed group, and n = 52 in the control group) that had large round or oval cell bodies, and Dogiel type I neurons (n = 9 in the inflamed group, none in the control group) that had a single axon and irregular lamellar dendrites (6, 11).

Responses of Dogiel type II neurons to brief tetanic stimulation of their synaptic inputs. Electrical stimulation of interganglionic connectives from one site by bursts of pulses at 20 Hz for 1 s evoked two types of responses in myenteric Dogiel type II neurons from the inflamed ileum, 1) slow EPSPs in 38 out of 77 (49%) tested neurons, and 2) maintained postsynaptic excitation in 10 out of 77 (13%) tested neurons (Fig. 1). In myenteric Dogiel type II neurons from control ileum, 20-Hz, 1-s stimulation evoked slow EPSPs in 11 out of 14 (78%) tested neurons but never caused maintained excitation. Moreover, maintained excitation in response to brief high-frequency presynaptic stimulation has not been reported in the literature although this method has been used in many investigations to elicit slow EPSPs. Slow EPSPs in myenteric Dogiel type II

Fig. 1. Effects of high- and low-frequency stimulation of synaptic inputs to neurons from the inflamed ileum. A: brief tetanic stimulation (20 Hz for 1 s) induced a slow excitatory postsynaptic potential (EPSP) characterized by membrane depolarization that lasted about 5 min and increased excitability of the neuron during membrane depolarization [measured by the number of action potentials (APs) elicited by 500-ms depolarizing current pulses]. Slow EPSPs were recorded in neurons from both the control and inflamed ileum. B: same stimulus, 20 Hz for 1 s, evoked long lasting (>40 min) depolarization of the membrane and increase in neuronal excitability (maintained excitation, ME) in a minority of neurons, only from the inflamed ileum. C: low-frequency stimulation (1 Hz for 4 min) induced sustained slow postsynaptic excitation (SSPE) characterized by long lasting (>30 min) depolarization of the membrane and increase in excitability in neurons from both control and inflamed ileum. Membrane hyperpolarizations and decreases in input resistance (\( R_n \)) seen at the beginnings of slow EPSPs or maintained excitations were due to summation of late AHPs following antidromic action potentials evoked by nerve stimulation.
neurons from the inflamed ileum were characterized by membrane depolarizations associated with increased excitability and increased input resistances ($R_{\text{in}}$) that peaked about 10–15 s after stimulation and lasted about 4 min (Fig. 1A). Parameters of slow EPSPs in neurons from the inflamed ileum were not significantly different from slow EPSPs recorded in neurons from the control ileum (Table 1). Maintained excitation recorded in neurons from the inflamed ileum was characterized by long-lasting depolarization of the membrane, increased excitability, and increased $R_{\text{in}}$ that lasted from 27 min to 3 h (Figs. 1 and 2, Table 1). This response was observed following the first stimulus applied to inputs to the neurons and was thus not a consequence of wind up of slow EPSPs. The increased excitability of neurons was measured as increases in the numbers of action potentials elicited by depolarizing current pulses. In many cases the firing of neurons reached a tonic state that lasted from 27 min to 3 h after the depolarizing current pulse, and prominent anode break action potentials are seen after the small hyperpolarizing current pulses from inflamed intestine that had slow EPSPs, maintained excitation, or no responses to presynaptic stimulation. In addition, there were no differences in excitability of neurons that had slow EPSPs and maintained excitation or no response to 20-Hz, 1-s stimulation. Values of the RMP, $R_{\text{in}}$, $C_{\text{in}}$, action potential amplitude and duration, late AHP amplitude and duration, summarized in Table 2, were analyzed by multiple-group comparison tests. There were no statistical differences in these properties between the three groups of neurons. Moreover, there were no differences in excitability of neurons, measured as responses to 500-ms depolarizing current pulses from inflamed intestine that had slow EPSPs, maintained excitation, or no responses to presynaptic stimulation. In addition, there were no differences between the groups of neurons from control intestine that had slow EPSPs or no responses to stimulation (Fig. 3, A and B). However, when pooled data from all groups of neurons tested with 20-Hz stimulation from the inflamed ileum were compared with the pooled data from all groups of neurons tested with 20-Hz stimulation from the control ileum, it was obvious that neurons from the inflamed ileum had lower thresholds to evoke action potentials and responded with more action potentials in response to the maximum (300 pA) depolarizing current pulse (Fig. 3, $A'$ and $B'$).

