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Platelet-Activating Factor Contributes to Immune Cell and Oxidant-Mediated Intestinal Secretion[1, 2]

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ABSTRACT

The sensitivity of the Ussing-chambered rat colon to stimulation of Cl^- secretion (as measured by the change in short-circuit current) by exogenous platelet-activating factor (PAF) was increased significantly by washing the colon *in vitro* with Ringer's solution containing fatty acid-free albumin. When the wash solution was extracted with chloroform/methanol and the lipid extract was added back to Ussing-chambered colons, inhibition of PAF-stimulated short-circuit current was observed, whereas short-circuit current responses to bradykinin or vasoactive intestinal peptide were not affected. Hypoxia appears to be an important trigger for the down-regulation of the PAF response. These data suggest that hypoxia releases PAF or an endogenous lipid PAF inhibitor that desensitizes PAF receptors on colonic epithelial or mucosal cells. The short-circuit current response of rabbit colon to the chemotactic peptide formyl-methionyl-leucyl-phenylalanine was not inhibited by any PAF antagonist devoid of cyclooxygenase inhibitory activity but was strongly inhibited by indomethacin. In contrast, anti-IgE- or H_2O_2-stimulated short-circuit current in rat colon was inhibited by specific PAF antagonists, and this inhibition was additive with indomethacin. Both anti-IgE and H_2O_2 significantly increased PAF production by rat colon. These data suggest that PAF plays an important role in oxidant (H_2O_2)- and anti-IgE-mediated colonic Cl^- secretion but not in Cl^- secretion mediated by formyl-methionyl-leucyl-phenylalanine-stimulated phagocytes.

PAF (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a phospholipid inflammatory mediator that is synthesized by mesenchymal cells such as endothelial cells, macrophages, mast cells, neutrophils, eosinophils and possibly fibroblasts (Arnoux *et al.*, 1980; Braquet *et al.*, 1987; Casmussi *et al.*, 1983; Chao and Olson, 1993; Lee *et al.*, 1984; Oda *et al.*, 1985). It is not stored preformed in these cells but rather is synthesized and released after activation of phospholipase A_2. The production of this bioactive substance is tightly regulated by a key enzyme, GPC acetyl transferase, and its degradation is regulated by 1-alkyl-2-acetyl-GPC-acetylhydrolase. Although its name is derived from its ability to activate platelets from certain species, many other proinflammatory actions have been attributed to it, including vasoconstriction (Hsueh *et al.*, 1988), enhanced adhesion of neutrophils to endothelial cells (Kubes *et al.*, 1990a, 1990b) and activation of the inducible prostaglandin synthase gene (Bazan *et al.*, 1994). PAF is proposed to have a role in the inflammation of inflammatory bowel disease, ischemic colitis and necrotizing enterocolitis (Ferraris *et al.*, 1993; Resnick *et al.*, 1995; Sobhani *et al.*, 1992; Travis and Jewell, 1994). Unlike lyso-PAF, PAF also has the ability to stimulate water and electrolyte secretion by the intestine (Bern *et al.*, 1989; Hanglow *et al.*, 1989; MacNaughton and Gall, 1991).

PAF stimulation of intestinal Cl^- secretion is due predominantly to the release of leukotrienes and prostaglandins in the intestine (Bern *et al.*, 1989; Hanglow *et al.*, 1989; MacNaughton and Gall, 1991), although there is some controversy regarding this point (Buckley and Hoult, 1989). In most laboratories in which this has been studied, the PAF-stimulated Cl^- secretory responses of rat intestine and simultaneously measured colonic PGE_2 and PGI_2 (measured as 6-keto-$PGF_{1\alpha}$) production are inhibited 75% to 90% by cyclooxygenase inhibitors but not by inhibitors of 5-lipoxy-

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ABBREVIATIONS: I_{sc}, short-circuit current; PAF, platelet-activating factor; GPC, 1-alkyl-lyso-glycerophosphocholine; FMLP, formyl-methionyl-leucyl-phenylalanine; PD, potential difference; BK, bradykinin; VIP, vasoactive intestinal polypeptide; fafBSA, fatty acid-free bovine serum albumin/Ringer's; DMSO, dimethylsulfoxide; INDO, indomethacin; PG, prostaglandin.

genase, histamine or serotonin (Bern *et al.*, 1989; MacNaughton and Gall, 1991).

The intestinal secretory response to PAF is often inconsistent (Bern *et al.*, 1989; Hanglow *et al.*, 1989). The dose response may vary considerably, and on occasion, the response may be entirely absent. To accomplish the primary goal of this study (*i.e.*, understanding whether PAF may play a role in the Cl^- secretion stimulated by immune cells), it was necessary to first understand the cause of this erratic secretory response to exogenous PAF. We postulated that it may be mediated by endogenous desensitization or by release of a PAF inhibitor by the intestine. After determining how to obtain consistent responses to exogenous PAF, specific stimuli were used to determine whether PAF is involved in the intestinal Cl^- transport response to immune system-mediated secretion. The following stimuli were used as models of immune cell-mediated secretions: chemotactic peptide FMLP, which is specific for phagocytes (Marasco *et al.*, 1984); anti-rat IgE, which is a potent but not entirely specific stimulus of mast cells (Ishizaka and Ishizaka, 1984); and H_2O_2, which is released by phagocytes during the respiratory burst and is a potent stimulant of prostaglandin and PAF secretion by mesenchymal cells (Karayalcin *et al.*, 1990; Lewis *et al.*, 1986).

Methods

Transport studies. Male Sprague-Dawley rats weighing 350 to 480 g and New Zealand White rabbits weighing 2.5 to 3 kg were killed by cervical dislocation or intravenously administered pentobarbital sodium (60 mg/kg), respectively. The colon was removed, opened longitudinally and washed of contents with oxygenated Ringer's solution. The entire rat colon or a 5-cm segment of distal rabbit colon was stripped of its outer muscle layers through a combination of blunt and sharp dissection as previously described (Bern *et al.*, 1989). Segments of colon were mounted in Lucite Ussing chambers with a 0.5-cm^2 aperture, with each side incubated with 10 ml of Ringer's solution maintained at 37°C and pH 7.4 when gassed with 95% O_2/5% CO_2. The Ringer's solution contained (in mM): Na^+ 140, K^+ 5.2, Ca^{++} 1.2, Mg^{++} 1.2, Cl^- 119.8, HCO_3^- 25, $H_2PO_4^-$ 0.4, HPO_4^- 2.4 and glucose 10. The bathing solutions were connected *via* agar bridges to calomel electrodes to measure the electrical PD across the tissue. The tissues were short-circuited to zero PD with an automatic voltage clamp (World Precision Instruments, Sarasota, FL) using Ag-AgCl electrodes connected to the bathing solution *via* agar bridges. Tissues were continuously short-circuited except for 5-sec intervals every 15 to 30 min when the open-circuit PD was read. Pilot studies showed no region-specific differences in rat colon in its response to PAF stimulation.

