PAF-like lipids- and PAF-induced gallbladder muscle contraction is mediated by different pathways in guinea pigs

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Guarino, Michele P. L., Zuo-Liang Xiao, Piero Biancani, and Jose Behar. PAF-like lipids- and PAF-induced gallbladder muscle contraction is mediated by different pathways in guinea pigs. Am J Physiol Gastrointest Liver Physiol 285: G1189–G1197, 2003.—H2O2 stimulates gallbladder muscle contraction and scavengers of free radicals through the generation of PGE2. Oxidative stress causes lipid peroxidation and generation of platelet-activating factor (PAF) or PAF-like lipids. The present studies therefore were aimed at determining whether either one induced by H2O2 mediates the increased generation of PGE2. Dissociated muscle cells of guinea pig gallbladder were obtained by enzymatic digestion. Both PAF-like lipids and PAF-induced muscle contraction was blocked by the PAF receptor antagonist CV-3988. This antagonist also blocked the increased PGE2 production caused by PAF-like lipids or PAF. Actions of PAF-like lipids were completely inhibited by indomethacin, but those of PAF were only partially reduced by indomethacin or by nordihydroguaiaretic acid and completely blocked by their combination. PAF-like lipids-induced contraction was inhibited by AACOCF3 (cystolic phospholipase A2 inhibitor), whereas the actions of PAF were blocked by MJ33 (secretory phospholipase A2 inhibitor). Receptor protection studies showed that pretreatment with PAF-like lipids before N-ethylmaleimide protected the contraction induced by a second dose of PAF-like lipids or PGE2 but not by PAF. In contrast, pretreatment with PAF protected the actions of PAF and PGE2 but not that of PAF-like lipids. Both PAF-like lipids and PAF-induced contractions were inhibited by anti-Gα11 antibody and by inhibitors of MAPK and PKC. In conclusion, PAF-like lipids seem to activate a pathway different from that of PAF probably by stimulating a different PAF receptor subtype.

REACTIVE OXYGEN SPECIES (ROS) such as H2O2 are increased during acute inflammatory processes (9, 25). The initial injury in the gallbladder muscle caused by ROS appears to be confined to the plasma membrane (27). These free radicals induce nonenzymatic oxidation of phospholipids (lipid peroxidation) and damage membrane protein constituents by oxidizing transmembrane receptors and calcium channels (3, 9). H2O2 impairs the contraction induced by receptor-dependent agonist CCK-8 and ACh as well as KCl, which is receptor-G protein independent (25).

H2O2 causes muscle cell contraction and increases PGE2 formation (27). The pathways responsible for these cellular responses are not known. Lipid peroxidation results in the generation of isoprostanes, platelet-activating factor (PAF)-like lipids (10), and oxidized byproducts. Isoprostanes do not appear to be plausible candidates of stimulating the pathways that mediate the contraction and cytoprotective responses, because they tend to behave like prostaglandin receptor antagonists; particularly against PGE2 (12–14, 16). Other oxidation products of phosphatidylcholine bind and activate PAF receptors (19). These PAF-like lipids are potent, selective inflammatory mediators formed by nonenzymatic mechanisms resulting in an unregulated accumulation in the plasma membranes (10, 20). The precursors of PAF synthesis are susceptible to oxidative attack resulting in the fragmentation of the esterified polyunsaturated fatty acid residue at the sn-2 position (11). Fragmentation of the sn-2 residue is the result of the oxidation of phospholipid byproducts that structurally resemble PAF. Some of the oxidized phospholipids have the ether bond at the sn-1 position of the glycerol backbone that is an important determinant for recognition by the PAF receptor (30). Thus the oxidative byproducts complete the molecular mimicry of PAF by creating a residue that in some cases is only a little longer than the acetyl residue of PAF. Therefore, the present studies were aimed at determining whether H2O2 induced muscle cell contraction and PGE2 formation are mediated by PAF or PAF-like lipids acting on PAF receptors.

