Platelet-activating factor in the enteric nervous system of the guinea pig small intestine

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Platelet-activating factor (PAF) is among the most potent of the lipid-derived proinflammatory mediators (1, 5, 23). It is synthesized by and released from a variety of tissue sources including platelets, neutrophils, monocytes, eosinophils, and mast cells (29, 32). Like eicosanoids, PAF is not preformed and stored in cells. It is synthesized and released in response to stimulation. Thrombin, chemotactic peptides, and antigen-antibody reactions are among the factors that stimulate its synthesis and release. Oral allergen-induced diarrhea in mice is mast cell, IgE, serotonin, and PAF dependent (4). Synthesis starts with the action of phospholipase A2 and the formation of 1-O-alkyl-2-lysoglycerophosphocholine (lyso-PAF) and arachidonate or another free fatty acid (8). PAF is formed in a second step when lyso-PAF is acetylated by acetyl-CoA.

PAF is associated with inflammatory states in the digestive tract. Gastritis, gastric erosions, and gastric ulceration, whether associated with hemorrhagic shock, stress, or mucosal irritants (e.g., ethanol), are linked to the elevation of PAF (11, 12, 31, 39, 48, 49). Likewise, an association has been reported for ulcerative colitis (10, 37, 42), Crohn’s disease (9), necrotizing enterocolitis (7, 30), and ischemia-reperfusion injury (5, 41). The chemotactant action of PAF for immune/inflammatory cells into the walls of the gut from the circulation has been reflected by the results of a study (38) showing an inducement of human eosinophils to migrate across a sheet of confluent intestinal epithelium in vivo.

Exposure of the serosal side of muscle-stripped preparations of the intestinal mucosa to PAF in Ussing flux chambers evokes chloride secretion, which is reflected by increases in short-circuit current under voltage-clamp conditions for the human colon and animal models (2, 3, 27, 47). This action of PAF includes an opening of the mucosal barrier and can be evoked by nanomolar concentrations. The available evidence suggests that the mucosal secretory action of PAF is secondary to the stimulation and release of prostaglandin E2 in the human colon and rat jejunum (3, 27) and thromboxane A2 in the rabbit colon (43). Mucosal mast cells appear to be a major source of the PAF-evoked release of prostaglandin E2 and of the release of PAF itself (36, 47).

Stimulation of short-circuit current in Ussing chamber studies with the rabbit colon is unchanged by the presence of tetrodotoxin in the bathing medium and in preparations stripped of the submucosal plexus. This suggests that the stimulation of enteric secretomotor neurons is not involved in the stimulation of short-circuit current by PAF. On the other hand, pretreatment with tetrodotoxin suppressed PAF-evoked increases in short-circuit current in the rat colon and jejunum and suggests that secretomotor neurons are stimulated either directly or indirectly in the presence of PAF (2, 15).

The application of PAF to muscle preparations obtained from the rat gastric fundus or corpus evokes contractions that are unaffected by tetrodotoxin or antagonists at receptors for...
neurotransmitters and suggestive of non-neural action at the musculature (22, 24, 28). The exposure of the guinea pig ileum or rat colon to PAF in vitro also evokes contraction of the musculature, which reflects a non-neural action at the musculature (45, 46). A direct action on intestinal muscle is suggested by findings that exposure of enzymatically dissociated muscle fibers from the guinea pig ileum to PAF stimulates shortening (20). In conscious rats, intraperitoneal injections of PAF stimulate intestinal transit and increase the liquidity of the stools (34, 35). In studies of isolated organs or muscle strips and in conscious animals, the actions of PAF on contractile behavior appear to involve the induction of the synthesis of prostaglandin E<sub>2</sub>. Jouet and Sarna (21) injected PAF by a close intrarterial injection into the small intestine of conscious dogs and found an increase in the number of giant migrating contractions that reflects a motility pattern called “power propulsion” (51). After the induction of an inflammatory state by an intraluminal application of an ethanol-acetic acid mixture, the intra-arterial injection of PAF evoked a significant increase in the number of power propulsive contractions. Intestinal power propulsion in humans and dogs is associated with cramping abdominal pain, diarrhea, and fecal urgency (40, 51).

