Digestive Diseases and Sciences, Vol. 38, No. 11 (November 1993), pp. 2071–2080

Phospholipase Activity of *Helicobacter pylori* and Its Inhibition by Bismuth Salts

Biochemical and Biophysical Studies

ANNA OTTLECZ, MD, PhD, JIMMY J. ROMERO, BS, STUART L. HAZELL, MD, DAVID Y. GRAHAM, MD, and LENARD M. LICHTENBERGER, PhD

In this study we measured phospholipase A (PLA) and C (PLC) activity of media filtrates and French Press lysates of the gastritis-inducing bacteria Helicobacter pylori. *We report here that both* H. pylori *lysates and filtrates contain PLA_1, PLA_2, and C enzymes, which readily hydrolyze a radiolabeled dipalmitoylphosphatidylcholine (DPPC) and phosphorylcholine substrates, respectively. The specific activity of both PLA and C enzymes were greatest in the 6.5–7.0 and 8.4–8.8 pH ranges, respectively. Colloidal bismuth subcitrate (CBS) induced a dose-dependent inhibition of PLA_2 and C activity of both* H. pylori *lysates and filtrates. This inhibitory effect of CBS on PLA_2 was antagonized in a dose-dependent fashion by the addition of $CaCl_2$ to the incubation mixture, suggesting that calcium and bismuth may be competing for the same site on the enzyme. In contrast, the ability of bismuth salts to inhibit PLC activity of* H. pylori *lysates was not antagonized by $CaCl_2$. Employing a biophysical assay system for surface wettability, it was determined that* H. pylori *lysates had the capacity to remove a synthetic phospholipid monolayer off a glass in a dose-dependent fashion. This ability of the bacterial lysates to catalyze the transformation of a hydrophobic surface to a wettable state was significantly attenuated in the presence of bismuth salts. Our experimental results are, therefore, consistent with the possibility that* H. pylori *colonization compromises the stomach's barrier to acid by eroding a phospholipid lining, possibly a monolayer, on the surface of the gastric mucus gel and that this process is blocked in response to bismuth therapy.*

KEY WORDS: phospholipase; *H. pylori;* hydrophobicity; bismuth; phospholipids; monolayer.

In recent years information has accumulated at a rapid rate in support for the concept that the bacterium *Helicobacter pylori* is an important etiological factor in the development of antral gastritis and is strongly associated with peptic ulcer disease (1–8). However, our understanding is at a rudimentary level concerning the pathogenic mechanism by which *H. pylori* induces mucosal inflammation and injury. Recently attention has focused on investigating proteolytic and lipolytic enzymes elaborated by the bacteria. It has been reported that *H. pylori* releases a protease enzyme that may have the capacity of degrading the glycoprotein constituents of mucin, thus weakening the barrier properties of the mucus gel layer of the stomach (9, 10). Phospholipase enzyme activity has also been measured in

Manuscript received August 5, 1992; revised manuscript received January 11, 1993; accepted January 20, 1993.

From the Department of Physiology and Cell Biology, The University of Texas Medical School at Houston; Department of Medicine, Baylor College of Medicine, and VAMC, Houston, Texas; and School of Microbiology, University of New South Wales, Kinsington, N.S.W. Australia.

Dr. Ottlecz's present address is: College of Optometry, University of Houston, 4901 Calhoun, Houston, Texas 77204.

Address for reprint requests: Lenard M. Lichtenberger, Department of Physiology and Cell Biology, The University of Texas Medical School, P.O. Box 20708, Houston, Texas 77225.

extracellular products of *H. pylori* (11–13). This lipolytic family of enzymes, which are present in many other pathogenic microorganisms, may act either directly on the cellular membranes of the gastric epithelium or on the lipidic constituents of the extracellular mucus gel layer, which provides the underlying tissue with a protective hydrophobic luminal lining. In previous studies we have obtained biochemical, biophysical, and ultrastructural evidence that are consistent with the possibility that the nonwettable lining of the stomach may be attributable to the presence of a phospholipid monolayer adsorbed to the luminal surface of the mucus gel layer (14–16). In the present study we confirmed and extended earlier reports (11–13) on the presence of phospholipase activity in lysates and media filtrates of *H. pylori.* New information is also presented on both the ability of these enzymes to remove a preformed phospholipid monolayer from a surface and the inhibitory mode of action of bismuth salts on these enzymes as demonstrated with both biochemical and biophysical assay systems.

