The contractile action of platelet-activating factor on gallbladder smooth muscle

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Platelet activating factor (PAF) is a biologically active phospholipid initially described for its platelet-aggregating effects and as a product released from basophils (2). PAF is now known to be produced by many inflammatory cells, including leukocytes, platelets, macrophages, and endothelial cells. PAF has been implicated as a mediator of acute inflammatory processes, systemic responses to shock, and allergic reactions (12). PAF has been shown to have proinflammatory actions, including stimulation of eicosanoid formation, superanion production, calcium uptake, and phospholipase $A_2$ activity (16).

PAF has been suggested to be involved in the pathophysiology of various inflammatory disorders of the gastrointestinal tract. PAF produces inflammation and alterations of intestinal motility that are similar to those observed during endotoxic shock (15, 22). PAF is produced in the gallbladder during acute cholecystitis and has been suggested to be a mediator of some of the sequelae of cholecystitis. Some studies suggest that PAF may play a role in the development of acute acalculous cholecystitis following shock (11). PAF infusion produces acute gallbladder inflammation in the cat (12). In addition, endotoxin, specifically lipopolysaccharide, produces acute gallbladder inflammation with increased PAF and prostanoid levels (13).

We have been interested in the gallbladder motility changes of acute cholecystitis (18). In acute acalculous cholecystitis, there is overall gallbladder hypomotility in humans and in animal models (24). The effects of PAF on gallbladder contractility are not known. The aims of this study were twofold: 1) to determine the effect of PAF on gallbladder muscle and 2) to determine the mechanism of this effect.

MATERIALS AND METHODS

Gallbladder muscle strip preparation. Guinea pigs (Ace Animals, Boyertown, PA) weighing 400–450 g were fasted overnight before the study. The gallbladder was removed after the guinea pig had been killed by CO$_2$ asphyxiation. The gallbladders were opened along the longitudinal axis and rinsed with Krebs-bicarbonate buffer (composition in mM: 120 NaCl, 4.6 KCl, 2.5 CaCl$_2$, 1.2 MgCl$_2$, 22 NaHCO$_3$, 1.2 NaH$_2$PO$_4$, and 11.5 glucose, oxygenated with 95% O$_2$-5% CO$_2$, pH 7.4). Gallbladder muscle strips were prepared as previously described (20). Full-thickness strips (2 $\times$ 10 mm) containing mucosa, smooth muscle, and serosa were cut along the longitudinal axis of the gallbladder. The strips were suspended in 10-ml organ baths containing Krebs-bicarbonate buffer (37°C). Muscle tension was measured along the longitudinal axis using an isometric force transducer (FT .03C; Grass Instruments, Quincy, MA). After a 60-min equilibration period, the preparations were stretched until the contractile force to 100 $\mu$M ACh was maximal.

Experimental protocols. For dose-response curves of PAF, increasing concentrations of each peptide were applied over the range of 0.1–10,000 ng/ml. Preliminary experiments revealed that there was a decrease in responsiveness with sequential applications of each muscle strip to PAF despite washing in between (see RESULTS). For this reason, the dose-
response curves for PAF were performed with only the initial dose for each muscle strip.

Submaximal doses of PAF-16 and PAF-18 were used to investigate the contractile mechanism of PAF. For receptor antagonism experiments, the antagonist was administered followed in 7 min by application of the control dose of agonist in the continued presence of the antagonist. For the experiments with pertussis toxin, the procedure of Kowal et al. (14) was used, with incubation of the tissue with pertussis toxin at 200 ng/ml for 3 h. A higher dose of pertussis toxin (1 μg/ml) for 3 h was also used. For the experiments with zero calcium, a procedure we have previously used was employed (19), with switching the Krebs solution surrounding the muscle strip to zero calcium with 0.1 mM EGTA for 4 min before the addition of PAF. The response to PAF in the presence of the antagonist was compared with the response of PAF alone (without the antagonist) in a separate muscle strip from the same gallbladder. ACh was also studied as an agonist to help avoid nonspecific effects of receptor antagonists. We have previously shown that ACh contracts the guinea pig gallbladder muscle by using a combination of intracellular and extracellular calcium (19, 23).