Information for actions of PAF on peripheral neurons in the gastrointestinal tract is limited. An infusion of PAF into the gastric artery of rats increases firing rates recorded from gastric mechanosensitive vagal afferents (33). Application of PAF in this manner is accompanied by increases in contractile activity of the stomach and a mucosal inflammatory response. Consequently, whether PAF acts directly to stimulate firing of vagal afferent terminals or is secondary to changes in tension in the musculature is unclear.

The only published study (5) of PAF actions on enteric neurons was done with myenteric neurons grown in cell cultures prepared from the small intestine of newborn rats. Application of PAF to the cultured neurons resulted in depolarization of the membrane potential and a discharge of action potentials. PAF (0.1–50 μM) is toxic to the cultured neurons, and maximum cell death occurred during a 2-min exposure to 20 μM PAF.

In the present study, we tested how PAF might act if released as a paracrine signal substance in the intestine by investigating the actions of PAF on electrical and synaptic behavior of morphologically and neurochemically identified neurons, including secretomotor and sympathetic neurons, in the guinea pig enteric nervous system (ENS). Identification of expression of the receptor responsible for the actions of PAF was an aim of the study. Preliminary reports (53, 58) have been published in abstract form.

**MATERIALS AND METHODS**

Immunohistochemistry, Western blot analysis, and RT-PCR studies were done with intestinal preparations from guinea pigs. For the electrophysiological studies, preparations of the submucosal or myenteric plexus were obtained from the ileum, jejunum, and colon of male and female Hartley-Dawley guinea pigs (0.3–0.6 kg). Guinea pigs were killed by rapid stunning and immediate exsanguination from the cervical vessels according to procedures reviewed and approved by the Ohio State University Laboratory Animal Care and Use Committee and United States Department of Agriculture veterinary inspectors. Flat sheet preparations of the myenteric and submucosal plexus (2.0 × 1.0 cm) were obtained by microdissection as described previously (52, 57). Preparations were pinned to Sylgard resin at the bottom of a 2.0-ml electrophysiological recording chamber that was perfused at a rate of 10–15 ml/min with Krebs solution warmed to 37°C and gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub> to buffer at pH 7.3–7.4. The composition of the Krebs solution was (in mM) 120.9 NaCl, 5.9 KCl, 1.2 MgCl<sub>2</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 14.4 NaHCO<sub>3</sub>, 2.5 CaCl<sub>2</sub>, and 11.5 glucose. Nifedipine and scopolamine (1 μM) were added to the Krebs solution to suppress muscle movements during electrophysiological recording from the enteric neurons. Myenteric and submucosal ganglia in the electrophysiological studies were visualized microscopically with differential interference contrast optics and epilumination. Ganglia selected for microelectrode recording were immobilized with 100-μm-diameter L-shaped stainless steel wires placed on either side of the ganglion (52).

**Electrophysiological recordings.** Our methods for intracellular recordings from neurons in the myenteric and submucosal plexuses have been described in detail elsewhere (52, 57). Transmembrane electrical potentials were recorded with conventional “sharp” microelectrodes filled with 2% biocytin in 2 M KCl buffered with 0.05 M Tris at pH 7.4. Resistances of the electrodes ranged between 80 and 120 MΩ. The low-frequency response of the recorders was blunted by the low-frequency response of the recorders. All data were recorded initially in digital format on magnetic tape for later analysis.

**Histochemical methods.** The morphology of each of the neurons in the electrophysiological studies was determined by passing hyperpolarizing current (0.5 nA for 10–30 min) to inject biocytin into the neuron from the microelectrode and later histochemical development of the intraneuronal biocytin. At the end of a recording session, the whole mount preparations were transferred to a disposable chamber filled with fixative containing 4% formaldehyde and 15% of a saturated solution of picric acid and stored at 4°C overnight. The preparations were cleared in three changes of DMSO and three 10-min washes with PBS, reacted with fluorescein streptavidin (Vector, Burlingame, CA) diluted 1:200 for 30 min at 37°C, and then examined with a Nikon Eclipse-1000 fluorescence microscope.