MATERIALS AND METHODS

Chemicals. L-α-Dipalmitoyl-(2[1-^{14}C]palmitoyl)phosphatidylcholine (50–55 mCi/mmol) and L-α-dipalmitoyl-(1,2[1-^{14}C]palmitoyl)phosphatidylcholine (110 mCi/mmol) were purchased from New England Nuclear, Boston, Massachusetts. The radiochemical purity of the labeled lecithin was greater than 98% as determined by thin-layer chromatography.

Dipalmitoylphosphatidylcholine (DPPC), phospholipase A_2 of *Naja naja* venom, phospholipase C of *Cl. perfringens,* *p*-chloromercuribenzoic acid sodium salt (PCMB), *p*-nitrophenylphosphorylcholine (PNP-PC), phenylmethylsulfonylfluoride (PMSF), sodium fluoride, and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, Missouri. 3-Isobutyl-1-methyl-xanthine was purchased from Aldrich, Milwaukee, Wisconsin. *N*-Tosyl-L-phenylalaninechloromethyl ketone (TPCK) was a product of Boehringer Mannheim Biochemicals, Indianapolis, Indiana. All other chemicals and solvents were analytical reagent grade. Colloidal bismuth subcitrate (CBS) or DeNol was kindly provided by Dr. Neville Yeomans, Department of Medicine, University of Melbourne, Australia.

Procedure of *H. pylori* Lysate and Filtrate. Strain 8826 of *H. pylori* was kindly donated by Dr. D.G. Evans, VAMC, Houston and Baylor College of Medicine, Houston, Texas. The bacteria were cultured either on 7% (v/v) blood–agar plates (17) (Difco) or in suspension (18) at 37° C in a microaerophilic atmosphere of 10% CO_2 in air and 99% relative humidity for two to three days. The bacterial outgrowths appeared as curved-shaped rods with a maximal length of 0.5–1.0 mm under high power (1000×) light microscopy. Further, they were Gram-negative and urease-, oxidase-, and catalase-positive, all documented characteristics of *H. pylori.* The plates with the bacterial colonies were repeatedly washed with 0.9% NaCl (1 ml/plate) and the obtained solution was centrifuged at 15,000 *g* for 10 min at 4° C. The supernatant was filtered through a Nalgene sterilization filter (0.2 μm), dialyzed against distilled water at 4° C, and lyophilized. The resulting powder was dissolved in distilled water, resulting in a 20× concentrated sample that was aliquoted and stored frozen (−80° C) until enzyme determination. This fraction is referred to as the media filtrate of *H. pylori.* The resultant *H. pylori* pellet was resuspended in 0.9% NaCl (0.5 ml/plate) and thereafter disrupted at a pressure of 1000 lb/cm^2 in a French Press. This cell homogenate was centrifuged at 15,000 *g* for 15 min at 4° C and the supernatant (referred to as the lysate) after filtering (Nalgene, 0.2 μm) was aliquoted and stored frozen (−80° C).

Phospholipase A Assay. The phospholipase A activity of *H. pylori* lysate of media filtrate was determined by following the release of 1-^{14}C-labeled palmitic acid from a labeled DPPC substrate employing a modification of the method of Shakir (19). In these studies isotopic DPPC was labeled either with [1-^{14}C]palmitate at the sn-2 position only (for PLA_2 activity) or both the sn-1 and sn-2 positions (total PLA activity = PLA_1 + PLA_2 activity). Briefly, labeled (0.4 μCi) and unlabeled DPPC (7.5 mg) were dissolved in 2 ml of chloroform–methanol (2:1). The solvent was then removed under a nitrogen stream and the tubes were placed in a vacuum overnight. The substrate was resuspended in the incubation mixture containing 0.5% Triton X-100 (v/v) and subjected to 2–3 min of sonication (bath-type sonicator, Laboratory Supplies Co., Inc. Hicksville, New York). The standard incubation mixture contained 200 μl of Tris-maleate (0.2 M, pH 4–6) or Tris-HCl (0.2 M, pH 7–9) buffer containing 2×10^{-3} M $CaCl_2$ and 2×10^{-3} M sodium deoxycholate, 50 μl of PLA enzyme, 50 μl of substrate (0.25 μmol about 30,000 cpm) in a final volume of 400 μl by additional 0.9% NaCl. When *H. pylori* lysate or filtrate was used as enzyme source (300–400 μg protein), it was protected by serine protease inhibitors [8 μl of PMSF (50 mM) and TPCK (25 mM), and 40 μl of PCMB (4–40 nmol) was added to the assay mixture to inhibit acyltransferase activity according to Kröner et al (20).