Agonists were added to the serosal bathing solution after stabilization of the base-line I_{sc} and ≥20 min after the addition of various antagonists or inhibitors. Dose-response curves were obtained by mounting six pieces of tissue from a single animal and adding various agonists or antagonists to the serosal bathing solution. The maximal change within 3 to 6 min in I_{sc} above base line (ΔI_{sc}) was then recorded. If the response was biphasic, a second peak response was measured between 15 and 30 min. This I_{sc} response to PAF has previously been shown to be due to electrogenic Cl^- secretion (Bern *et al.*, 1989; Hanglow *et al.*, 1989; MacNaughton and Gall, 1991). In experiments involving exogenous inhibitors, tissues pretreated with inhibitors were compared with simultaneously studied control tissues from the same animal. Dose-response curves to exogenous PAF were determined in washed and unwashed tissues (see below). In other studies, the ΔI_{sc} value of washed and unwashed intestine previously exposed to hypoxia was determined in response to various secretagogues, such as theophylline, BK and VIP.

Studies of PAF desensitization or inhibition. To investigate the influence of hypoxia on intestinal desensitization to PAF or the possible release of endogenous PAF inhibitors, tissues were rendered hypoxic for 5- to 20-min intervals by bubbling argon in the Ringer's solution before mounting. In these hypoxia experiments, great care was taken to keep the time interval between the preparation and the mounting procedure as short and consistent as possible. Untreated tissues or washed hypoxic tissue was used as controls in experiments to study the response to exogenous PAF, to PAF antagonists and to theophylline, BK or VIP.

Washing procedure. In an attempt to remove PAF or any putative endogenous lipid PAF inhibitor(s), Ussing-chambered intestine was incubated with a wash solution consisting of 0.01% faf BSA. This wash solution was added to both sides of the tissue and drained after 10 min. Both sides were then rinsed twice with 50 ml of prewarmed, oxygenated Ringer's solution. During the rinsing procedure, which required 15 min, the fluid of both bathing solutions was carefully removed through continuous suction and replaced at the same time to maintain a constant fluid level with minimal hydrostatic or mechanical disturbances of the tissue. The washed and unwashed tissues were then allowed an additional incubation period of 30 min before the addition of agonists.

Extraction of lipids from wash solution. Wash fluid from the serosal compartment of 22 Ussing-chambered tissues, rendered hypoxic for 5 to 20 min, was extracted according to the Bligh and Deyer method using four volumes of chloroform/methanol/concentrated HCl (100:200:1) (Bligh and Dyer, 1959). The nonaqueous phase was then subjected to two extractions with 1:3 volumes chloroform/0.1 N HCl, and the extract was dried in a vacuum-centrifuge (Jouan Inc., Winchester, VA). The samples were dissolved in 2 ml of ethanol (95%) and chromatographed on a C18 Silica column (Sep-Pak, Waters, Milford, MA). Methanol (70%) was used to elute a prostaglandin-containing fraction from the column. The column was then eluted twice with 2 ml of 100% methanol (PAF-containing fractions 1 and 2). The methanol fractions were then dried as described above and redissolved in DMSO (100%). In mock experiments, 90% of ^3H-PGE_2 was extracted by the 70% methanol elution, and 62% of ^{14}C-PAF was eluted from the column with 100% methanol (data not shown). Both PAF-containing fractions were used separately for experiments in which ~10% of these fractions were added back to the serosal bathing solution of a single intestine mounted in 6 to 10 chambers.

Prostaglandin measurements. After the tissues were mounted in Ussing chambers, 1-ml samples were removed from the serosal bathing solution at varying intervals, put into plastic vials, gassed with argon and stored at −70°C until they were analyzed for prostanoids PGE_2 and 6-keto-$PGF_{1\alpha}$ by direct radioimmunoassay on 100- to 300-μl samples. Internal standards were assayed in the presence of the various inhibitors and agonists that were used. Prostaglandin production rate was calculated from concentrations measured at 15-min time periods. All values were expressed as ng/15 min/cm^2.

PAF measurements. After the colon was mounted in Ussing chambers bathed in Ringer's solution containing 0.05% faf BSA, 1-ml samples of the sersosal bathing solution were obtained from control and agonist-treated tissues before and at 5- to 15-min intervals after the addition of the agonist. Preagonist and postagonist samples were extracted with chloroform/methanol before radioimmunoassay as recommended by the manufacturer. PAF concentrations were expressed as pg/100 μl of serosal bathing solution.

Materials. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Sheep anti-rat IgE was purchased from ICN Immunobiologicals (Lisle, IL). Radioimmunoassay reagents for PGE_2 and 6-keto-$PGF_{1\alpha}$ assays were obtained from Advanced Magnetics, Inc. (Cambridge, MA). The ^{125}I radioimmunoassay kit for PAF was purchased from New England Nuclear Research Products (Boston, MA). FMLP was dissolved in DMSO, divided into aliquots and stored at −20°C until use. Lyophilized anti-IgE sera were reconstituted with sterile water and stored at 4°C. PAF (L-α-lysophosphatidylcholine,β-

acetyl-γ-o-hexadecyl) was obtained as dry powder, dissolved in 2.5% faf BSA in water and stored in aliquots at 20°C for single-time use. INDO was dissolved as a stock solution in DMSO. PAF antagonists WEB 2086, BN 52021, Ro 24-0238 and Ro 24-4736 were dissolved in DMSO; these were graciously supplied by Hoffmann-LaRoche, Inc. (Nutley, NJ).

Statistics. The n in these studies refers to the number of animals. Experiments were performed on paired tissue from the same animal. In some instances, 4 to 12 Ussing chambers were run simultaneously to compare a control tissue with other treatments on tissues from the same animal. In these instances of multiple comparisons with rat colon, it was not possible to obtain more than four to eight pieces of colon from a single animal; therefore, a control tissue was always paired with one to seven treatments and the data were normalized to percentage of control response. Statistical significances of differences were determined by paired *t* test when single paired comparisons were made. When multiple comparisons were made, the significance of differences were determined by parametric or nonparametric analysis of variance.

Results

Variability of rat colonic I_{sc} response to PAF. The additions of exogenous PAF to the serosal bathing solution of rat colon in the Ussing chamber elicits a biphasic ΔI_{sc} response (Cl^- secretion), with a first peak at 3 to 5 min and a second peak at 15 to 30 min (fig. 1). This response has been noted previously, although the cause of its biphasic nature has not been determined (Bern *et al.*, 1989). This secretory response requires a high concentration of PAF (10^{-5} M) for a maximal effect. Furthermore, the magnitude of the response is variable; occasionally, there is no response to PAF. Because such erratic responses to PAF may be due to the release of endogenous PAF with resulting desensitization or to the presence of endogenous inhibitors of PAF, rat colon was washed with 0.01% faf BSA, as described above, and the dose response to PAF was determined in washed and unwashed tissue. As shown in figure 2, the ED_{50} value for the ΔI_{sc} response to PAF in washed colon (500 nM) was decreased by 2 logs compared with unwashed colon (50 μM).