**Immunohistochemical studies.** Were done on whole mounts preparations that were incubated in 10% normal donkey serum in PBS for 1 h at room temperature before exposure to primary antiserum (Table 1), which were diluted in PBS containing 10% normal donkey serum, 0.3% Triton X-100, and 0.1% sodium azide for 18–24 h at room temperature. After incubation with the primary antibodies, tissues were washed (3 × 10 min) in PBS, transferred to humidified chambers, and incubated at 37°C for 30 min with secondary antibodies conjugated with FITC or indocarbocyanine (Cy3). The biocytin-labeled preparations were reacted with fluorescein streptavidin (Vector). Tissues were then rinsed in PBS and coverslipped with Vectorshield (Vector). All preparations were examined with an eclipse microscope (Nikon Eclipse-1000, Nikon) and analyzed using a filter combination that enabled separate visualization of multiple fluorophores. Digital images were obtained with a SPOT RT, cooled
charge-coupled device digital camera (Diagnostic Instruments, Sterling Heights, MI) and analyzed with SPOT III software.

**Western blot analysis.** Proteins in guinea pig and human small intestinal submucosal or myenteric plexus preparations were extracted with lysis buffer containing 50 mmol/l Tris·HCl (pH 7.5), 5 mmol/l EDTA, 250 mmol/l NaCl, 1% Triton X-100, and a protease inhibitor cocktail consisting of 0.2 mmol/l phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 5 mmol/l dithiothreitol, and 1 mmol/l Na3VO4 on ice for 1 h. Ganglia were isolated by enzymatic digestion as described in detail elsewhere (54, 56). Equivalents of 50 µg of extracted proteins were electrophoresed and then transferred to a 10% acrylamide gel for 2 min. A total of 30 cycles was followed by an extension at 72°C for 1 min. PCR products were analyzed by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. Reverse transcription without AMV reverse transcriptase was the negative control.

**Reagents and antibodies.** PAF was obtained from Cayman Chemical (Ann Arbor, MI) and Sigma Biochemicals (St. Louis, MO). Carbamyl-PAF (C-PAF; a stable analog of PAF) and CV-3988 were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). BD-52021 (ginkgolide B) was from Tocris (Ellisville, MO). WEB-2086 was kindly provided as a gift of Dr. Zhishang Zhuo of Yale University. Tetrodotoxin and hexamethonium were purchased from Sigma Biochemicals. The antibodies and antisera that were used are shown in Table 1.

**Data analysis.** Results are expressed as means ± SE; n refers to the numbers of neurons examined. Statistical significance was determined with a paired Student’s t-test between control and experimental populations. Differences were considered significant at P < 0.05. Concentration-response curves for drug-evoked responses were constructed using the following least-squares fitting routine: V = Vmax/[1 + (EC50/C)^H].

**RESULTS**

**Western blot analysis and RT-PCR.** A purified goat anti-PAFR polyclonal antibody (sc-8744 and sc-8742, Santa Cruz Biotechnology) was used for Western blot analysis of the expression of PAFR protein in myenteric ganglia after 3 days in culture and in longitudinal muscle-myenteric plexus whole mount preparations from the ileum. Immunoblots, obtained simultaneously for the isolated ganglia and whole mounts, identified protein bands with a molecular mass of 55 kDa for PAFR. The primers used were J upstream 5'-CTT CTG CAA CCT GGT CAT CA-3' and 2) downstream 5'-TGT GGT GTA TTG GAA TGG CC-3'.