**SSPE in Dogiel type II neurons.** Low-frequency stimulation of interganglionic connectives at 1 Hz for 4 min evoked SSPE in 36 out of 47 (77%) tested Dogiel type II neurons from the inflamed ileum and in 29 out of 38 (76%) Dogiel type II neurons from the control ileum. The SSPE in neurons from the inflamed ileum were characterized by membrane depolarizations associated with increased excitability and increased input resistances ($R_{\text{in}}$) that peaked about 10–15 s after stimulation and lasted about 4 min (Fig. 1A). Parameters of slow EPSPs in neurons from the inflamed ileum were not significantly different from slow EPSPs recorded in neurons from the control ileum (Table 1). Maintained excitation recorded in neurons from the inflamed ileum was characterized by long-lasting depolarization of the membrane, increased excitability, and increased $R_{\text{in}}$ that lasted from 27 min to 3 h (Figs. 1 and 2, Table 1). This response was observed following the first stimulus applied to inputs to the neurons and was thus not a consequence of wind up of slow EPSPs. The increased excitability of neurons was measured as increases in the numbers of action potentials elicited by depolarizing current pulses. In many cases the firing of neurons reached a tonic state that lasted from 27 min to 3 h after the depolarizing current pulse, and prominent anode break action potentials are seen after the small hyperpolarizing current pulses from inflamed intestine that had slow EPSPs, maintained excitation, or no responses to presynaptic stimulation. In addition, there were no differences in excitability of neurons that had slow EPSPs and maintained excitation or no response to 20-Hz, 1-s stimulation. Values of the RMP, $R_{\text{in}}$, $C_{\text{in}}$, action potential amplitude and duration, late AHP amplitude and duration, summarized in Table 2, were analyzed by multiple-group comparison tests. There were no statistical differences in these properties between the three groups of neurons. Moreover, there were no differences in excitability of neurons, measured as responses to 500-ms depolarizing current pulses from inflamed intestine that had slow EPSPs, maintained excitation, or no responses to presynaptic stimulation. In addition, there were no differences between the groups of neurons from control intestine that had slow EPSPs or no responses to stimulation (Fig. 3, A and B). However, when pooled data from all groups of neurons tested with 20-Hz stimulation from the inflamed ileum were compared with the pooled data from all groups of neurons tested with 20-Hz stimulation from the control ileum, it was obvious that neurons from the inflamed ileum had lower thresholds to evoke action potentials and responded with more action potentials in response to the maximum (300 pA) depolarizing current pulse (Fig. 3, $A'$ and $B'$).

**Table 1. Effects of brief tetanic stimulation (20 Hz for 1 s) on electrophysiological properties of myenteric Dogiel type II neurons from the control and inflamed ileum**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Slow EPSP (11/14, 79%)</th>
<th>Maintained Postsynaptic Excitation (0/14, 0%)</th>
<th>Slow EPSP (38/77, 49%)</th>
<th>Maintained Postsynaptic Excitation (10/77, 13%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude of depolarization, mV</td>
<td>13±2</td>
<td>—</td>
<td>11±2</td>
<td>—</td>
</tr>
<tr>
<td>Time to peak of depolarization, s</td>
<td>15±2</td>
<td>—</td>
<td>28±5</td>
<td>39±11</td>
</tr>
<tr>
<td>Duration, min</td>
<td>4.3±0.5</td>
<td>—</td>
<td>4.6±0.4</td>
<td>27 to 180</td>
</tr>
<tr>
<td>Increase in $\Delta R_{\text{in}}$, MΩ</td>
<td>94±24</td>
<td>—</td>
<td>66±12</td>
<td>71±23</td>
</tr>
</tbody>
</table>

Means ± SE are shown. The numbers and percentages of neurons exhibiting different types of slow postsynaptic excitations are given in parentheses. EPSP, excitatory postsynaptic potential; $R_{\text{in}}$, input resistance.