Compounds. PAF-16 (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine, C_{36}H_{54}NO_{7}P, molecular weight 523.7) and PAF-18 (1-O-stearyl-2-acetyl-sn-glycero-3-phosphocholine, C_{38}H_{62}NO_{7}P, molecular weight 551.8) were obtained from Calbiochem-Novabiochem (La Jolla, CA). ACh, atropine, hexamethonium bromide, TTX, nifedipine, BAY K 8644, indomethacin, pyrilamine, and cimetidine were obtained from Sigma Chemical (St. Louis, MO). Thioperamide maleate was obtained from Research Biochemicals International (Natick, MA). The PAF receptor antagonist ginkolide B (BN-52021) (17) was obtained from Biomol Research Laboratories (Plymouth Meeting, PA) as was PAF receptor antagonist CV-3988 (28). Pertussis toxin was obtained from Calbiochem-Novabiochem.

Data analysis. For dose-response curves, the amplitude of each contraction was expressed as the absolute contractile response in grams of tension and was also expressed as a percentage of the maximal muscle strip contractile response to 100 μM ACh under control conditions in normal Krebs solution. For receptor antagonism studies, each preparation after receptor antagonist was compared with a strip without the antagonist from the same guinea pig and expressed as a percentage of this control response in normal Krebs solution.

Data are expressed as means ± SE. ANOVA and Student’s t-test were used to test whether the effects after administration of receptor antagonists or calcium modulators were significantly different from control. A P value of <0.05 was considered statistically significant.

RESULTS

Effect of PAF. PAF-16 and PAF-18 caused dose-dependent contractions of gallbladder muscle strips (see Figs. 1 and 2). PAF-16 was more potent in causing gallbladder contraction than PAF-18, with the threshold dose for causing gallbladder muscle contraction being 1 ng/ml for PAF-16 and 10 ng/ml for PAF-18. Values are means ± SE from 5–16 muscle strips from different animals.

Fig. 1. Tracing of the effects of platelet-activating factor (PAF)-16 (A) and PAF-18 (B) on gallbladder muscle contractility. Each agent caused gallbladder muscle strip contraction. ●, Time of addition of each agent.

Fig. 2. Dose-response curves for effect of PAF-16 and PAF-18 on gallbladder smooth muscle contractility. The amplitude of the PAF contractile response is graphed as the absolute contractile response in grams of tension. PAF-16 and PAF-18 caused dose-dependent contractions of gallbladder muscle strips. PAF-16 was more potent in causing gallbladder contraction than PAF-18, with the threshold dose for causing gallbladder muscle contraction being 1 ng/ml for PAF-16 and 10 ng/ml for PAF-18. Values are means ± SE from 5–16 muscle strips from different animals.
application of 1,000 ng/ml was 55 ± 8% of the first application (P < 0.01; n = 7 muscle strips).

We examined whether PAF can downregulate gallbladder motor function to conventional stimulation by evaluating whether low doses of PAF impair the response to ACh. Prolonged (45 min) incubation of the gallbladder muscle strips in varying concentrations of PAF-16, from 0.1 ng/ml to 10,000 ng/ml, had no effect on the contractile responses to ACh. For example, incubation with 10 ng/ml PAF-16 for 45 min did not alter the contractile response to 5 μM ACh (98 ± 5% of control ACh contractile response; n = 7 muscle strips).

**Effect of receptor antagonists.** Submaximal doses of PAF-16 and PAF-18 were used to investigate the contractile effect of PAF. A PAF-16 dose of 1,000 ng/ml caused 53 ± 10% of the maximal contractile response to 100 μM ACh, and a PAF-18 dose of 1,000 ng/ml caused 19 ± 5% of the maximal contractile response to ACh.

The PAF-16- and PAF-18-induced contractions were slightly decreased by ~20% by either 1 μM TTX or 1 μM atropine; however, the decrease did not reach statistical significance (Table 1). There was no effect of hexamethonium on the PAF-induced contractions.

The PAF receptor antagonist (17) ginkolide B (BN-52021) at 100 μM inhibited the PAF-16 contractile response by 92 ± 8% (P < 0.01) and the PAF-18 contractile response by 65 ± 9% (P < 0.01) (Table 1 and Fig. 3A). Ginkolide B had no effect on the contractile response to 100 μM ACh (4 ± 3% inhibition; P > 0.10), an agent that contracts the gallbladder predominantly by smooth muscle muscarinic receptors (19).

Another PAF receptor antagonist, CV-3988, was also used (28). At 10 μM, CV-3988 dose-dependently inhibited the PAF-induced contractile response. At 10 μM, CV-3988 inhibited the PAF-16 contractile response by 72 ± 9% (P < 0.01; Table 1) and inhibited the PAF-18 contractile response by 44 ± 10% (P < 0.05) but had no effect on the contractile response to 100 μM ACh (5 ± 3% inhibition; P > 0.10).