MATERIALS AND METHODS

Animals. Adult male guinea pigs were purchased from Elm Hill breeding Laboratory (Chelmsford, MA). The Animal Welfare Committee of Rhode Island Hospital approved their use. Animals were housed in thermoregulated rooms and had free access to food and water. After an overnight fast, the animals were anesthetized with an intramuscular injection of ketamine hydrochloride (30 mg/kg), followed by pentobarbital (30 mg/kg ip). The gallbladder was removed and rinsed with ice-cold, oxygenated Krebs solution (in mM: 116.6 NaCl, 4.7 KCl, 25 NaHCO3, 1.2 MgSO4, 1.2 CaCl2, 11.1 glucose, and 1.25 KH2PO4). The gallbladder was cut open longitudinally, and one part was tied to the other with fine sutures to prevent outflow of Krebs solution. All experiments were performed in a warm, humidified incubator at 37°C. Animals were allowed to recover for 1 h before the start of the experiment. The gallbladder was isolated and then placed in the organ bath containing Krebs solution gassed with 95% O2-5% CO2.

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3.4 KCl, 21.9 NaHCO₃, 1.2 Na₂HPO₄, 2.5 CaCl₂, 1.2 MgCl₂, and 5.4 glucose). Muscosa and serosa were carefully peeled off under a dissecting microscope. The muscle layer was further cleaned by gently removing the remaining connective tissue.

**Isolation of single cells from gallbladder muscle.** Gallbladder muscle cells were prepared as previously described (24, 25, 27, 28). After the gallbladder was removed from the animal, it was immediately placed in ice-cold preoxygenated Krebs buffer solution, washed, and transferred to a dissecting pan containing the same buffer continuously aerated with 95% O₂-5% CO₂. The Krebs buffer solution contained (in mM) 116.6 NaCl, 3.4 KCl, 21.9 NaHCO₃, 1.2 Na₂HPO₄, 2.5 CaCl₂, 1.2 MgCl₂, and 5.4 glucose. Under a dissecting microscope, the mucosa and then the serosa were carefully peeled off. The muscle layer was further cleaned by removing the remaining connective tissue and blood vessels. It was minced to 2 × 2-mm squares and digested in HEPES and in a buffered nutrient solution containing 150 units of type II collagenase per milliliter for 2.5–3.5 h at 31°C. HEPES buffer solution contained (in mM) 112.5 NaCl, 5.5 KCl, 2.0 KH₂PO₄, 24 HEPES, 1.9 CaCl₂, 0.6 MgCl₂, and 10.8 glucose, as well as 0.08 mg/ml soybean trypsin inhibitor and 2% ethanol (vol/vol) basal Eagle's medium (50×) amino acids without Tris buffer (5 mM) and creatine phosphokinase (10 U/ml). Cells were then equilibrated in modiﬁed cytosolic buffer with appropriate concentrations of an agonist (24, 25, 27, 28). Receptor protection studies were performed in muscle cells first incubated with PAF or H₂O₂ for 2 min, followed by a 15-min incubation with 5 μM of N-ethylmaleimide (NEM) (5). The cells were then washed by superfusion with HEPES buffer for 20 min. Their control cell length was measured before the contraction studies were performed with the same or different agonist. Cells were then exposed to agonists for 30 s, and their response was stopped by the addition of acrolein at a ﬁnal concentration of 1%. A drop of ﬁxed cells was placed on a microscope slide and covered with a coverslip. Edges of the coverslip were sealed with nail enamel to prevent evaporation. Lengths of 30 consecutive cells from control and experimental samples were measured.

**Preparation of enriched plasma membranes.** Enriched plasma membranes from muscle squares were prepared and puriﬁed by sucrose gradient centrifugation (17, 23). Muscle squares were homogenized in sucrose-HEPES buffer by using a tissue tearer (Biospec Products, Racine, WI). This sucrose-HEPES buffer contains 0.25 M sucrose, 10 mM HEPES (pH 7.4), 0.01% soybean trypsin inhibitor, 0.1 mM PMSF, 0.1 mM 1,10-phenanthroline, and 1 mM 2-mercaptoethanol. The homogenates were centrifuged at 600 g for 5 min; the supernatant was collected in a clean tube and again centrifuged at 150,000 g for 45 min. The pellet was resuspended in sucrose-HEPES buffer, layered over a linear 9–60% sucrose gradient, and centrifuged at 90,000 g for 3 h. The plasma membranes were collected at ~24% sucrose and stored at 70°C.