Fig. 2. Maintained postsynaptic excitation induced by brief tetanic stimulation in a Dogiel type II neuron from the inflamed ileum. A: stimulation of synaptic inputs at 20 Hz for 1 s caused depolarization of the membrane and increase in neuronal excitability that lasted > 40 min. B and $B'$: neuronal excitability measured by 500-ms depolarizing current pulses of the same intensity before stimulation (B) and after stimulation ($B'$) shows that after induction of maintained excitation the neuron starts firing throughout the depolarizing current pulse, and prominent anode break action potentials are seen after the small hyperpolarizing pulse. Action potentials followed by the late afterhyperpolarizing potentials (AHPs) recorded before (C) and after stimulation ($C'$). D: camera lucida drawing of the Dogiel type II neuron from which these records were taken.
from the inflamed ileum was characterized by smaller amplitude of depolarization, shorter latency to peak of depolarization, and smaller increase in $R_{in}$ compared with neurons from the control ileum (Table 3). Although the baseline excitability of neurons from the inflamed ileum was higher than that of neurons from the control ileum, the increase in neuronal excitability caused by SSPE was not significantly different from that of control neurons (Fig. 4).

Baseline properties of the neurons from the inflamed ileum that had SSPE were compared with the properties of neurons with no response to a low frequency of stimulation. No significant differences between neuronal properties were found (Table 4).

### Table 2. Baseline parameters of Dogiel type II neurons from the control and inflamed ileum tested with 20 Hz, 1 s stimulation

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control ($n = 14$)</th>
<th>Inflamed ($n = 77$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No slow EPSP (3/14)</td>
<td>Slow EPSP (11/14)</td>
</tr>
<tr>
<td>RMP, mV</td>
<td>$-65 \pm 1$</td>
<td>$-62 \pm 1$</td>
</tr>
<tr>
<td>$R_{m}$, MΩ</td>
<td>$88 \pm 14$</td>
<td>$165 \pm 20$</td>
</tr>
<tr>
<td>$C_{in}$, pf</td>
<td>$41 \pm 13$</td>
<td>$33 \pm 3$</td>
</tr>
<tr>
<td>AP amplitude, mV</td>
<td>$93 \pm 4$</td>
<td>$90 \pm 3$</td>
</tr>
<tr>
<td>AP duration, ms</td>
<td>$-6.0 \pm 1.6$</td>
<td>$-8.7 \pm 0.8$</td>
</tr>
<tr>
<td>AHP amplitude, mV</td>
<td>$7.0 \pm 0.8$</td>
<td>$7.0 \pm 0.9$</td>
</tr>
<tr>
<td>Neurons exhibiting spontaneous APs</td>
<td>$0/3 (0%)$</td>
<td>$1/11 (9%)$</td>
</tr>
<tr>
<td>Neurons exhibiting anode break APs</td>
<td>$0/3 (0%)$</td>
<td>$1/11 (9%)$</td>
</tr>
<tr>
<td></td>
<td>Slow EPSP (29/77)</td>
<td>Slow EPSP (38/77)</td>
</tr>
<tr>
<td>RMP, mV</td>
<td>$-60 \pm 1$</td>
<td>$-62 \pm 1$</td>
</tr>
<tr>
<td>$R_{m}$, MΩ</td>
<td>$198 \pm 17$</td>
<td>$167 \pm 12$</td>
</tr>
<tr>
<td>$C_{in}$, pf</td>
<td>$38 \pm 3$</td>
<td>$40 \pm 2$</td>
</tr>
<tr>
<td>AP amplitude, mV</td>
<td>$86 \pm 2$</td>
<td>$89 \pm 1$</td>
</tr>
<tr>
<td>AP duration, ms</td>
<td>$2.0 \pm 0.1$</td>
<td>$1.8 \pm 0.1$</td>
</tr>
<tr>
<td>AHP duration, ms</td>
<td>$-9.6 \pm 0.8$</td>
<td>$-8.2 \pm 0.6$</td>
</tr>
<tr>
<td>Neurons exhibiting spontaneous APs</td>
<td>$1/29 (3.4%)$</td>
<td>$2/38 (5.3%)$</td>
</tr>
<tr>
<td>Neurons exhibiting anode break APs</td>
<td>$13/29 (44.8%)$</td>
<td>$3/38 (7.9%)$</td>
</tr>
</tbody>
</table>

Means ± SE are shown, with numbers of neurons analyzed and the proportions of neurons exhibiting the given property in parentheses. RMP, resting membrane potential; $C_{in}$, input capacitance; AP, action potential; AHP, afterhyperpolarizing potential.