Because there appeared to be a relationship between the time at which the animal was killed to the time at which the tissue was mounted in the Ussing chamber and the subsequent response to PAF, we formally studied the influence of hypoxia on PAF responsiveness. Tissues were rendered hypoxic in Ringer's solution bubbled with argon for different time intervals before they were mounted in Ussing chambers. Figure 3 demonstrates the effect of hypoxia time on the response to PAF by comparing the response in previously hypoxic tissues with that in tissue mounted immediately after the stripping procedure. All tissues were allowed to reoxygenate for 30 min before the addition of PAF. A time-dependent decrease in the response to PAF was seen. No difference was seen in the ΔI_{sc} response to theophylline at the end of the experiment. Furthermore, hypoxia of 5 min had no

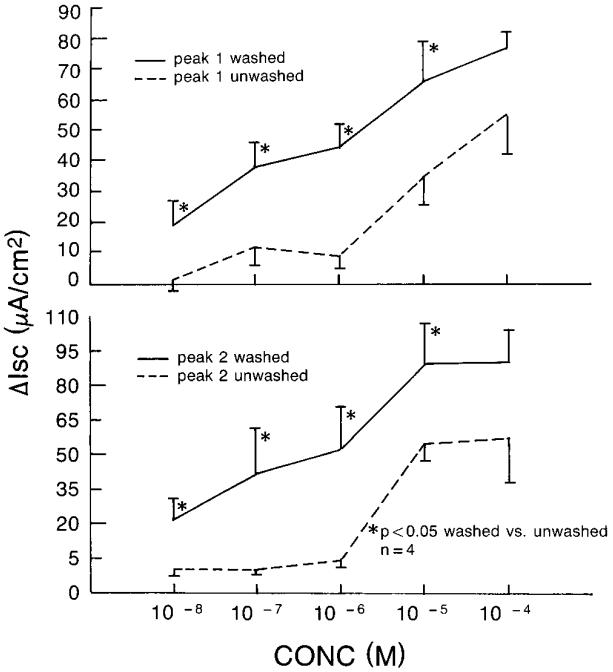

Fig. 2. The PAF-induced ΔI_{sc} of rat colon stripped of muscularis propria and mounted in Ussing chambers is biphasic with early (peak 1) and later (peak 2) increases in I_{sc}. Washing rat colon with 0.01% fafBSA (——) shifts the dose response to exogenous PAF ~2 two logs to the left of that observed in unwashed (– – –) colon.

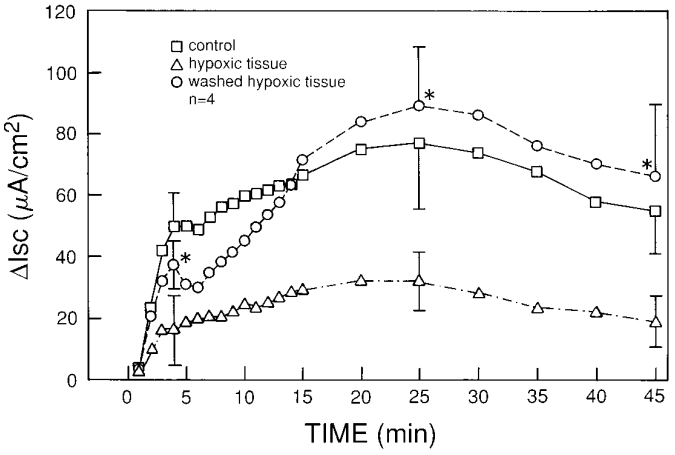

Fig. 1. The maximum ΔI_{sc} to exogenous 10^{-5} M PAF in control (minimally hypoxic) tissues, hypoxic (10 min bubbling with argon) tissues and washed hypoxic (10 min bubbling with argon followed by faf BSA wash) tissues are shown. *, Significance of difference ($P < .05$) between washed hypoxic tissue and hypoxic unwashed tissue.

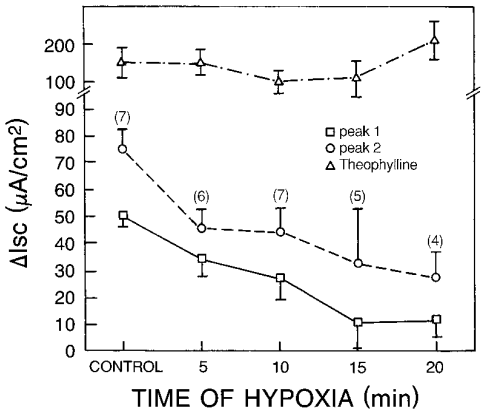

Fig. 3. Maximum ΔI_{sc} to 10^{-5} M PAF of control intestine (minimal hypoxia) or intestine rendered hypoxic by bubbling in argon for 5, 10, 15, and 20 min before mounting in the Ussing chamber. The peak 1 (□) and peak 2 (○) ΔI_{sc} values are shown as well as the subsequent response of each tissue to stimulation with 5 mM theophylline added to both mucosal and serosal bathing solutions. n is number of animals.

inhibitory effect on the ΔI_{sc} response to BK (10^{-6} M) or VIP (10^{-7} M) (fig. 4).

To further clarify the effect of the washing procedure on the response of hypoxic tissue to PAF, the ΔI_{sc} response to PAF was compared with hypoxic (10 min) tissues, washed hypoxic tissues and unwashed control (minimally hypoxic) preparations (fig. 1). Although 10 min of prior hypoxia significantly diminished the ΔI_{sc} response to PAF compared with previously nonhypoxic (control) tissues, washing the tissue restored the ΔI_{sc} response of hypoxic tissue.

To gain some insight into the nature of the substance inhibiting the PAF response, the fluid from the washing procedure was extracted with chloroform/methanol/HCl, and the effect of this lipid extract on the colonic ΔI_{sc} response was determined. Figure 5 demonstrates the ΔI_{sc} response of washed rat colon to the extract and subsequent exogenous PAF. The extract alone had no or only minimal stimulatory effect on the basal ΔI_{sc} of rat colon. In contrast, prior addition of the extract (fraction 1 or 2) significantly inhibited the I_{sc} response to PAF. The second extraction of the wash fluid (fraction 2) reproducibly inhibited the PAF response to a lesser but not significantly different degree than the first (fraction 1) extraction, which is consistent with the idea that fraction 2 contained less of the inhibitor. The specificity of the inhibiting substance was tested by determining the effect of the extract on the I_{sc} response to BK or VIP. Nonhypoxic intestine had a ΔI_{sc} value BK of 45 ± 20 $\mu A/cm^2$, whereas hypoxic tissues demonstrated a ΔI_{sc} value of 77 ± 24 $\mu A/cm^2$ ($n = 3$). Similarly, the ΔI_{sc} value of nonhypoxic and hypoxic tissues to VIP was 79 ± 20 and 58 ± 17 $\mu A/cm^2$, respectively ($n = 3$).