The assay was started by the addition of substrate followed by 10 min of sonication. The incubation was performed in a shaking waterbath at 37° C for a designated period of time (40 min for *Naja naja* venom and 24 hr for *H. pylori* PLA activity). The procedure of fatty acid extraction was carried out as previously described (19). Tubes with the incubation mixture plus 40 μl of EDTA (100 mM) were included in each experiment in order to calculate the PLA enzyme activity, which was expressed as nanomoles of labeled free fatty acid released per hour per milligram of protein.

Phospholipase C Assay. The assay is based on the hydrolysis of the water-soluble substrate, *p*-nitrophenylphosphorylcholine (PNP-PC), with the release of the chromogen, *p*-nitrophenol (PNP) as described by Kurioka and Matsuda (21). The incubation mixture (700 μl) contained 20 mM PNP-PC as substrate, 1 mM $ZnCl_2$ as

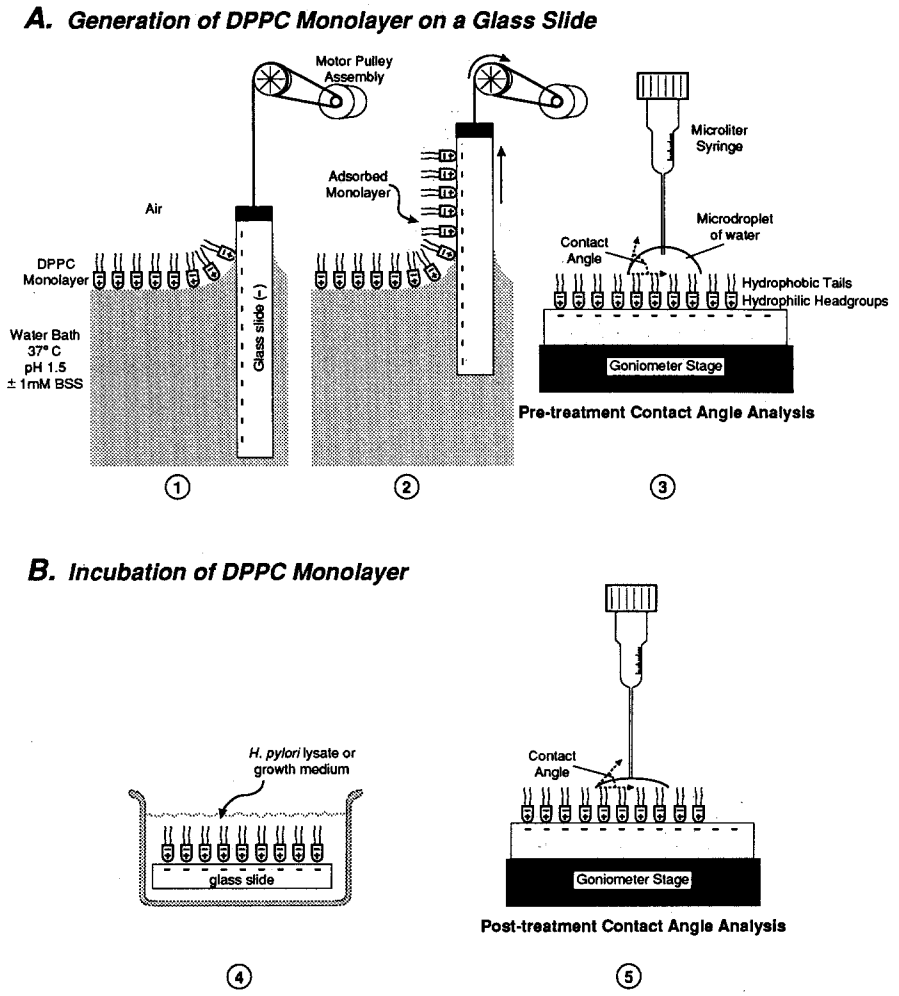

Fig 1. Schematic representation of the methods employed to: (A) transfer DPPC monolayer from the air–liquid interface of water contained in a Langmuir trough to a glass slide carrying a negative surface charge as confirmed by contact angle analysis; and (B) to assess the ability of *H. pylori* lysates to remove the hydrophobic DPPC monolayer by *in vitro* incubation followed by contact angle analysis.

cofactor, 10 mM NaF as phosphatase inhibitor, 60% glycerol in Tris-HCl buffer (0.25 M, pH 7.2) and 1 unit of PLC enzyme (20 μl) of *Cl. perfringens*. When *H. pylori* lysates of the washed samples were tested as enzyme source (50 μl, 300–400 μg protein) $ZnCl_2$ was generally omitted from the incubation mixture, but 14 μl of PMSF (50 mM) and TPCK (25 mM) were applied as protease inhibitors. The tubes were incubated at 37° C for 60–120 min. The reaction was stopped by addition of 70 μl of EDTA (1×10^{-1} M), and the yellow color reaction, indicative of the presence of PNP, was read in a spectrophotometer at 405 nm. Tubes with incubation mixture plus 70 μl of EDTA (1 mM) were also included in each experiment to calculate the PLC enzyme activity as nanomoles per hour per milligram of protein.