The PAF-16-induced contraction was reduced by 47 ± 10% (P < 0.05) with the prostaglandin synthase inhibitor indomethacin (Table 1). The PAF-16-induced contraction was not, however, inhibited by histamine receptor antagonists pyrilamine (100 nM; H1 receptor antagonist), cimetidine (10 μM; H2 receptor antagonist), or thioperaimide (10 nM; H3 receptor antagonist).

**Effect of agents regulating calcium availability.** Preventing influx of extracellular calcium by 4-min incubation in a calcium-free Krebs solution with 0.1 mM EGTA solution inhibited the PAF-16 response by 97 ± 3% (P < 0.01) and the PAF-18 response by 87 ± 7% (P < 0.01) (Table 2 and Fig. 3B). We have previously shown that ACh utilizes both intracellular and extracellular calcium for its contractile effect on the guinea pig gallbladder (19). A 4-min incubation in calcium-free/0.1 mM EGTA solution inhibited the 100 μM ACh contraction by only 38 ± 6% (P < 0.01 vs. control and P < 0.01 vs. the inhibition to PAF).

Nifedipine, a dihydropyridine compound that inhibits calcium influx through voltage-dependent calcium channels (26), at 1 μM inhibited the PAF-16 contractile response by 51 ± 7% (P < 0.01) and the PAF-18 contractile response by 53 ± 16%. This dose of nifedipine inhibited the 100 μM ACh contractile response by 43 ± 5% (Table 2).

Ryanoine, a compound that inhibits intracellular calcium mobilization (23), at 30 μM had no effect on the PAF-16 contractile response (113 ± 27% of control; P > 0.10; Table 2 and Fig. 3C). A lower dose of ryanoine (1 μM) similarly had no effect on the PAF-16-induced contractile effect (115 ± 30% of control; P > 0.10). Ryanoine (30 μM) also had no effect on the PAF-18 contractile response, whereas it inhibited the 100 μM ACh contractile response by 43 ± 5% (P < 0.01 vs. control and P < 0.01 vs. the effect on PAF).

BAY K 8644, a dihydropyridine that enhances calcium influx through cell membrane voltage-dependent calcium channels (25), at 100 nM increased the PAF contractile response by 139 ± 48% (P < 0.05) and the PAF-18 response by 268 ± 55% (Table 2).

In other gastrointestinal tissues, PAF causes contraction by activation of PAF receptors linked to G proteins and resultant influx of extracellular calcium (7). Pretreatment of the muscle strips with pertussis toxin (200 ng/ml), the inactivator of inhibitory guanine nucleotide-binding proteins, for 3 h (14) reduced the PAF-16 contractile response by 15 ± 7% (P = 0.05) and reduced the PAF-18 response by 20 ± 8% (P < 0.05; Table 2). A higher dose of pertussis toxin (1 μg/ml) for 3 h reduced the PAF-16 contractile response by 21 ± 8% (P < 0.025).

**DISCUSSION**

Our studies demonstrate that the exogenous administration of PAF causes gallbladder smooth muscle contraction. This contractile effect of PAF is dose dependent. Additional experiments were designed to investigate the mechanism of the PAF-induced contractile effect. Determining the mechanism of the contractile action of PAF by pharmacological techniques, however, has been somewhat difficult to study (28). The contractile effects after exogenous adminis-

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**Table 1. Effects of receptor antagonists on the PAF-16 and PAF-18 contractile responses of guinea pig gallbladder muscle strips**

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>PAF-16 (μM)</th>
<th>PAF-18 (μM)</th>
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<tbody>
<tr>
<td>Ginkolide B</td>
<td>8 ± 8**</td>
<td>35 ± 9**</td>
</tr>
<tr>
<td>CV-3988, 10 μM</td>
<td>28 ± 9**</td>
<td>56 ± 10*</td>
</tr>
<tr>
<td>TTX, 1 μM</td>
<td>78 ± 23</td>
<td>81 ± 18</td>
</tr>
<tr>
<td>Atropine, 1 μM</td>
<td>79 ± 12</td>
<td>72 ± 17</td>
</tr>
<tr>
<td>Hexamethonium, 100 μM</td>
<td>105 ± 33</td>
<td>125 ± 15</td>
</tr>
<tr>
<td>Indomethacin, 10 μM</td>
<td>53 ± 10*</td>
<td>63 ± 18**</td>
</tr>
<tr>
<td>Pyrilamine, 0.1 μM</td>
<td>111 ± 20</td>
<td>ND</td>
</tr>
<tr>
<td>Cimetidine, 10 μM</td>
<td>124 ± 22</td>
<td>ND</td>
</tr>
<tr>
<td>Thioperaimide, 0.1 μM</td>
<td>115 ± 14</td>
<td>ND</td>
</tr>
</tbody>
</table>