Amplification of cDNA was done with an iCycler (Bio-Rad Laboratories) in PCR buffer solution containing 5 µl of cDNA product, 25 µl of PCR master mix (Roche Diagnostics), 2.0 µl of PAFR primers, and 18 µl of sterile H2O. PCR cycles consisted of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 2 min. A total of 30 cycles was followed by an extension at 72°C for 7 min. PCR products were analyzed by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. Reverse transcription without AMV reverse transcriptase was the negative control.

**Immunohistochemistry.** The localization of PAFR immunoreactivity (IR) and colocalization with calbindin IR, cal-
retinin IR, ChAT IR, nitric oxide synthase (NOS) IR, neuropeptide Y (NPY) IR, and vasoactive intestinal peptide (VIP) IR was studied for 44 submucosal and 51 myenteric preparations from the intestinal tract. PAFR-IR neurons were found in both submucosal (Fig. 2A) and myenteric plexuses (Fig. 2B) of all intestinal regions, including the duodenum, jejunum, ileum, cecum, colon, and rectum (Table 2). The specificity of neuronal localization was confirmed by double labeling with anti-human neuronal protein (Hu; HuC/HuD) mouse monoclonal antibody, which has been shown to label all enteric neurons (16, 25) (Fig. 3, A and B). Double labeling of PAFR IR with anti-Hu IR revealed that all PAFR-IR cells were immunopositive for anti-Hu IR. PAFR IR was expressed by 6.9% of anti-Hu-IR myenteric neurons and 14.5% of anti-Hu-IR submucosal neurons (Table 2).

Calbindin is a chemical code that identifies most guinea pig enteric neurons with AH-type electrophysiological behavior and AH-Dogiel type II multipolar morphology (13). PAFR IR was expressed by 1.5% of calbindin-IR myenteric neurons and 6.1% of calbindin-IR submucosal neurons; conversely, calbindin IR was expressed by 2.4% of PAFR-IR myenteric neurons and 0.4% of PAFR-IR submucosal neurons (Fig. 3, C and D, and Table 2). IR for calretinin, which is another calcium-binding protein expressed by enteric neurons, was not

Fig. 1. Platelet-activating factor (PAF) receptor (PAFR) expression in guinea pig longitudinal muscle-myenteric plexus whole mounts and dissociated myenteric neurons in culture. A: Western blot analysis for a longitudinal muscle-myenteric plexus whole mount. B: Western blot analysis for enzymatically dissociated myenteric neurons in culture. C: RT-PCR analysis for longitudinal muscle-myenteric plexus whole mounts and enzymatically dissociated myenteric neurons in culture. Lane 1, cDNA ladder; lane 2, longitudinal muscle-myenteric plexus whole mount; lane 3, dissociated neurons; lane 4, negative control with RNA omitted from RT-PCR amplification.

Fig. 2. Regional expression of PAFR immunoreactivity (IR) in the myenteric plexus (A) and submucosal plexus (B).
expressed by PAFR-IR neurons (data not showed). ChAT IR, a neurochemical code for cholinergic neurons, was expressed by 73.2% of PAFR-IR myenteric neurons and 64.5% of PAFR-IR submucosal neurons; conversely, 32.9% of ChAT-IR myenteric neurons and 30.6% of ChAT-IR submucosal neurons were PAFR IR (Fig. 4, A and B, and Table 2). NOS IR, a neurochemical code for enteric inhibitory musculomotor neurons in the guinea pig, was not coexpressed with PAFR IR (data not shown). PAFR IR was expressed by 51.1% of NPY-IR myenteric neurons and 35.8% of NPY-IR submucosal neurons; conversely, NPY IR was expressed by 5.7% of PAF-IR myenteric neurons and 66.3% of PAF-IR submucosal neurons (Fig. 4, C and D, and Table 2). VIP IR, which is a neurochemical code for inhibitory musculomotor neurons in the myenteric plexus and secretomotor neurons in the submucosal plexus, was expressed by 4.4% of PAFR-IR submucosal neurons; PAFR IR and VIP IR did not colocalize in the myenteric plexus (Fig. 4, E and F).