Inhibition of prostaglandin synthesis by PAF inhibitors. Previous studies have shown that ≥90% of the secretory effect of PAF on rat intestine is due to prostaglandin release by PAF (Guerrant *et al.*, 1994; Guthrie *et al.*, 1991; Hanglow *et al.*, 1989). The remainder of the secretory response could be due to PAF stimulation of PAF receptors on colonic epithelial cells or perhaps on PAF receptors on other inflammatory cells in the lamina propria, causing release of other Cl^- secretagogues, such as histamine, serotonin or H_2O_2. To dissect the role of PAF in mast cell (anaphylactic)-mediated or phagocyte (inflammatory)-mediated secretion, stimulants of mast cells (anti-IgE) and phagocytes (the chemotactic peptide FMLP) were studied in conjunction with PAF antagonists.

To prove that PAF is being released after mast cell or phagocyte stimulation and accounts for part of the subsequent Cl^- secretory response, the inhibitory effect of a PAF antagonist must be distinct from any inhibitory effect on cyclooxygenase-mediated prostaglandin production. To determine the cyclooxygenase inhibitory activity of PAF antagonists, we measured PGE_2 and PGI_2 (measured as 6-keto-$PGF_{1\alpha}$) production by rat colon in response to H_2O_2, a potent stimulant of intestinal prostaglandin secretion, in the presence and absence of INDO and four different PAF antagonists (table 1). Neither WEB 2086, BN 52021 nor Ro 24-4736 blocked prostaglandin formation at concentrations used in this study, whereas Ro 24-0238 at 10^{-5} M had inhibitory activity approaching that of 10^{-6} M INDO.

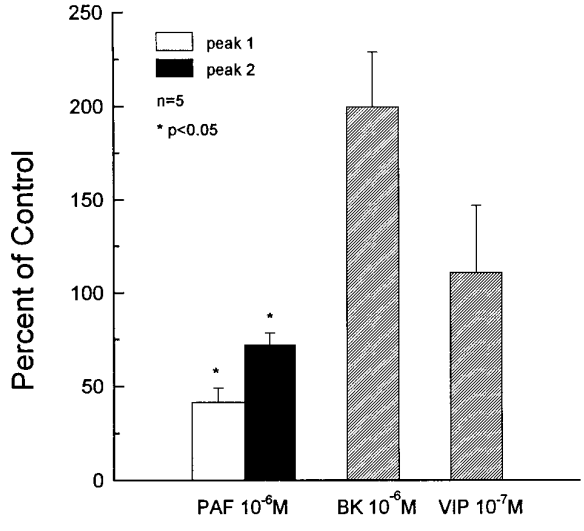

Fig. 4. The ΔI_{sc} response of hypoxic colon (5 min) is shown as a percent of control (nonhypoxic). The response to PAF (two peaks) was significantly inhibited by prior hypoxia, whereas the single peak response to BK and VIP was not. *, $P < .05$, significantly different from control.

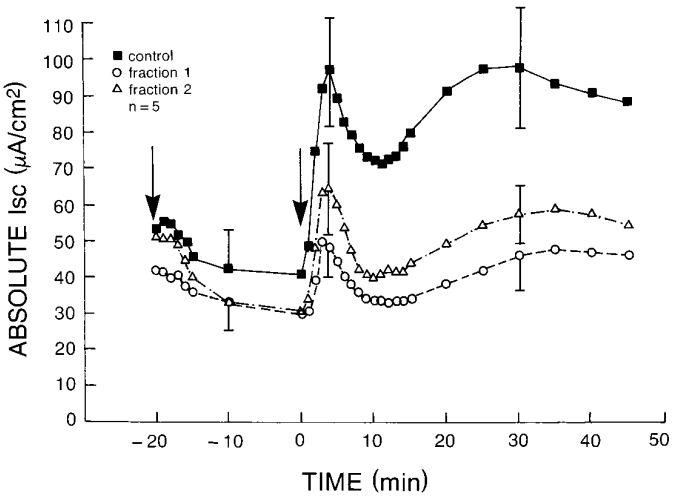

Fig. 5. The effect of addition of chloroform/methanol extract of wash solution back to washed rat colon (left arrow) on subsequent ΔI_{sc} to 10^{-5} M PAF (right arrow) is demonstrated. Both fraction 1 and fraction 2 had a significant inhibitory effect on the subsequent ΔI_{sc} response to PAF. Note also the lack of any stimulatory effect of the chloroform/methanol extract on basal ΔI_{sc}.

TABLE 1

PGE_2 and prostacyclin (measured as 6-keto-$PGF_{1\alpha}$) production by rat colon in response to H_2O_2 (5×10^{-4} M) and effect of PAF antagonists and 1 μM INDO

PG production rate (ng/15 min/cm^2) in the serosal solution of Ussing-chambered rat colon measured 30 min before and 30 min after the addition of H_2O_2.

Agonist + antagonist	PGE_2	6-Keto-$PGF_{1\alpha}$
Control	4.5 ± 1.8	40.8 ± 4.8
+INDO	0.2 ± 0.2[b]	4.1 ± 1.8[b]
+WEB 2086 (10^{-4} M)	2.4 ± 1.6	39.0 ± 3.7
+INDO	0.2 ± 0.7[b]	7.0 ± 2.4[b]
BN 52021 (10^{-4} M)	3.0 ± 1.6	28.0 ± 2.7
+INDO	0.3 ± 0.7[b]	7.8 ± 2.7
Ro 24-0238 (10^{-5} M)	0.5 ± 0.7[a]	9.2 ± 2.4[a]
+INDO	−1.0 ± 0.4[b]	3.7 ± 1.6[b]
Ro 24-4736 (10^{-6} M)	4.2 ± 0.2	43.2 ± 2.8
+INDO	0.1 ± 0.3[b]	5.4 ± 1.0[b]

[a] $P < .05$ control *vs.* PAF antagonists.
[b] $P < .05$ control *vs.* PAF antagonist + INDO.
$n = 4$.