Fig. 3. Comparison of the thresholds for action potential generation and neuronal excitability of Dogiel type II (DII) neurons tested with 20-Hz, 1-s stimulation. Neurons that had maintained postsynaptic excitation (ME) in response to 20-Hz, 1-s stimulation were compared with neurons that had either slow (s) EPSP or no response to 20-Hz, 1-s stimulation from both control and inflamed ileum. A: threshold currents for action potential generation in DII neurons with maintained excitation from the inflamed ileum were not significantly different compared with other groups. A': thresholds for action potential generation in DII neurons tested with 20-Hz, 1-s stimulation from all groups of neurons of the inflamed ileum, combined, were significantly lower than from all neurons tested from the control ileum ($P < 0.05$). B: excitability of DII neurons with maintained excitation from the inflamed ileum, measured as the numbers of action potentials elicited by 500-ms depolarizing pulses, was not significantly different compared with other groups of neurons from the inflamed and control intestine. B': excitability of all groups of DII neurons, combined, from the inflamed ileum tested with 20-Hz, 1-s stimulation was significantly increased compared with all groups of DII neurons, combined, from the control ileum ($P < 0.05$). Values of means ± SE and numbers of neurons analyzed for each parameter are incorporated into the histograms. *Significantly different from Dogiel type II neurons from the control ileum ($P < 0.05$). TNBS, trinitrobenzene sulfonic acid.
Effects of PKC and PKA inhibitors on slow postsynaptic events. PKC and PKA are second messengers that are necessary for the generation of the slow EPSP and SSPE; inhibitors of PKC block or substantially reduce both these slow events in myenteric neurons in normal conditions (4, 30, 35, 44). In the present study, we investigated the effect of PKC and PKA inhibition on the two types of slow postsynaptic excitation induced by high-frequency stimulation.

In the inflamed ileum, application of PKC inhibitors bisindolylmaleimide (100 nM) or chelerythrine (1–3 μM) reduced slow EPSP amplitudes from 14.1 ± 2.6 mV to 5.4 ± 2.2 mV (paired t-test, P < 0.05) and durations from 5.2 ± 0.9 min to 2.2 ± 0.9 min (paired t-test, P < 0.05) in 7 out of 8 tested neurons and had no effect on the amplitude and duration of slow EPSP in one neuron from the inflamed ileum (Fig. 5A). PKC inhibition had no effect on the amplitude and duration of maintained postsynaptic excitation recorded in AH neurons from the inflamed ileum (Fig. 5B). In the control ileum, PKC inhibitors suppressed the amplitude of slow EPSPs from 9.0 ± 2.0 mV to 6.3 ± 2.5 mV (paired t-test, P < 0.05) and duration from 3.1 ± 0.4 min to 2.2 ± 0.5 min in 3 out of 4 neurons.

Application of the PKA inhibitor, H-89 (1 μM), had no effect on slow EPSPs in neurons from control ileum (n = 5) but reduced the amplitude of slow EPSPs in neurons from the inflamed ileum (n = 11) (Fig. 6A). In the latter group, H-89 reduced the amplitude of slow EPSPs from 11.2 ± 1.8 mV to 8.1 ± 1.4 mV (paired t-test, P < 0.05) but had no significant inhibitory effect on the duration of slow EPSPs (6.0 ± 0.7 min before and 4.4 ± 0.8 min after the application of H-89, paired t-test, P > 0.05). In contrast, application of H-89 (1 μM) during the maintained excitation in neurons from the inflamed ileum caused further augmentation of the excitatory response associated with further membrane depolarization and increased neuronal excitability (Fig. 6B).

Fig. 4. Excitability tested by depolarizing current steps before and during SSPE in Dogiel type II neurons from the control and inflamed ileum. Excitability was measured as the number of action potentials elicited by 500-ms depolarizing current pulses at intensities of 100, 200, and 300 pA before and during SSPEs evoked by 1-Hz, 4-min stimulation of synaptic inputs in Dogiel type II neurons from the inflamed (n = 21) and control (n = 7) ileum. The baseline excitability of the neurons from the inflamed ileum was higher compared with the neurons from the control ileum. The responses to depolarizing current pulses after the induction of SSPEs were similar in neurons from the inflamed and control ileum. In all cases, the membraneous potential before depolarization steps was held at −60 mV.