Results are expressed as percentage of control (means ± SE) from 4–16 muscle strips. Concentration of platelet-activating factor (PAF)-16 and PAF-18 was 1,000 ng/ml. *P < 0.05 and **P < 0.01; ND, not done.
tration of PAF to a muscle bath often persist after washing for some time. Furthermore, marked tachyphylaxis or desensitization occurs where the tissues once exposed to PAF often became refractory to subsequent challenge with PAF. This has been described in guinea pig ileum (3, 27), rat colon (28), and rat gastric fundus (10). Our preliminary studies showed a similar phenomenon in the gallbladder, with reduced responses upon repeated application of PAF. We circumvented this problem by using the first application of PAF for the control response and compared this to other tissue strips from the same animal treated with antagonists before the first application of PAF. Other investigators have found that tachyphylaxis does not develop with washing with bovine serum albumin (28), which has been reported to trap PAF.

Our studies demonstrate that the contractile effect in the gallbladder appears to largely involve a direct smooth muscle effect. TTX, atropine, or hexamethonium did not significantly affect the contractile responses to PAF. The PAF-induced contraction involves specific PAF receptors because the PAF receptor antagonists ginkolide B and CV-3988 inhibited the PAF contractile response. The inhibitory effect by indomethacin suggests that the PAF contractile effect is mediated through a prostaglandin-mediated mechanism. A similar prostaglandin-mediated effect of PAF has been shown in other tissues (8). In some tissues, PAF causes histamine release (21); however, this was not present in the gallbladder because histamine receptor antagonists did not alter the response in our studies.

Table 2. Effects of modulation of intracellular and extracellular calcium utilization on the PAF-16 and PAF-18 contractile responses of guinea pig gallbladder muscle strips

<table>
<thead>
<tr>
<th></th>
<th>PAF-16</th>
<th>PAF-18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium free/0.1 mM EGTA for 4 min</td>
<td>3 ± 3**</td>
<td>13 ± 7**</td>
</tr>
<tr>
<td>Nifedipine, 1 μM</td>
<td>49 ± 7**</td>
<td>47 ± 16**</td>
</tr>
<tr>
<td>Ryanodine, 30 μM</td>
<td>113 ± 27</td>
<td>88 ± 19</td>
</tr>
<tr>
<td>BAY K 8644, 100 nM</td>
<td>238 ± 48**</td>
<td>368 ± 55**</td>
</tr>
<tr>
<td>Pertussis toxin, 200 ng/ml for 3 h</td>
<td>85 ± 7#</td>
<td>80 ± 8**</td>
</tr>
</tbody>
</table>

Results are expressed as percentage of control (means ± SE) from 6–18 muscle strips. Concentration of PAF-16 and PAF-18 was 1,000 ng/ml. *P < 0.05, **P < 0.01, and #P = 0.05.

PAF has been shown to cause contraction of other gastrointestinal smooth muscles, including guinea pig
ility of pertussis toxin was small, inhibitory effect on the PAF-induced contraction. The inhibitory studies demonstrate that pertussis toxin has an inhibitory mechanism occurs in the gallbladder, since our activation of a pertussis toxin-sensitive G protein (7). A influx into the cell and that this is signaled through the myocytes also suggested that the effect of PAF is mediated through specific receptors located on the muscle itself. Recent studies suggest that there might be different types of PAF receptors (5). We do not have evidence from our studies for different subtypes of PAF receptors.

Our experiments carefully investigated the calcium utilization for the contractile effect of PAF in the guinea pig gallbladder. Gastrointestinal smooth muscle contraction, in general, is initiated by an increase of cytosolic calcium, derived from influx of extracellular calcium and/or release of calcium from intracellular stores, which then activates the contractile proteins actin and myosin. The contribution of these different calcium sources to smooth muscle contraction is dependent on the particular agent stimulating the muscle. In the guinea pig gallbladder, potassium causes gallbladder contraction by influx of extracellular calcium, whereas ACh uses calcium derived from both extracellular calcium influx and release from intracellular calcium stores (19, 23).