Effect of PAF antagonists on colonic Cl^- secretion. To choose the proper concentration of antagonist for studies of immune cell agonists, the response (ΔI_{sc}) of unwashed but minimally hypoxic rat colon to PAF 10^{-5} M was determined in the presence of varying concentrations of the PAF antagonists (fig. 6). A relative ED_{50} value of 0.05 μM was calculated for Ro 24-4736, followed by Ro 24-0238 (0.3 μM), WEB 2086 (10 μM) and BN 52021 (50 μM). At 10^{-4} M WEB 2086 and BN 52021 and at 10^{-7} M Ro 24-4736, a 100-fold higher concentration of PAF (10^{-3} to 10^{-5} M) was necessary to obtain the same maximal ΔI_{sc} response in colons without an antagonist present (data not shown). The inhibitory effect of compound Ro 24-0238 (10^{-5} M) could not be completely overcome by increasing concentrations of PAF ($\leq 10^{-3}$ M), suggesting a partially noncompetitive inhibitory action of this compound, a finding that is in keeping with its cyclooxygenase inhibitory activity. In subsequent studies, a concentration of antagonists greater than these calculated ED_{50} values was used.

H_2O_2, like PAF, causes a biphasic I_{sc} response in rat colon (Bern *et al.*, 1989). Each of the PAF antagonists inhibited the I_{sc} response to 500 μM H_2O_2 (table 2). For example, the peak 1 response to H_2O_2 was 65 $\pm$ 13 $\mu A/cm^2$ in controls, but when 10^{-6} M INDO was present alone, the peak 1 response was 21 $\pm$ 5% of the control response. Furthermore, when 10^{-4} M WEB 2086 was present alone, the response to H_2O_2 was 61 $\pm$ 16% of the control response. The addition of INDO to the PAF antagonists further inhibited the H_2O_2 response. For example, in presence of both WEB 2086 and INDO, the response was 36 $\pm$ 14% of the control response. This suggests that both PAF and eicosanoids are released by H_2O_2 from lamina propria cells and contribute to the H_2O_2-induced ΔI_{sc} response of rat colon. WEB 2086 failed to inhibit the second I_{sc} peak. This may be due to the strong oxidizing properties of H_2O_2, as suggested by the fact that considerably higher concentrations (10–100-fold) of these PAF antagonists were necessary to inhibit the ΔI_{sc} seen in response to a higher (1000 μM) concentration of H_2O_2 (data not shown). At the concentration used, 10^{-5} M, Ro 24-0238 has been shown to be a potent inhibitor of eicosanoid production (see table 1); therefore, its inhibitory action may be in part due to this as well as to PAF antagonism.

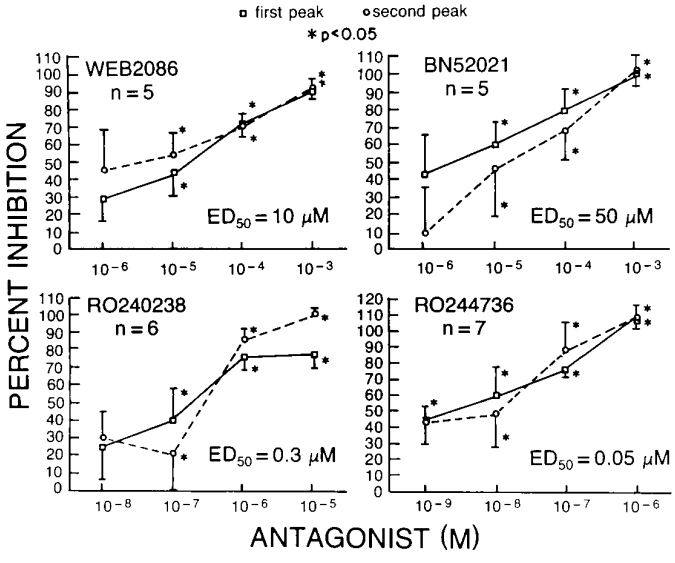

Fig. 6. Response curves to 10^{-5} M PAF in the presence of varying concentrations of four different PAF antagonists. Maximum inhibition of ΔI_{sc} (normalized to percentage) of the first (□) and second peak (○) is shown for each antagonist. The calculated, relative ED_{50} value for each antagonist is shown in the bottom right of each graph. These do not represent true ED_{50} values because the data are normalized and the degree of inhibition of peak 1 is different from peak 2.

Table 2 also demonstrates that the ΔI_{sc} response to anti-IgE was significantly inhibited by all four PAF antagonists. The addition of INDO to the PAF antagonists had no additive inhibitory effect. However, the combination of diphenhydramine and INDO with compounds Ro 24-0238 or Ro 24-4736 significantly reduced the ΔI_{sc} response, suggesting that PAF, histamine and eicosanoids are released by degranulating mast cells and together cause the ΔI_{sc} response to anti-IgE. This interpretation must be viewed with caution; when four compounds are used together, there is the possibility of nonspecific inhibitory effects.

Rat phagocytes have few FMLP receptors; therefore, the response of rat colon to FMLP is very inconsistent, with occasionally no response occurring (Bern *et al.*, 1989). Consequently, we used rabbit colon to study the role of PAF in colonic secretion stimulated by FMLP. Of four PAF antagonists used, only Ro 24-0238 (the compound with antiprostaglandin activity) significantly inhibited the ΔI_{sc} response to FMLP (table 2). Although indomethacin alone inhibited 90% of the FMLP response, only a small additional inhibition was seen when INDO was used in conjunction with any PAF antagonist.

Release of PAF by immune cell agonists. To obtain direct evidence that PAF is released by H_2O_2 and anti-IgE in rat colon, as suggested by the antagonist studies above, PAF concentrations were measured in the serosal bathing solution before and after stimulation with H_2O_2 or anti-IgE. As shown in figure 7, both immune cell agonists significantly increased PAF production by rat colon.

Discussion

PAF is one of the many inflammatory mediators synthesized and released by activated immune cells that are located in a subepithelial location in the lamina propria of intestine. PAF is known to stimulate intestinal Cl^- secretion by rat small and large intestine, although the ED_{50} value for this response (20 μM) is several logs higher than is necessary to stimulate chemotaxis and degranulation of phagocytes *in vitro* (0.01–100 nM) (Bern *et al.*, 1989; Hanglow *et al.*, 1989; MacNaughton and Gall, 1991). Furthermore, as observed by our laboratory and others, the ΔI_{sc} response of intestinal tissues to exogenous PAF may be inconsistent (Bern *et al.*, 1989; Buckley and Hoult, 1989; Hanglow *et al.*, 1989; MacNaughton and Gall, 1991). This suggests prior desensitization due to endogenous PAF release or the presence of an endogenous PAF antagonist. There is evidence of endogenous PAF inhibitors coexisting *in situ* with PAF in different organ systems (Miwa *et al.*, 1987; Nakayama *et al.*, 1987). Among the inhibitors discovered are polyunsaturated free fatty acids, such as oleic acid, as well as lyso-glycero-phospholipids and sphingophospholipids, which have chemical structures similar to PAF (Miwa *et al.*, 1987; Nakayama *et al.*, 1987; Nunez *et al.*, 1990; Smiley *et al.*, 1991; Tokumura *et al.*, 1989). PAF and PAF-like substances bind avidly to albumin (Ludwig *et al.*, 1985); therefore, we washed Ussing-cham-