### Table 3. Comparison of the effects of SSPE on electrophysiological properties of myenteric Dogiel type II neurons from the control and inflamed ileum

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n = 29)</th>
<th>Inflamed (n = 36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude of depolarization, mV</td>
<td>9.2 ± 3.1</td>
<td>6.0 ± 1.0*</td>
</tr>
<tr>
<td>Time to peak of depolarization, s</td>
<td>243 ± 17</td>
<td>165 ± 20*</td>
</tr>
<tr>
<td>Duration, min</td>
<td>20 ± 2</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>Increase in R_m, ΔR_m, MΩ</td>
<td>135 ± 27</td>
<td>63 ± 11*</td>
</tr>
</tbody>
</table>

Means ± SE are shown. The numbers of neurons analyzed are given in parentheses. *Statistically significant at P < 0.05. SSPE, sustained slow postsynaptic excitation.

### Table 4. Baseline parameters of Dogiel type II neurons from the inflamed ileum tested with 1 Hz, 4 min stimulation

<table>
<thead>
<tr>
<th>Parameters</th>
<th>No SSPE</th>
<th>SSPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>R_M, MΩ</td>
<td>175 ± 24</td>
<td>182 ± 12</td>
</tr>
<tr>
<td>C_M, pF</td>
<td>43 ± 5</td>
<td>54 ± 4</td>
</tr>
<tr>
<td>AP amplitude, mV</td>
<td>85 ± 3</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>AP duration, ms</td>
<td>2.0 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>AHP amplitude, mV</td>
<td>−9.3 ± 1.2</td>
<td>−7.5 ± 0.5</td>
</tr>
<tr>
<td>AHP duration, ms</td>
<td>6.4 ± 0.8</td>
<td>4.5 ± 0.6</td>
</tr>
<tr>
<td>Neurons exhibiting spontaneous APs</td>
<td>0/11 (0%)</td>
<td>1/36 (2.8%)</td>
</tr>
<tr>
<td>Neurons exhibiting anode break APs</td>
<td>7/11 (64%)</td>
<td>13/36 (36%)</td>
</tr>
</tbody>
</table>

Means ± SE are shown with numbers of neurons analyzed and the proportions of neurons exhibiting the given property in parentheses.
cases the membrane potential and neuronal excitability did not return to the baseline level after the first stimulation. Nevertheless, in two cases when the membrane potential returned to the baseline after the first stimulation, we were able to induce a second maintained excitation after 20-min application of the antagonists. In these cases, application of NK1 and NK3 receptor antagonists had very little or no effect on maintained excitations induced by 20-Hz, 1-s stimulations of synaptic inputs (Fig. 7, B and B').

The effects of the NK3 receptor antagonist SB223412 (1 μM) were tested in neurons that displayed SSPEs in response to 1-Hz, 4-min stimulation. SB223412 (1 μM) had no effect on sustained excitation that lasted more than 30 min following stimulation in the neurons from the inflamed ileum (Fig. 8). These results are similar to those reported previously in the Dogiel type II neurons from the control ileum (2).

Slow excitatory postsynaptic transmission induced in Dogiel type I neurons with late AHPs. Late AHPs following action potentials were seen in 9 uniaxonal neurons from the inflamed ileum in this study. These cells had Dogiel type I morphology with anally projecting axons (n = 7) or a short axon broken close to the cell body (n = 2). All Dogiel type I neurons with late AHPs had prominent fast EPSPs. We did not encounter Dogiel type I neurons from the control ileum with late AHPs.