Our studies in the gallbladder suggest that the PAF contractile response is largely mediated through the utilization of extracellular calcium influx through voltage-dependent calcium channels because nifedipine and depleting extracellular calcium reduced the contractile effects of PAF. Prior studies in isolated ileal myocytes also suggested that the effect of PAF is mediated through the triggering of extracellular calcium influx into the cell and that this is signaled through the activation of a pertussis toxin-sensitive G protein (7). A similar mechanism occurs in the gallbladder, since our studies demonstrate that pertussis toxin has an inhibitory effect on the PAF-induced contraction. The inhibitory effect of pertussis toxin was small, ~20%; it was surprising that there was not more of an inhibitory effect of pertussis toxin on PAF. It is likely that a longer incubation with pertussis toxin would have reduced the PAF contractile effect. Alternatively, there could be an alternative pathway that does not involve pertussis toxin-sensitive G proteins in the gallbladder.

Neural actions of PAF have been demonstrated in some tissues by recording neuronal electrical activity in vitro directly with intracellular microelectrodes. PAF has been shown to have effects on neurons of the guinea pig small intestine. PAF causes membrane depolarization and increase in firing as well as a presynaptic inhibitory action to suppress release of norepinephrine for sympathetic neurons (29). In our studies, neither TTX nor atropine significantly inhibited the PAF-induced contraction of the gallbladder.

PAF has been implicated as a mediator primarily of acute inflammatory processes, especially in inflammation of the gallbladder and colon (12, 16). In intestinal inflammation, in addition to possibly playing a role in the pathogenesis of the inflammation, PAF has been suggested to mediate the abnormal motor activity that occurs with inflammation. In in vivo studies with dog colon, exogenous infusion of PAF stimulated phasic contractions and giant migrating contractions in control animals, an effect that was enhanced during ileal inflammation (9). This response to PAF was inhibited by atropine, hexamethonium, and TTX, suggesting that presynaptic enteric neurons are needed for the stimulated contractions from PAF (9). This suggests that PAF may be one of the inflammatory response mediators that stimulate giant migrating contractions during ileal inflammation. In the rat, the intestinal motor abnormalities that develop with endotoxin are prevented by the administration of a PAF receptor antagonist (22). In experimental models of colitis, there is a shift in the dose-response curve for PAF of isolated colonic myocytes during inflammation that is reverted with either in vivo administration with PAF antagonist or indomethacin, suggesting desensitization of PAF receptors by endogenous PAF produced during inflammation (8).

Acute acalculous cholecystitis is a condition consisting of acute gallbladder inflammation occurring in the absence of gallstones, characterized clinically by fever, leukocytosis, and right upper quadrant abdominal pain (4). The pathogenesis of this disorder is not fully understood and is probably multifactorial. Biliary stasis and nonocclusive ischemia of the gallbladder have been suggested as major pathogenic factors (1). Impaired gallbladder smooth muscle contractility may also be important. The role of PAF in mediating some of the sequelae of cholecystitis has been under investigation by several investigators, especially in the role of PAF in acute acalculous cholecystitis following shock. Animal studies suggest that PAF infusion produces prosta- glandin formation and acute gallbladder inflammation (12). These abnormalities were reduced with the cyclooxygenase inhibitor indomethacin, suggesting a prostaglandin-mediated mechanism. Studies using common bile duct ligation in rabbits, a model for acute cholecystitis, suggest that PAF stimulates release of PGI2 from inflamed rabbit gallbladder cell cultures (16). Studies have also shown that endotoxin, specifically lipopolysaccharide, produces acute gallbladder inflammation with increased PAF and prostanoid levels (11, 13). These proinflammatory changes in the gallbladder produced by lipopolysaccharide were prevented by indomethacin. However, the inflammatory changes and prostanoid increase were not prevented (and actually slightly increased) by a PAF antagonist alprazolam. This suggests that PAF may not have a mediator role but may have a counterregulatory role in acute cholecystitis. Our studies also raise this possibility. In contrast to the decreased gallbladder contractility in acute and chronic cholecystitis (24), the inflammatory mediator PAF, at least with acute exogenous administration as performed in this study, increases gallbladder contractility. We also found that incubation in PAF did not alter the cholinergic contractile
responses of gallbladder muscle strips. These observations suggest that the gallbladder motor dysfunction during acute cholecystitis may not be mediated through PAF. The role of PAF in acute cholecystitis could be further investigated by inhibiting the effects of endogenous PAF in vivo by administering a PAF receptor antagonist in vivo during evolving acute cholecystitis and determining if gallbladder motor function is preserved. In experimental colitis models, administration of a PAF antagonist in vivo has reduced inflammation and preserved colonic smooth muscle contractility (8).

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