We were able to identify the morphology of some of the neurons by injecting biocytin from the microelectrode during

### Table 2. **PAFR IR in relation to enteric neurochemical codes**

<table>
<thead>
<tr>
<th>Code</th>
<th>Submucosal Plexus</th>
<th>Myenteric Plexus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAF/code</td>
<td>Code/PAF</td>
</tr>
<tr>
<td>Anti-Hu</td>
<td>14.52 (651/4,482)</td>
<td>100.00 (651/651)</td>
</tr>
<tr>
<td>Calbindin</td>
<td>6.12 (3/49)</td>
<td>0.39 (3/753)</td>
</tr>
<tr>
<td>NPY</td>
<td>35.80 (459/1,282)</td>
<td>66.32 (459/692)</td>
</tr>
<tr>
<td>ChAT</td>
<td>30.56 (387/1,266)</td>
<td>64.50 (387/600)</td>
</tr>
<tr>
<td>VIP</td>
<td>1.96 (15/762)</td>
<td>4.39 (15/341)</td>
</tr>
</tbody>
</table>

Values are percentages, with numbers of neurons in parentheses.

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Fig. 3. Colocalization of PAFR IR with anti-Hu IR and calbindin IR in myenteric and submucosal plexuses of the guinea pig small intestine. **A**: colocalization with anti-Hu IR in the submucosal plexus. **B**: colocalization with anti-Hu IR in the myenteric plexus. **C**: colocalization with calbindin IR in the submucosal plexus. **D**: colocalization with calbindin IR in the myenteric plexus.

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electrical recording and studying immunohistochemistry localization of the PAFR in the same whole mount preparation.

Three biocytin-filled neurons had uniaxonal morphology and electrophysiological behavior characteristic for S-type enteric neurons. Four biocytin-filled neurons had multipolar Dogiel type II morphology and electrophysiological behavior characteristic for AH-type enteric neurons.

**Electrophysiology.** Conventional recording with “sharp” intracellular microelectrodes was used to study the actions of C-PAF on electrophysiological behavior in 57 submucosal and 49 myenteric plexus preparations. Exposure to C-PAF resulted in depolarization of the membrane potential associated and discharge of action potentials in neurons in both the myenteric and submucosal plexuses (Figs. 5 and 6). In submucosal neurons, bath application of C-PAF (200–600 nM) evoked membrane depolarization of 8.2 ± 3.8 mV in 12.4% (12 of 97) of the neurons with uniaxonal morphology and S-type electrophysiological behavior (Fig. 5) and 12.5% (1 of 8) of the neurons with Dogiel type II morphology and AH-type electrophysiological behavior (Fig. 6). Depolarizing responses and elevated excitability were evoked by C-PAF in 12.0% (14 of 117) of the myenteric neurons with Dogiel type II morphology and AH-type electrophysiological behavior and 9.5% (4 of 42) of the myenteric neurons with uniaxonal morphology and S-type electrophysiological behavior.

The amplitude of C-PAF-evoked depolarizing responses increased in a concentration-dependent manner with an EC50 of 98.7 ± 16.8 nM (Fig. 7). Progressively enhanced excitability was reflected by concentration-dependent increases in the frequency of the action potential discharge during the depolarizing responses. Concentration-response relations for the depolarizing action of C-PAF could not be fully evaluated for all neurons because the responses in most neurons desensitized rapidly. With second or third applications, higher concentra-
tions of 400 nM to 1 μM were required to evoke a response and longer washout periods ranging from 40 min to 1 h were required for restoration of the response to control levels in 19 of 31 of neurons. Furthermore, the responses never recovered in 5 of 31 neurons.