Stimulation of interganglionic strands at 20 Hz for 1 s induced slow EPSPs in 7 out of 9 neurons and maintained excitation in 1 out of 9 Dogiel type I neurons with late AHPs. Slow EPSPs in Dogiel type I neurons with late AHPs had amplitudes of 15 ± 2 mV and durations of 173 ± 31 s, increases in $R_{in}$ ($\Delta R_{in}$) of 87 ± 19 MΩ, and time to maximum depolarization of 16 ± 9 s (n = 7). Other properties of these 7
Dogiel type I neurons with late AHPs were action potential amplitude 56 ± 5 mV, action potential duration 1.7 ± 0.2 ms, RMP −60 ± 1.5 mV, $R_{in}$ 242 ± 37 MΩ, late AHP amplitude −6.6 ± 1.4 mV, late AHP duration 4.4 ± 0.9 s, $C_{in}$ 15.5 ± 2.0 pF, threshold to evoke the first action potential 70 ± 10 pA, and response to 300-pA depolarizing current pulse 19.4 ± 3.3 action potentials/500-ms pulse. The maintained excitation, which was seen in one Dogiel type I neuron with late AHPs, had an amplitude of 8 mV and duration of 40 min, increase in $R_{in}$ from 150 to 230 MΩ, time to the maximum depolarization of 20 s, and increase in the number of action potentials in response to 100-pA, 500-ms depolarizing current pulse from 4 to 25. Characteristics of this neuron were action potential amplitude 48.2 mV, action potential duration 1.5 ms, RMP −55 mV, $R_{in}$ 139 MΩ, AHP amplitude −7.7 mV, AHP duration 6.5 s, $C_{in}$ 18.6 pF, threshold to evoke the first action potential 50 pA, and response to 300 pA depolarizing current pulse 12 action potentials/500-ms pulse. This neuron had a single axon with descending projection.

Low-frequency stimulation was tested in 3 Dogiel type I neurons with late AHPs. None of these cells had SSPEs in response to brief stimuli at 20 Hz and SSPEs in response to stimulation at 1 Hz for 4 min. Slow EPSPs and SSPEs have been previously described in studies of enteric neurons of normal ileum (11). Maintained excitation has never before been reported, despite the fact that many studies have utilized brief tetanic stimuli to evoke postsynaptic events in neurons of normal ileum (3, 28, 31, 45). Maintained excitation thus appears to be a new phenomenon that is revealed after inflammation.

Maintained postsynaptic excitation in AH neurons from the inflamed ileum. Maintained excitation that lasted from 27 min up to 3 h occurred in 10 out of 77 AH neurons from the inflamed ileum, but not in the neurons from the control ileum. These long-term excitations were associated with membrane depolarization, increased neuronal excitability, and increased input resistance. Other properties of the neurons with maintained excitations were not different from the properties of AH neurons with slow EPSPs or AH neurons that had no response to 20-Hz stimulation. We investigated whether the occurrence of maintained excitation was associated with a different level of baseline excitability of these neurons. We did not find any significant differences in the responses to depolarizing current pulse and thresholds for action potential induction between AH neurons of inflamed intestine with and without maintained excitation (Fig. 3). The properties of Dogiel type II neurons are heterogeneous, and it is possible that a particular type of
Dogiel type II neuron shows maintained excitation. For example, some Dogiel type II neurons have a Cl\(^{-}\) component of slow EPSPs (3), about 10% do not express a significant hyperpolarization-activated cation current (\(I_h\)) (13, 38), and about 20% do not contain detectable calbindin (37). We have not yet had the opportunity to test which of these subtypes might exhibit maintained excitation.

The maintained excitation indicates that a brief synaptic activation can trigger a long period of hyperexcitability in Dogiel type II neurons after they have been exposed to an inflamed environment. This is one of a number of sustained changes in these neurons after inflammation. Even without the additional effect of a tetanic synaptic activation, the neurons are hyperexcitable, a change that persists for at least 2 mo, well beyond the period of detectable inflammation (17, 22). The maintained excitation was induced in all cases after the first stimulation and was not a result of multiple trains of stimuli. Therefore it cannot be due to a “run-up” phenomenon in which neurons fail to return to baseline following several rounds of 20-Hz stimulation. However, it is possible that the slow EPSPs induced by 20-Hz stimulation trigger activation of other second messenger mechanisms, leading to long-term persistent excitation in a small population of neurons that were modified by inflammation. We did not find a correlation between the occurrence of maintained excitation and the level of inflammation.

Slow EPSPs and SSPE in AH neurons from the inflamed ileum. Previous studies have reported that inflammation caused no changes in the incidence and amplitudes of slow EPSPs in AH neurons from the guinea pig distal colon (19, 20). Consistent with this, our results show that there were no changes in the amplitudes, durations, and incidence of slow EPSPs in the myenteric AH neurons from the inflamed ileum compared with the control ileum.