**[35S]GTPγS binding and immunoprecipitation.** [35S]GTPγS binding was assayed by immunoprecipitation (22). Enriched plasma membranes were solubilized for 1 h at 4°C with 1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate in HEPES buffer (in mM: 10 HEPES, pH 7.4, 0.1 EDTA, and 10 MgCl₂). Solubilized membranes at a concentration of 2.5 mg protein/ml were incubated with 60 nM [35S]GTPγS in the presence or absence of agonists at 37°C for 10 min (total volume, 300 μl). For nonspeciﬁc binding, 6 μM GTP was used. The reaction was stopped with 10 volumes of ice-cold Tris buffer (in mM: 100 Tris-HCl, pH 8.0, 10 MgCl₂, 100 NaCl, and 20 GTP). Aliquots (200 μl) of the reaction mixture were added to pretreated ELISA wells. The ELISA wells were prepared by initially coating with an anti-rabbit immunoglobulin antibody (1:2,000) at 4°C for 1 h, by washing out the trace of the antibody, and then by coating with specific G protein subunit antibodies (1:2,000) at 4°C for 1 h. The specific G protein subunit antibodies of anti-Go₁₂, Go₁₃, Go₅₁₁, and Go₁₅ were used. After incubation with the reaction mixture in ELISA wells at 4°C for 2 h, the wells were washed three times with phosphate buffer (in mM: 1 K₂HPO₄, pH 7.4, 10 Na₂HPO₄, 137 NaCl, and 2.7 KCl) with saponin (see above) has been shown to allow the large molecules such as inositol 1,4,5-trisphosphate to diffuse across the plasma membrane freely, whereas it does not affect the intracellular contractile mechanisms.

**Experimental procedure and cell measurements.** After muscle cells were equilibrated for 20 min, 0.25-ml aliquots of cell suspension were added to test tubes containing 0.1 ml of buffer with appropriate concentrations of an agonist (24, 25, 27, 28). Permeabilization studies were performed in muscle cells first incubated with PAF or H₂O₂ for 2 min, followed by a 15-min incubation with 5 μM of N-ethylmaleimide (NEM) (5). The cells were then washed by superfusion with HEPES buffer for 20 min. Their control cell length was measured before the contraction studies were performed with the same or different agonist. Cells were then exposed to agonists for 30 s, and their response was stopped by the addition of acrolein at a final concentration of 1%. A drop of fixed cells was placed on a microscope slide and covered with a coverslip. Edges of the coverslip were sealed with nail enamel to prevent evaporation. Lengths of 30 consecutive cells from control and experimental samples were measured.
containing 0.05% Tween 20. The radioactivity of each well was counted by a scintillation counter. Data were expressed as percent increase over basal levels (without agonist stimulation).

Measurement of PGE₂ levels. The content of PGE₂ was measured by using a radioimmunoassay kit from New England Nuclear Life Science Products (Boston, MA) (4, 28). Muscle cells were homogenized and the suspension was centrifuged at 10,000 g for 15 min. PGE₂ was extracted from the supernatant (8) and PGE₂ was converted into its methyl oximate derivative by using the methyl oximation reagent. The determination of PGE₂ content was achieved by following the kit’s protocol. Briefly, samples or different concentrations of purified PGE₂ (standard curve) were incubated with ¹²⁵I-labeled PGE₂ and PGE₂ antiserum overnight at 4°C. Separation of bound form was achieved by adding precipitating reagent and centrifuged for 30 min at 4°C. Samples were counted in a gamma counter and the content of PGE₂ was calculated from the standard curve and expressed as nanograms per milligrams protein.

Protein determination. Protein content of muscle membranes was measured by using a protein assay kit (Bio-Rad Laboratories, Hereules, CA). Values for each sample were means of triplicate measurements.

Drugs and chemicals. H₂O₂ was obtained from Fisher Scientific (Pittsburgh, PA); CV-3988 was obtained from Bio-Mol Research Laboratories (Plymouth Meeting, PA); antibodies against G protein subunits [G₁₁-₁₂ (COOH terminus 345–354 and 346–355), G₁₁ (COOH terminus 345–354), G₁₁ (internal 283–300), G₁β (COOH terminus 385–394)] were purchased from CytoSignal (Irvine, CA); [³⁵S]GTP·S and PGE₂ radioimmunoassay kit were obtained from New England Nuclear Life Science Products; type F collagenase, papain, NEM, and other reagents were purchased from Sigma (St. Louis, MO).