TABLE 2

Inhibition of rat or rabbit colonic Cl^- secretory response [ΔI_{sc} ($\mu A/cm^2$)] induced by H_2O_2, anti-IgE or FMLP (rabbit) with PAF antagonists, INDO alone or combinations of PAF antagonist, INDO and diphenhydramine

Agonist	Maximum ΔI_{sc} control response (*n*)	INDO 10^{-6} M	WEB 2086 10^{-4} M	BN 52021 10^{-4} M	Ro 24-0238 10^{-5} M	Ro 24-4736 10^{-6} M
	$\mu A/cm^2$			% of Control response		
H_2O_2						
Peak 1[e]	65 ± 13 (5)	21 ± 5	61 ± 16[a]	38 ± 7[a]	15 ± 16[a]	49 ± 4[a]
+INDO[f]			36 ± 14[c]	13 ± 4[c]	3 ± 4[c]	12 ± 6[c]
Peak 2[e]	46 ± 6 (5)	53 ± 6[a]	73 ± 21	48 ± 11[a]	17 ± 9[a]	77 ± 4[a]
+INDO[f]			87 ± 18	43 ± 12	1 ± 4[c]	43 ± 11[c]
Anti-IGE	51 ± 14 (6)	50 ± 7[a]	62 ± 12[a]	52 ± 14[a]	36 ± 12[a]	43 ± 10[a]
+INDO[f]			51 ± 29	31 ± 13	19 ± 12	25 ± 14
+INDO + DPH[d]					3 ± 4[b]	13 ± 7[b]
FMLP[e]	64 ± 2 (6)	13 ± 8[a]	92 ± 9	86 ± 1	49 ± 9[a]	95 ± 20
+INDO[f]			8 ± 5[c]	7 ± 7[c]	12 ± 8[c]	10 ± 11[c]

[a] $P < .05$ agonist *vs.* agonist + antagonist or INDO.
[b] $P < .05$ agonist + antagonist *vs.* agonist + antagonist + INDO + diphenhydramine.[d]
[c] $P < .05$ agonist + antagonist *vs.* agonist + antagonist + INDO.
[e] The percentage of control response on this line refers to the inhibition observed with INDO alone or with the PAF antagonist alone.
[f] The percentage of control response on this line refers to the inhibition observed with a combination of INDO and the PAF antagonist.
INDO, 10^{-6} M; diphenhydramine, 10^{-6} M; FMLP, 10^{-7} M; H_2O_2 500 μM; anti-IgE 90–120, μg of protein/ml.

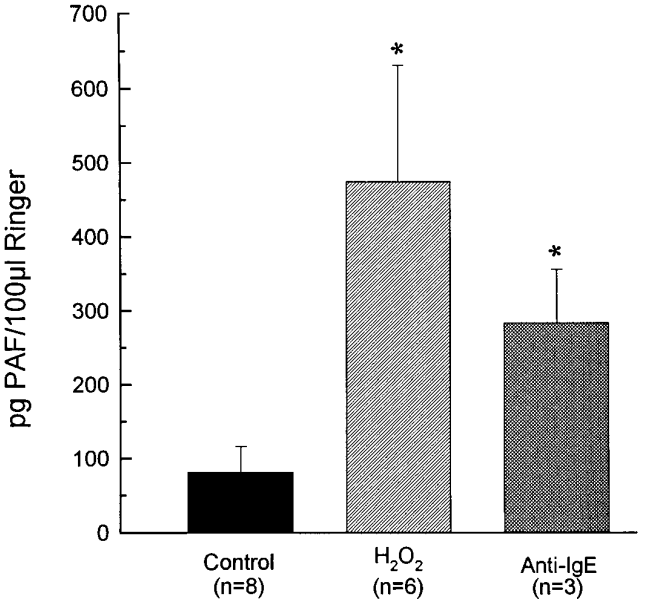

Fig. 7. PAF production by rat colon in response to 500 μM H_2O_2 and 120 μg/ml anti-IgE. The measurements were made on pooled samples of serosal bathing solution collected 5 to 15 min after agonist addition in the Ussing chamber. *, $P < .05$ compared with control.

bered rat colon with fafBSA and demonstrated a significant decrease of 2 logs in the ED_{50} value of the ΔI_{sc} response to exogenous PAF. Because PAF is released by the hypoxic intestine (Caplan *et al.*, 1990; Kubes *et al.*, 1990a), the role of hypoxia in generating the putative PAF antagonist or antagonists was investigated. A hypoxia time-dependent reduction in the ΔI_{sc} response was observed to exogenous PAF but not to BK or VIP. To further explore the hypothesis that PAF or an endogenous inhibitor was present and down-regulating the PAF response, a chloroform/methanol/HCl extract of the wash fluid was added back to the serosal bathing solution. The extract had little effect on the ΔI_{sc} but recreated the blunted response to exogenous PAF. Thus, we hypothesize that PAF or a lipid inhibitor of PAF is released from the rat large intestine in response to hypoxia and that this putative substance desensitizes PAF receptors. The fact that the lipid extract failed to stimulate the I_{sc} when added back to the Ussing chamber is a point against PAF-induced desensitization as the mechanisms of the blunted response. However, we have not determined whether the inhibitor substance is PAF or one of the reported inhibitor substances with a similar structure. A similar desensitization of PAF receptors has been demonstrated in guinea pig ileal smooth muscle in response to inflammation (Jeanneton *et al.*, 1995).

In this study, BN 52021, an alkaloid derived from the Gingko tree, and three synthetic compounds, WEB 2086 (a triazolodiazepine) (Casals-Stenzel *et al.*, 1987; Dent *et al.*, 1989), Ro 24-0238 (a pentadienylamide) (Guthrie *et al.*, 1991) and Ro 24-4736 (thienodiazepine) (Crowley *et al.*, 1991), were tested for their ability to block PAF-induced intestinal Cl^- secretion stimulated by diverse agonists. WEB 2086 (Dent *et al.*, 1989) and Ro 24-4736 (Crowley *et al.*, 1991) are specific PAF antagonists devoid of cyclooxygenase inhibitory activity (table 1). In dose-response studies to exogenous PAF, all four antagonists showed significant inhibition of the ΔI_{sc} response. Furthermore, these PAF antagonists inhibited the Cl^- secretion induced by H_2O_2 and anti-IgE but not the Cl^- secretion induced by FMLP.