Long-term hyperexcitability of enteric neurons (up to 4 h) following stimulation of synaptic inputs, SSPE, has been reported previously after low frequency of stimulation for 4 min or more in control guinea pig ileum. In the present study, we found that the amplitude of depolarization and input resistance were less affected during the SSPE in Dogiel type II neurons from the inflamed ileum, compared with the neurons from the control ileum. The baseline excitability of neurons from the inflamed ileum was higher compared with neurons from the control ileum, but the excitability of neurons from the inflamed ileum during SSPE was
similar to the excitability of neurons from the control ileum during SSPE. This may be a consequence of nonlinear summation of excitatory events. In the control ileum, SSPEs were observed only in neurons with Dogiel type II morphology (1). Our data from neurons in the inflamed ileum are consistent with this previous observation.

Thus there is a selectivity of the effect of inflammation on slow synaptic events in the small intestine; slow EPSPs were not detectably changed; SSPEs were smaller, and a new phenomenon, maintained excitation, was observed.

Comparison of maintained excitation with SSPE and slow EPSP. The intensity and duration of neuronal hyperexcitability, as well as the duration of membrane depolarization during maintained excitation, were similar to those observed during SSPEs. Similar to SSPEs, but unlike slow EPSPs, maintained excitation was not inhibited by NK1 and NK3 receptor antagonists in neurons from the inflamed ileum. Previous studies, confirmed by our data, have shown that the primary transmitters of slow EPSPs are tachykinins acting at NK1 and NK3 receptors (2, 15). Previous studies indicate the involvement of 5-HT (25, 27, 46), ATP acting at P2Y1 receptors (14, 26), and CCK (40) in the generation of slow EPSPs in myenteric AH neurons. Moreover, peptides such as calcitonin gene related peptide, gastrin releasing peptide, pituitary adenyl cyclase-activating peptide, and vasoactive intestinal peptide (8, 24, 47) can induce responses that mimic slow EPSPs. Any of these substances can be candidates that initiate maintained excitation and therefore need to be further investigated in the inflamed ileum. In contrast to SSPE, which was confined to Dogiel type II neurons from both control and inflamed ileum, maintained excitation was induced in both Dogiel type II neurons and a Dogiel type I neuron with AH characteristics from the inflamed ileum, providing some evidence that these events might be induced by different neurotransmitters.

It has been reported that inhibition of both PKA and PKC can suppress slow EPSPs in enteric neurons from the control intestine (5, 35, 44), which we have confirmed. Inhibition of PKC also suppresses the SSPE in neurons from the control ileum (30). The results of our study show that the maintained excitation was not influenced by PKC inhibitors.

Several studies point to an involvement of kinases in the sustained changes that occur following inflammation. In the previously inflamed distal colon, PKA activity in nerve terminals increases and contributes to the facilitation of fast synaptic transmission, possibly through an inhibition of big conductance K+ (BK) channels and an increase in the readily releasable pool of synaptic vesicles (16). In other studies, inflammation of the small intestine induced by Trichinella spiralis caused increased adenyl cyclase activity of myenteric neurons 6–9 days after infection (7), and, in submucosal neurons, increased PKC activation was apparent at 6–7 days after TNBS (36). Our results show that inhibition of PKA in the inflamed ileum enhanced hyperexcitability during maintained excitation and also reduced the hump on the action potential repolarization and inhibiting the late AHP. The reduced hump was probably due, in part, to inhibition of the Ca2+ component of the action potential, which reduces the Ca2+ availability to activate intermediate conductance Ca-activated K+ channels (IKCa) that underlie the late AHPs. This leads to increase of neuronal excitability and membrane depolarization. In normal neurons, inhibition of PKA enhances the AHP, whereas activation of PKA reduces the AHP, without affecting the repolarization of the action potential (43). Thus our results point to profound alterations in the regulation of neuronal properties by kinases following inflammation.

Conclusion. This study has revealed a type of postsynaptic event, a prolonged hyperexcitability after a brief stimulus (20 Hz for 1 s) that lasted up to 3 h. Such long-term hyperexcitability is not encountered in enteric neurons of normal intestine. The maintained excitation was observed in intrinsic primary afferent neurons that form synapses with each other to create self-reinforcing networks (18). It thus can be anticipated that an intense stimulation of these neurons could lead to a long period of
activation of many primary afferent neurons and of down-stream interneurons and motor neurons. This generalized neuronal activation may contribute to the dysmotility, pain, and discomfort of irritable bowel syndrome, which is commonly a postinflammatory pathology (23, 29, 39, 42).

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