Statistics. One- and two-factorial repeated ANOVA or Student’s t-tests (unless otherwise noted) were used for statistical analysis. P values of <0.05 were considered significant.

RESULTS

Enzymatically dissociated muscle cells were treated with PAF receptor antagonist CV-3988 (10⁻⁵ M) for 15 min, followed by agonist for 30 s.Buffered, treated muscle cells contract an average of 20% in response to agonists (25). Increasing concentrations of PAF₁₆ and PAF₁₈ (10⁻¹⁵–10⁻⁸ M) induced a similar dose-dependent muscle contraction (Fig. 1). Maximal contractions of 20.4 ± 1.5% (PAF₁₆) and 21.3 ± 1.4% (PAF₁₈) were reached with a dose of 10⁻⁸ M. PAF₁₆- and PAF₁₈-induced contractions were completely blocked by the PAF receptor antagonist CV-3988 (10⁻⁶ M) to 3.1 ± 0.7 and 3.5 ± 0.1% (*P < 0.001 and #P < 0.001 by ANOVA).

H₂O₂ also induced a dose-dependent muscle contraction with a maximal contraction of 13.3 ± 1.9% achieved with a dose of 70 μM (Fig. 2). H₂O₂-induced muscle contraction was also blocked by pretreatment with the PAF receptor antagonist CV-3988 (10⁻⁵ M) (*P < 0.001 by Student’s t-test). H₂O₂ generates PAF-like lipids by acting on plasma membrane phospholipids (10). These data suggest that PAF-like lipids are one of the byproducts of lipid peroxidation acting on membrane receptors.

![Fig. 1. Effect of specific platelet-activating factor (PAF) receptor antagonist CV-3988 on PAF (10⁻¹⁵ to 10⁻⁶ M)-induced muscle contraction. Both PAF₁₆ and PAF₁₈ (two isoforms of PAF) caused a dose-dependent muscle contraction of ≤20%. Their effects were blocked by pretreatment with CV-3988 (10⁻⁵ M)(*P < 0.001, #P < 0.001 by ANOVA). Values are means ± SE of 3 experiments.](http://ajpgi.physiology.org/)

To determine whether PAF and PAF-like lipids are generated by lipid peroxidation, the signal transduction responsible for muscle contraction caused by exogenous PAF and by PAF-like lipids was examined. Muscle cells were pretreated with indomethacin (cyclooxygenase inhibitor) and nordihydroguaiaretic acid (NDGA; a lipoxygenase inhibitor) for 15 min before agonist stimulation (Fig. 3). PAF-induced contractions were partially inhibited by either indomethacin (20.2 ± 2.4 to 8.4 ± 1.3%; *P < 0.001 by Student’s t-test) or NDGA (20.2 ± 2.4 to 12.2 ± 0.9%; **P < 0.001 by Student’s t-test) and completely blocked by both (20.2 ± 2.4 to 1.5 ± 1.0%; ***P < 0.001 by Student’s t-test). However, H₂O₂-induced contractions were only blocked by indomethacin (14.6 ± 1.2 to 8.4 ± 1.3%; #P < 0.001, ##P < 0.001 by Student’s t-test) and unaffected by NDGA. These data suggest that prostaglandins are involved in the pathways that mediate PAF-like lipid-induced muscle cell contractions. In contrast, the PAF-induced contraction is mediated by prostaglandins and probably also by leukotrienes. The finding that PAF and PAF-like lipids stimulate different phospholipases (PLAs) supports these results. In these studies, the inhibitors of cytosolic PLA₂ (cPLA₂; AACOCF₃) and secretory PLA₂ type I (sPLA₂; MJ₃₃) were tested against the contraction induced by these agonists (Fig. 4). The highly selective sPLA₂ type I inhibitor MJ₃₃ was used in permeabilized muscle cells. PAF-induced contractions were only blocked by MJ₃₃ (20.2 ± 2.4 to 4.1 ± 0.9%; *P < 0.001 by Student’s t-test). H₂O₂-induced contractions were only inhibited by AACOCF₃ (14.2 ± 1.2 to 0.9 ± 0.3%; #P < 0.001 by Student’s t-test). Levels of PGE₂ production were then measured by radioimmunoassay in muscle homogenates stimulated with PAF or H₂O₂ pretreated with buffer alone or with PAF receptor antagonist CV-3988 (10⁻⁵ M) (Fig. 5). PAF and H₂O₂ increased PGE₂ production from 7.5 ±
0.8 to 16.1 ± 1.4 ng/mg protein (*P < 0.001 by Student’s t-test) or to 14.7 ± 0.6 ng/mg protein (#P < 0.001 by Student’s t-test), respectively. The rise in PGE2 levels was prevented by PAF receptor antagonist CV-3988 (10⁻⁵ M) pretreatment from 16.1 ± 1.4 to 7.4 ± 0.6 ng/mg protein (**P < 0.001 vs. PAF alone by Student’s t-test) and to 8.1 ± 0.9 ng/mg protein (##P < 0.001 vs. H₂O₂ alone by Student’s t-test). These data suggest that PAF receptors are involved in the increased PGE₂ levels induced by PAF and H₂O₂.