Protein Determination. Protein was determined using the Bradford method (Bio-Rad) employing bovine serum albumin (A grade, Calbiochem) as standard.

Biophysical Assay System for Phospholipase Activity. An assay system was developed to investigate the ability of phospholipase enzymes to remove a phospholipid monolayer from an inert surface, as determined by contact angle analysis with the use of a goniometer (see Figure 1 for schematic representation.) A monolayer of DPPC was initially transferred from an air–liquid interface of water contained in a Langmuir trough to a negatively charged surface of an acid-cleaned glass slide in accordance to a technique described elsewhere (22), which was modified from the original monolayer transfer method of Blodgett (23) and Gaines (24). The successful transfer of the DPPC monolayer to the acid-cleaned glass slide carrying a negative surface charge was confirmed by contact angle analysis, as the contact angle increased from <15° before transfer to >50° after monolayer transfer. The slides containing an adsorbed monolayer of DPPC were then incubated for varying periods of time (0–4 hr) in 0.1M Tris buffer (pH 7.4) containing 2–5 mM $CaCl_2$ either alone or in the presence of *H. pylori* lysates or filtrates. In

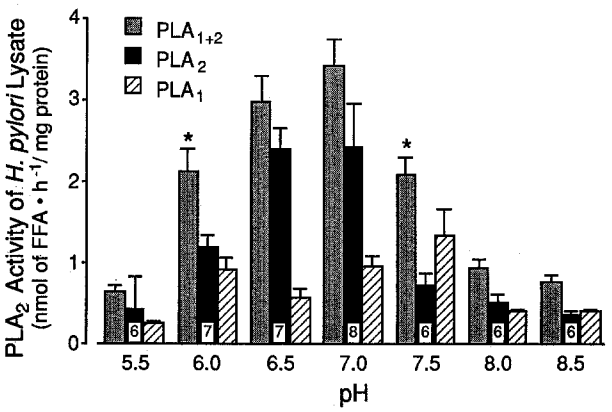

Fig 2. Phospholipase A activity of *H. pylori* lysate after 24-hr incubation with 2[1-^{14}C]DPPC (filled bars = PLA_2) or with 1,2[1-^{14}C]DPPC (shaded bars = PLA_{1+2}) at various pH values. PLA_1 was computed as the difference between $PLA_{1,2}$ and PLA_2 and is represented by the hatched bars. Vertical lines represent SE of the mean for seven separate determinations. *$P < 0.001$ versus the PLA_2 activity at the same pH.

certain experiments bismuth subsalicylate (BSS) at a concentration of 1 mM (kindly provided by Dr. Donna Morgan Murray of Procter and Gamble Co.) was added to acidified water (pH 1.5) contained in a Langmuir Trough prior to monolayer transfer. Use of BSS in those studies was necessitated by our limited supply of CBS. At the completion of the incubation period, the slides were returned to the stage of the goniometer for a postincubation contact angle reading.