Decreases in input resistance, which reflected increased membrane conductance, occurred during the PAF-evoked depolarizing responses in neurons with uniaxonal morphology and S-type electrophysiological behavior (Fig. 5). Input resistance either increased or remained unchanged in neurons with multipolar Dogiel type II morphology and AH-type electrophysiological behavior (Fig. 6). CV3988 (2–10 μM), a selective PAFR antagonist, suppressed the depolarizing responses to C-PAF in 27 neurons (Figs. 5 and 6). The PAFR antagonist BD-52021 (ginkgolide B, 5 μM) or WEB-2086 (5 μM) abolished the depolarizing responses in 14 and 11 neurons, respectively.

**Synaptic transmission.** Focal electrical stimulation (20 Hz, 2 ms, 0.6 mA) of interganglionic nerve tracts in the submucosal plexus evoked both slow IPSPs and slow excitatory postsynaptic potentials (EPSPs) in uniaxonal neurons with S-type electrophysiological behavior. Slow IPSPs were suppressed or abolished by 2 μM idazoxan (Fig. 8). An application of C-PAF (300–600 nM) in the presence of idazoxan enhanced both the

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**Fig. 5.** Excitatory action of carbamyl-PAF (C-PAF) on an uniaxonal neuron in the submucosal plexus. *A:* exposure to C-PAF evoked membrane depolarization associated with action potential discharge and decreased input resistance. *B:* presence of the selective PAFR antagonist CV3988 in the bathing solution abolished the excitatory action of C-PAF. *C:* excitatory response to C-PAF recovered after washout of the antagonist. The downward deflections on each trace are electronic potentials evoked by repetitive injections of rectangular constant-current hyperpolarizing pulses. *D:* neuron from which the records were obtained.

**Fig. 6.** Excitatory action of C-PAF on a neuron with Dogiel type II morphology and AH-type electrophysiological behavior in the myenteric plexus. *A:* exposure to C-PAF evoked membrane depolarization associated with action potential discharge and increased input resistance. *B:* presence of the selective PAFR antagonist CV3988 in the bathing solution abolished the excitatory action of C-PAF. *C:* excitatory response to C-PAF recovered after washout of the antagonist. The downward deflections on each trace are electronic potentials evoked by repetitive injections of rectangular constant-current hyperpolarizing pulses. *D:* neuron from which the records were obtained.

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**Fig. 7.** Concentration-response relation for the depolarizing action of PAF.
amplitude of the slow EPSPs and the frequency of the action potential discharge during the slow EPSP in 3 of 21 submucosal neurons (Fig. 8C). C-PAF also enhanced the slow EPSP amplitude and spike discharge in 4 of 47 myenteric neurons. The presence of CV3988 (2 μM) in the bathing solution suppressed the stimulatory action of C-PAF on neuronal slow EPSPs in seven neurons (Fig. 8D).

Stimulus-evoked IPSPs occurred in conjunction with slow EPSPs that were suppressed or abolished by the P2Y1 receptor antagonist MRS-2179 (data not shown), as reported by Hu et al. (18). Investigation of the action of C-PAF on the slow IPSPs was done in the presence of 10 μM MRS-2179 to avoid interference of the purinergic slow EPSP with the IPSP. The presence of 500 nM C-PAF in the bathing medium significantly suppressed the amplitude of stimulus-evoked slow noradrenergic IPSPs to 86 ± 2% of the control value in five submucosal neurons (Fig. 9). The PAFR antagonist CV3988 (2 μM) reversed the inhibitory action of 500 nM C-PAF on the IPSP in the same five neurons.

DISCUSSION

Our results suggest that PAF can be added to the list of eight or more inflammatory/immune mediators that might be released in a paracrine fashion within the intestinal wall and interact with intrinsic neural elements of the ENS or extrinsic sympathetic nerve terminals in the ENS (51). Nevertheless, the numbers of enteric neurons responding to C-PAF were small relative to the numbers for some of the other immunoneural mediators, such as histamine (44), mast cell proteases (14), bradykinin (17, 19), leukotrienes (26), and cytokines (55).

No greater than 12.5% of the sampled neuronal populations responded to C-PAF, and expression of PAFR IR was in the same range with 7% of myenteric neurons and 14.5% of...
REFERENCES