Because PAF and PAF-like lipids stimulate different PLAs, we examine whether PAF and PAF-like lipids act on the same PAF receptor, by performing experiments with NEM, a nonspecific membrane receptor inactivator (Fig. 6). This compound inactivates all transmembrane receptors unprotected by their specific ligands. Agonists are capable of protecting their own receptors if muscle cells are treated with them before NEM (5). Pretreatment of muscle cells with half-maximal doses of PAF (10⁻¹¹ M) before NEM protected the actions of a second dose of PAF and the functions of PGE₂, but it did not protect those receptors stimulated by H₂O₂. PAF and PGE₂ induced a full contraction in muscle cells treated with PAF and followed by NEM. However, the H₂O₂-induced contraction was significantly reduced (*P < 0.001 by Student’s t-test). In contrast, pretreatment of muscle cells with H₂O₂ (that Fig. 2. Effect of PAF receptor (PAF-F) antagonist CV-3988 (10⁻⁵ M) on muscle contraction induced by increasing concentrations of H₂O₂. H₂O₂ (17.5 to 140 μM) induced a dose-dependent increase with a maximal contraction achieved at a dose of 70 μM. This effect was blocked by pretreatment with CV-3988 (⁎P < 0.001 by Student’s t-test). Values are means ± SE of 3 experiments.

Fig. 3. Effect of inhibitors of cyclooxygenase [indomethacin (Indo) 5 μM] and lypoxygenase [nordihydroguaiaretic acid (NDGA); 10 μM] on PAF (10⁻⁹ M) and H₂O₂ (70 μM)-induced muscle contraction. Pretreatment of muscle cells with indomethacin or NDGA for 15 min was followed by PAF or H₂O₂ for 30 s. PAF-induced contraction was partially inhibited by either indomethacin (⁎⁎P < 0.001 by Student’s t-test) or NDGA (⁎⁎⁎P < 0.001 vs. PAF alone by Student’s t-test) but was completely blocked by their combination (⁎⁎⁎P < 0.001 by Student’s t-test). However, H₂O₂-induced contractions were completely blocked by indomethacin alone (#P < 0.001, ###P < 0.001 vs. Student’s t-test) and not affected by NDGA. Values are means ± SE of 3 experiments.

Fig. 4. Effect of inhibitors of cyclooxygenase [indomethacin (Indo) 5 μM] and lypoxygenase [nordihydroguaiaretic acid (NDGA); 10 μM] on PAF (10⁻⁹ M) and H₂O₂ (70 μM)-induced muscle contraction. Pretreatment of muscle cells with indomethacin or NDGA for 15 min was followed by PAF or H₂O₂ for 30 s. PAF-induced contraction was partially inhibited by either indomethacin (⁎⁎P < 0.001 by Student’s t-test) or NDGA (⁎⁎⁎P < 0.001 vs. PAF alone by Student’s t-test) but was completely blocked by their combination (⁎⁎⁎P < 0.001 by Student’s t-test). However, H₂O₂-induced contractions were completely blocked by indomethacin alone (#P < 0.001, ###P < 0.001 vs. Student’s t-test) and not affected by NDGA. Values are means ± SE of 3 experiments.
G protein subunit antibodies (29). Permeabilized muscle cells were pretreated with antibodies against different G protein subunits for 15 min before PAF (Fig. 8). Antibodies themselves had no effect on muscle cell contraction. However, pretreatment of muscle cells with anti-Gq/11 antibody inhibited the PAF-induced contractions from 19.7 ± 1.2 to 3.1 ± 0.2% (*P < 0.001 by Student's t-test). All other antibodies had no effect on PAF-induced contractions. Similarly, muscle contraction induced by H2O2 was also inhibited by pretreatment with anti-Gq/11 antibody (*P < 0.001 by Student's t-test) (Fig. 9). These data suggest that receptors that mediate the actions of PAF and PAF-like lipids couple to the same G proteins.