H_2O_2, which may be created *in vivo* from the respiratory burst of primed phagocytes (Nathan, 1987), is capable of stimulating PAF and eicosanoid production by endothelial cells (Lewis *et al.*, 1986). We have shown here that H_2O_2 releases PAF from rat colon, and previously our laboratory demonstrated that H_2O_2 stimulates colonic prostaglandin production (Karayalcin *et al.*, 1990). Both PAF and H_2O_2 stimulate intestinal Cl^- secretion by releasing prostaglandins, which stimulate the enteric nervous system (Bern *et al.*, 1989; Hanglow *et al.*, 1989; Karayalcin *et al.*, 1990; MacNaughton and Gall, 1991). The experiments reported in table 2 suggest that both PAF and eicosanoid production is stimulated by H_2O_2 and that both contribute to the ΔI_{sc} response induced by H_2O_2. PGE_2 and PGI_2 production was not affected by the selective PAF antagonists, yet these antagonists were capable of inhibiting the ΔI_{sc} response to H_2O_2, indicating that PAF accounts for part of the response.

We have also shown here that PAF is released by the colon stimulated with anti-IgE and that it contributes to the ΔI_{sc} response of anti-IgE. Mast cell degranulation may result in Cl^- secretion through release of mediators such as hista-

mine, serotonin, adenosine and eicosanoids. Because exogenous PAF releases PGE_2 and PGI_2 in rat colon and ≥50% of the anti IgE-mediated ΔI_{sc} response is inhibited by INDO (table 2; Bern *et al.*, 1989), the lack of an additive effect of PAF antagonists to that of INDO suggests that most of the I_{sc} response to anti-IgE is due to release of prostaglandins by PAF. PAF may therefore mediate mast cell-induced Cl^- secretion through stimulation of prostaglandin production by intermediate targets in the lamina propria such as other immune cells or mesenchymal cells. The combination of a PAF antagonist, cyclooxygenase inhibitor and H_1 antagonist was additive, suggesting that all three agonists play some role in mast cell-mediated secretion. Others have presented evidence for PAF involvement in the electrolyte secretion stimulated during mast cell-mediated intestinal anaphylaxis, and our findings are consistent with those data (MacNaughton *et al.*, 1992).

The chemotactic peptide FMLP was used as a phagocyte stimulant in rabbit colon. It has been shown previously that almost 90% of the FMLP-mediated ΔI_{sc} response of rabbit colon was inhibited by INDO (Bern *et al.*, 1989). Of the four PAF inhibitors used, only compound Ro 24-0238 inhibited the response to FMLP, and at the concentrations used in our study, this compound was found to significantly inhibit prostaglandin synthesis by intestinal tissue. Therefore, we conclude that PAF does not contribute to the FMLP-stimulated phagocyte Cl^- secretory response in rabbit colon.

In summary, these experiments provide evidence that hypoxia releases PAF or, more likely, an endogenous lipophilic inhibitor of PAF action that desensitizes the *in vitro* rat colon. PAF also appears to contribute to some, but not all, immune cell-mediated intestinal water and electrolyte secretion. The Cl^- secretory responses to exogenous H_2O_2 and anti-IgE are partially PAF dependent, but FMLP-stimulated secretion is not. Also of interest is the recent report implicating PAF in intestinal prostaglandin production and secretory response to cholera toxin (Guerrant *et al.*, 1994). PAF has Cl^- secretory effects in part by releasing eicosanoids from lamina propria mesenchymal cells, and it may also directly stimulate epithelial cells and enteric nerves (Berschneider and Powell, 1992; Willard, 1992).

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References

Arnoux, B., Duval, D. and Benveniste, J.: Release of platelet-activating factor (PAF-acether) from alveolar macrophages by the calcium ionophore A23187 and phagocytosis. Eur. J. Clin. Invest. **10:** 437–441, 1980.

Bazan, N. G., Fletcher, B. S., Herschman, H. R. and Mukherjee, P. K.: Platelet-activating factor and retinoic acid synergistically activate the inducible prostaglandin synthase gene. Proc. Natl. Acad. Sci. U.S.A. **91:** 5252–5256, 1994.

Bern, M. J., Sturbaum, C. W., Karayalcin, S. S., Berschneider, H. M., Wachsman, J. T. and Powell, D. W.: Immune system control of rat and rabbit colonic electrolyte transport: Role of prostaglandins and enteric nervous system. J. Clin. Invest. **83:** 1810–1820, 1989.

Berschneider, H. M. and Powell, D. W.: Fibroblasts modulate intestinal secretory responses to inflammatory mediators. J. Clin. Invest. **89:** 484–489, 1992.

Bligh, E. G. and Dyer, W. J.: A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:** 911–917, 1959.

Braquet, P., Touqui, L., Shen, T. Y. and Vargaftig, B. B.: Perspectives in platelet-activating factor research. Pharmacol. Rev. **39:** 97–145, 1987.

Buckley, T. L. and Hoult, J. R.: Platelet activating factor is a potent colonic secretagogue with actions independent of specific PAF receptors. Eur. J. Pharmacol. **163:** 275–283, 1989.

Caplan, M. S., Sun, X.-M. and Hsueh, W.: Hypoxia causes ischemic bowel necrosis in rats: the role of platelet-activating factor (PAF-acether). Gastroenterology **99:** 979–986, 1990.

Casals-Stenzel, J., Muacevic, G. and Weber, K.-H.: Pharmacological actions of WEB 2086, a new specific antagonist of platelet activating factor. J. Pharmacol. Exp. Ther. **241:** 974–981, 1987.

Casmussi, G., Pawlowski, I., Bussolino, F., Caldwell, P. R. B., Brentjens, J. and Andres, G.: Release of platelet-activating factor in rabbits with antibody-mediated injury of the lung: The role of leukocytes and of pulmonary endothelial cells. J. Immunol. **131:** 1802–1807, 1983.

Chao, W. and Olson, M. S.: Platelet-activating factor: Receptors and signal transduction. Biochem. J. **292:** 617–629, 1993.

Crowley, H. J., Yaremko, B., Selig, W. M., Janero, D. R., Burghardt, C., Welton, A. F. and O'Donnell, M.: Pharmacology of a potent platelet-activating factor antagonist: Ro 24-4736. J. Pharmacol. Exp. Ther. **259:** 78–85, 1991.

Dent, G., Ukena, D., Sybrecht, G. W. and Barnes, P. J.: [^{3}H]WEB 2086 labels platelet activating factor receptors in guinea pig and human lung. Eur. J. Pharmacol. **169:** 313–316, 1989.

Ferraris, L., Karmeli, F., Eliakim, R., Klein, J., Fiocchi, C. and Rachmilewitz, D.: Intestinal epithelial cells contribute to the enhanced generation of platelet activating factor in ulcerative colitis. Gut **34:** 665–668, 1993.