GTPγS binding studies further supported these data (Fig. 10). Basal levels (without receptor activation) for the [35S]GTPγS binding to Goi1, Goi2, Goq1, and Goq2 were 2,064, 2,075, 2,030, and 2,106 counts/min, respectively. PAF caused a significant increase in [35S]GTPγS binding to Goi1 (68.0 ± 2.5%) and Goq1 (79.0 ± 4.5%). Pretreatment with the cyclooxygenase inhibitor indomethacin before PAF blocked the [35S]GTPγS binding to Goi1 (68.0 ± 2.5 to 17.0 ± 1.3%; *P < 0.001 by Student's t-test) but not that to Goq1. PAF receptor antagonist CV-3988 (10−5 M) abolished the [35S]GTPγS binding to both Goi1 (68.0 ± 2.5 to 12.0 ± 0.9%; #P < 0.001 by Student's t-test) and Goq1 (79.0 ± 4.5 ± 12.0 ± 0.2%; ##P < 0.001 by Student's t-test). H2O2 alone (Fig. 11) caused a significant increase in [35S]GTPγS binding to Goi1 (68.0 ± 2.5 to 75.0 ± 3.5 to 15.0 ± 1.3%; *P < 0.001 by Student's t-test), Goi3 (28.0 ± 6.5 to 12.0 ± 0.2%; ***P < 0.001 by Student's t-test) but not that to Goq1. PAF receptor antagonist CV-3988 (10−5 M) abolished [35S]GTPγS binding to Goq1 (56.0 ± 8.2 to 6.0 ± 2.4%; #P < 0.001 by Student's t-test) but not that to Goi1 and Goq2.

Binding of an agonist to membrane receptors activates specific G proteins and can be blocked by specific

Fig. 6. Protection of receptors by agonists from the actions of N-ethylmaleimide (NEM). Agonists are capable of protecting receptors from the inactivating of NEM. Pretreatment of muscle cells with a low dose of PAF (10−11 M) for before NEM (5 μM) protected the actions of a second dose of PAF and PGE2 on muscle cell contraction, but it did not protect that of H2O2. PAF and PGE2 induced a full contraction in muscle cells pretreated with PAF before NEM but H2O2-induced contractions were significantly reduced (*P < 0.001 by Student's t-test). Values are means ± SE of 3 experiments.

Fig. 7. Receptor protection by PAF-like lipids from the actions of NEM. Pretreatment of muscle cells with H2O2 (70 μM) (induction of PAF-like lipids) before NEM (5 μM) protected the actions of H2O2 and PGE2 but not that of PAF, because H2O2- and PGE2-induced contractions were similar to those of controls. In contrast, PAF (10−9 M)-induced contractions were reduced (*P < 0.001 by Student's t-test). Values are means ± SE of 3 experiments.

Fig. 8. Effects of specific antibodies against G protein subunits on PAF-induced muscle contraction. Antibodies themselves had no effect on muscle contraction. However, pretreatment of muscle cells with anti-Gq11 antibody (1:400 titer) inhibited PAF-induced contraction (*P < 0.001 by Student's t-test). All other antibodies had no effect on PAF-induced muscle contractions. Values are means ± SE of 3 experiments.

G protein subunit antibodies (29). Permeabilized muscle cells were pretreated with antibodies against different G protein subunits for 15 min before PAF (Fig. 8). Antibodies themselves had no effect on muscle cell contraction. However, pretreatment of muscle cells with anti-Gq11 antibody inhibited the PAF-induced contractions from 19.7 ± 1.2 to 3.1 ± 0.2% (*P < 0.001 by Student's t-test). All other antibodies had no effect on PAF-induced contractions. Similarly, muscle contraction induced by H2O2 was also inhibited by pretreatment with anti-Gq11 antibody (*P < 0.001 by Student's t-test) (Fig. 9). These data suggest that receptors that mediate the actions of PAF and PAF-like lipids couple to the same G proteins.

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Binding of an agonist to membrane receptors activates specific G proteins and can be blocked by specific...
Furthermore, pretreatment of muscle cells with inhibitors of PKC (0.1 μM chelerythrine) or MAP kinase (10 μM PD-98059) before PAF or H$_2$O$_2$ blocked their contraction (Fig. 12). PAF-induced muscle cell contraction was reduced by chelerythrine (21.2 ± 3.5 to 5.1 ± 1.2%; *P < 0.001 by Student’s t-test) and by PD-98059 (21.2 ± 3.5 to 1.0 ± 1.0%; *P < 0.001 by Student’s t-test). Similarly, H$_2$O$_2$-induced contractions were also blocked by chelerythrine (16.7 ± 2.4 to 3.1 ± 1.5%; **P < 0.001 by Student’s t-test) and by PD-98059 (16.7 ± 2.4 to 0.8 ± 0.7; *P < 0.001 by Student’s t-test). These data suggest that PAF and PAF-like lipids share a common final pathway.

On the basis of these results, possible pathways that mediate PAF- and H$_2$O$_2$-induced muscle cell contractions in guinea pig gallbladder muscle are illustrated in Fig. 13. Stimulation of muscle cells with PAF (through PAF receptors) or H$_2$O$_2$ (through possible PAF-like lipids receptors) activates $\text{G}i_1$ proteins, leading to activation of secreted PLA$_2$ (sPLA$_2$, for PAF) and cytosolic PLA$_2$ (cPLA$_2$, for H$_2$O$_2$) that mediate the hydrolysis of arachidonic acid to produce prostaglandins. Prostaglandins act on their membrane receptors to induced muscle cell contraction.

**DISCUSSION**

The present studies show that ROS (H$_2$O$_2$) stimulates PGE$_2$ synthesis in gallbladder muscle by increasing the formation of PAF-like lipids acting on receptor subtypes that couple to $\text{G}q/11$, which activates cPLA$_2$.
This appears to be a different receptor subtype from those stimulated by PAF, because its receptors are not protected by PAF-like lipids and activate a different signal transduction pathway, which although it couples to Gq/11, it includes sPLA2 and subsequent stimulation of COX and lipoxygenase.

Acute inflammatory processes affect the gallbladder muscle cells and impair the contraction induced by receptor-dependent agonists CCK and ACh and that of the receptor-independent KCl (25). These muscle cells showed evidence of oxidative stress characterized by increased generation of ROS and lipid peroxidation as well as synthesis of PGE2 and of scavengers of free radicals (25). Increased generation of ROS affect cell functions by oxidizing phospholipids and transmembrane proteins. ROS may also increase the formation of PGE2, they may stimulate the synthesis of scavengers of free radicals (27). However, the mechanisms by which ROS stimulate PGE2 synthesis resulting in muscle contraction and increase scavengers of free radicals are unknown. PAF and PAF-like lipids have been suggested as potential candidates to mediate these cell responses.

PAF is a potent phospholipid that is known to mediate several biological actions. PAF stimulates various effector functions in eosinophils, such as degranulation, production of superoxide anion and leukotriene C4, and formation of lipid bodies (7). A specific receptor for PAF has been identified in a number of cell types (7). Human and guinea pig PAF receptors are single polypeptides composed of 342 amino acids (6) present as seven transmembrane domains that couple to G proteins. PAF-like lipids are oxidized phospholipids induced by ROS by nonenzymatic mechanisms whose actions are blocked by specific PAF antagonists.