Guerrant, R. L., Fang, G. D., Thielman, N. M. and Fonteles, M. C.: Role of platelet activating factor in the intestinal epithelial secretory and Chinese hamster ovary cell cytoskeletal responses to cholera toxin. Proc. Natl. Acad. Sci. U.S.A. **91:** 9655–9658, 1994.

Guthrie, R. W., Tilley, J. W., Kierstead, R. W. and O'Donnell, M.: Ro 24-0238: PAF antagonist. Drugs Future **16:** 1103–1109, 1991.

Hanglow, A. C., Bienenstock, J. and Perdue, M. H.: Effects of platelet-activating factor on ion transport in isolated rat jejunum. Am. J. Physiol. **257:** G845–G850, 1989.

Hsueh, W., Gonzalez-Crussi, F. and Arroyave, J. L.: Sequential release of leukotrienes and norepinephrine in rat bowel after platelet-activating factor. Gastroenterology **94:** 1412–1418, 1988.

Ishizaka, T. and Ishizaka, K.: Activation of mast cells for mediator release through IgE receptors. Prog. Allergy **34:** 188–235, 1984.

Jeanneton, O., Delvaux, M., Frexinos, J. and Bueno, L.: Desensitization of platelet-activating factor receptors, induced by inflammation in guinea pig ileal smooth muscle cells. Gastroenterology **108:** 1666–1675, 1995.

Karayalcin, S. S., Sturbaum, C. W., Wachsman, J. T., Cha, J.-H. and Powell, D. W.: Hydrogen peroxide stimulates rat colonic prostaglandin production and alters electrolyte transport. J. Clin. Invest. **86:** 60–68, 1990.

Kubes, P., Ibbotson, G., Russell, J., Wallace, J. L. and Granger, D. N.: Role of platelet-activating factor in ischemia/reperfusion-induced leukocyte adherence. Am. J. Physiol. **259:** G300–G305, 1990b.

Kubes, P., Masayuki, S. and Granger, D. N.: Modulation of PAF-induced leukocyte adherence and increased microvascular permeability. Am. J. Physiol. **259:** G859–G864, 1990a.

Lee, T.-C., Lenihan, D. J., Malone, B., Roddy, L. L. and Wasserman, S. I.: Increased biosynthesis of platelet-activating factor in activated human eosinophils. J. Biol. Chem. **259:** 5526–5530, 1984.

Lewis, M. S., Whatley, R. E., Cain, P., McIntyre, T. M., Prescott, S. M. and Zimmerman, G. A.: Hydrogen peroxide stimulates the synthesis of platelet-activating-factor by endothelium and induces endothelial cell-dependent neutrophil adhesion. J. Clin. Invest. **82:** 2045–2055, 1986.

Ludwig, J. C., Hoppens, C. L., McManus, L. M., Mott, G. E. and Pinckard, R. N.: Modulation of platelet-activating factor (PAF) synthesis and release from human polymorphonuclear leukocytes (PMN): Role of extracellular albumin. Arch. Biochem. Biophys. **241:** 337–347, 1985.

MacNaughton, W. K., Catto-Smith, A. G. and Gall, D. G.: A role for phospholipid-derived inflammatory mediators in intestinal anaphylaxis in the rat. J. Pharmacol. Exp. Ther. **260:** 773–779, 1992.

MacNaughton, W. K. and Gall, D. G.: Mechanisms of platelet-activating factor-induced electrolyte transport in the rat jejunum. Eur. J. Pharmacol. **200:** 17–23, 1991.

Marasco, W. A., Phan, S. H., Krutzsch, H., Showell, H. J., Feitner, D. E., Nairn, R., Becker, E. L. and Ward, P. A.: Purification and identification of formylmethionyl-leucyl-phenylalanine as the major peptide neutrophil chemotactic factor produced by *Escherichia* coli. J. Biol. Chem. **259:** 5430–5439, 1984.

Miwa, M., Hill, C., Kumar, R., Sugatani, J., Olson, M. S. and Hanahan, D. J.: Occurrence of an endogenous inhibitor of platelet-activating factor in rat liver. J. Biol. Chem. **262:** 527–530, 1987.

Nakayama, R., Yasuda, K. and Saito, K.: Existence of endogenous inhibitors of platelet-activating factor (PAF) with PAF in rat uterus. J. Biol. Chem. **262:** 13174–13179, 1987.

Nathan, C. F.: Neutrophil activation on biological surfaces: Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. J. Clin. Invest. **80:** 1550–1560, 1987.

Nunez, D., Randon, J., Gandhi, C., Siafaka-Kapadai, A., Olson, M. S. and Hanahan, D. J.: The inhibition of platelet-activating factor-induced platelet activation by oleic acid is associated with a decrease in polyphosphoinositide metabolism. J. Biol. Chem. **265:** 18330–18338, 1990.

Oda, M., Satouchi, K., Yasunaga, K. and Saito, K.: Molecular species of platelet-activating factor generated by human neutrophils challenged with ionophore A23187. J. Immunol. **134:** 1090–1093, 1985.

RESNICK, M. B., COLGAN, S. P., PARKOS, C. A., DELP-ARCHER, C., MCGUIRK, D., WELLER, P. F. AND MADARA, J. L.: Human eosinophils migrate across an intestinal epithelium in response to platelet-activating factor. Gastroenterology **108:** 409–416, 1995.

SMILEY, P. L., STREMLER, K. E., PRESCOTT, S. M., ZIMMERMAN, G. A. AND MCINTYRE, T. M.: Oxidatively fragmented phosphatidylcholines activate human neutrophils through the receptor for platelet-activating factor. J. Biol. Chem. **266:** 11104–11110, 1991.

SOBHANI, I., HOCHLAF, S., DENIZOT, Y., VISSUZAINE, C., RENE, E., BENVENISTE, J., LEWIN, M. J. J. AND MIGNON, M.: Raised concentrations of platelet activating factor in colonic mucosa of Crohn's disease patients. Gut **33:** 1220–1225, 1992.

TOKUMURA, A., TAKAUCHI, K., ASAI, T., KAMLYASU, K., OGAWA, T. AND TSUKATANI, H.: Novel molecular analogues of phosphatidylcholines in a lipid extract from bovine brain: 1-Long-chain acyl-2-short-chain acyl-sn-glycero-3-phosphocholines. J. Lipid Res. **30:** 219–224, 1989.

TRAVIS, S. P. L. AND JEWELL, D. P.: Review: The role of platelet-activating factor in the pathogenesis of gastrointestinal disease. Prost. Leukot. Essent. Fatty Acids **50:** 105–113, 1994.

WILLARD, A.: Excitatory and neurotoxic actions of platelet-activating factor on rat myenteric neurons in cell culture. Ann. N.Y. Acad. Sci. **664:** 284–292, 1992.

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