Although a single receptor is currently thought to mediate their effects (1), our data suggest that the actions of PAF and PAF-like lipids are linked to two distinct pathways although the specific PAF receptor antagonist blocked the muscle contraction and increased PGE2 levels induced by both PAF and PAF-like lipids induced by H2O2. This conclusion is supported by the observations made from studies with PLA2 inhibitors and with receptor protection studies against the actions of the nonspecific receptor inactivator NEM. PAF-like lipids induced by H2O2 stimulate cytosolic PLA2, because the specific inhibitor AACOCF3 blocks their actions. This enzyme releases arachidonic acid that seems to be exclusively hydrolyzed by cyclooxygenase, resulting in the formation of prostaglandins, particularly PGE2, because their actions are almost completely antagonized by indomethacin. PGE2 causes gallbladder muscle contraction by acting on EP1 receptors (unpublished observations) and stimulates the formation of catalase and SOD (26). In contrast, the actions of PAF are antagonized by sPLA2-specific blocker MJ33 and unaffected by the cPLA2 inhibitor. Moreover, the PAF-induced contraction was only partially blocked by either COX or lipoxygenase antagonists and was only completely inhibited by their combination.

These results suggest the existence of two receptor subtypes, one activated by PAF and the other by PAF-like lipids, an assumption that is supported by studies with NEM. NEM inactivates all free transmembrane receptors (5). Receptors can be protected against the actions of NEM if they are occupied by their specific ligands. PAF-like lipids generated by the actions of PLA2, or H2O2 (through PAF receptors) or H2O2 (through PAF-like lipids receptors, unidentified) activates Goq/11 proteins, leading to activation of secreted PLA2 (sPLA2, for PAF) to likely produce prostaglandins and leukotrienes and cPLA2 (for H2O2) to produce PGs. PGs act on their membrane receptors to induced muscle cell contraction. AA, arachidonic acid.

Fig. 13. Model of the signaling and effector pathways that mediates the actions of PAF and H2O2 in guinea pig gallbladder muscle. Stimulation of muscle cells with PAF (through PAF receptors) or H2O2 (through PAF-like lipids receptors, unidentified) activates Goq/11 proteins, leading to activation of secreted PLA2 (sPLA2, for PAF) to likely produce prostaglandins and leukotrienes and cPLA2 (for H2O2) to produce PGs. PGs act on their membrane receptors to induced muscle cell contraction. AA, arachidonic acid.
the increase in PGE\(_2\) synthesis that results in contraction and formation of scavengers of free radicals (26), because the specific PAF antagonist blocked the muscle contraction and increased PGE\(_2\) induced by both PAF and by H\(_2\)O\(_2\).

Both receptor subtypes, however, couple to the same G protein, because the specific Goq/11 antibody blocks the contraction induced by PAF and PAF-like lipids. This receptor-G protein coupling was also demonstrated by determining the increase in [\(^{35}\)S]GTP\(^\gamma\)/binding to G proteins in response to both agonists. Both PAF and PAF-like lipids increased GTP binding to Goq/11 and to G\(_\beta\)\(_\gamma\). However, after the cells were pretreated with indomethacin, the [\(^{35}\)S]GTP\(^\gamma\)/binding was increased only to Goq/11, suggesting that the increased binding to G\(_\beta\)\(_\gamma\) proteins is due to the generation of lipid peroxides other than PAF-like lipids that stimulate the formation of prostaglandins. Moreover, a similar pathway that includes PKC and MAPK receptors stimulated by prostaglandins (21, 26) and probably by leukotrienes also mediates the muscle contraction induced by PAF and PAF-like receptors.

Activation of different PLA\(_2\)s by the same G protein (Goq/11) cannot be explained at this time, because there are no previous reports on this subject. These findings, however, are supported by two sets of experiments using antagonists. First, the contraction induced by PAF-like lipids is blocked by cPLA\(_2\) antagonist and by COX inhibitors. Second, the contraction induced by PAF is only blocked by sPLA\(_2\) antagonists and by the combined use of cyclooxygenase and lipoxygenase inhibitors. This new finding deserves further investigation. However, it should not be surprising, because a receptor is able to couple to different G proteins either sequentially or simultaneously as Murthy and coworkers (15) have repeatedly shown. It is conceivable that this may be due to the known fact that different receptors may have access to different sets of signaling molecules preassembled in caveolea structures that may include similar G proteins activating different PLAs.

Thus these studies demonstrate that ROS generate PAF-like lipids that appear to act on PAF receptor subtypes and establish the link between lipid peroxidation and PGE\(_2\) generation that contributes to the gallbladder muscle contraction and increased synthesis of scavengers of free radicals.

DISCLOSURES

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REFERENCES