RESULTS

PLA Activity of *H. pylori*. Figure 2 demonstrates that the maximal PLA_2 activity of *H. pylori* lysates was found at a slightly acidic pH range (6.5–7.0 pH), showing a sharp decrease at pH values of 7.5 or higher. We then measured total PLA activity using the double-labeled substrate 1,2[1-^{14}C]DPPC, which had a pH-dependent activity curve similar to that of PLA_2, reaching an optimum at a pH of 7.0. From these data, one can then assess PLA_1 activity indirectly by computing the difference between the $PLA_{1,2}$ and PLA_2 curves (see shaded bars). In this case PLA_1 displayed a wider pH range, where the optimal activity recorded encompassed pH values between 6 and 7.5 (Figure 2).

When parallel studies were performed on PLA activity released into media filtrates of *H. pylori*, it was determined that PLA_2, $PLA_{1,2}$, and PLA activity all displayed pH optima at or near neutral pH (data not shown).

PLC Activity of *H. pylori*. When $ZnCl_2$ was added to the incubation mixture at 1 mM as a necessary cofactor, as recommended in the original paper of Kurioka and Matsuda, a marginal PLC activity (0.58 nmol PNP released/hr/mg protein) was detected in *H. pylori* lysates (Table 1). However, as shown in Table 1 reduction of the $ZnCl_2$ induced a clear dose-dependent enhancement in enzyme activity, which was increased 100-fold in the absence of the metal. In contrast, removal of $ZnCl_2$ had little effect on the activity of commercially available PLC from *Cl. perfringens*. The PLC enzyme present in media filtrates of *H. pylori* also was inhibited by Zn^{2+}, and in the absence of metal, released 21.2 nmol PNP/hr/mg protein into the incubation buffer.

TABLE 1. PHOSPHOLIPASE C ACTIVITY* OF *H. pylori* LYSATES: EFFECT OF $ZnCl_2$

Conc of $ZnCl_2$ (M) in incubation buffer	*PNP released (nmol × hr^{-1}/mg protein)*	*Inhibition (%)*
0.0	51.82 + 2.90	0.0
1.0×10^{-4}	41.61 + 0.25†	19.7
2.0×10^{-4}	26.19 + 0.24†	49.4
1.0×10^{-3}	0.58 + 0.30†	98.8

**H. pylori* lysates (50 μl, 374 μg protein) were preincubated with 10^{-4} and 10^{-3} M $ZnCl_2$ in buffer solution as described in Materials and Methods for 10 min at room temperature, prior to the addition of PNP-PC substrate (30 μmol) and then incubated for 60 min at 37° C. Data represent mean ± SE for six different determination.

†Significant difference at $P < 0.001$ in comparison to PLC activity of lysates incubated in the absence of Zn.

A number of experiments were designed to study the pH dependency of the PLC enzyme of *H. pylori* lysates. The incubations were performed for 1 hr without the presence of $ZnCl_2$ in the assay mixture. By increasing the pH of the assay buffer, a parallel increase of optical density was measured, with maximum activity recorded at a pH range of 8.6–8.8, where the specific activities were found to be 107.4–111.0 nmol PNP released/hr/mg protein. It also should be noted that in contrast to our findings on PLA activity, only trace amounts of PLC were detected in the media filtrates.

Effect of CBS on PLA_2 Activity. The effect of CBS (DeNol), an effective antiulcer drug, on PLA_2 activity was determined by preincubating the enzyme preparation with various doses of the drug ($2–8 \times 10^{-4}$ M) in the incubation buffer (adjusted to pH 7) prior to the addition of substrate. Figure 3A and B demonstrate that CBS induced a clear dose-dependent inhibition in the PLA_2 activity of both the *H. pylori* lysate (A) and filtrate (B) with a significant 60–80% reduction in enzyme activity being recorded at a bismuth concentration of 0.8 mM.

Figure 4 demonstrates that a very similar and somewhat more pronounced inhibitory effect of

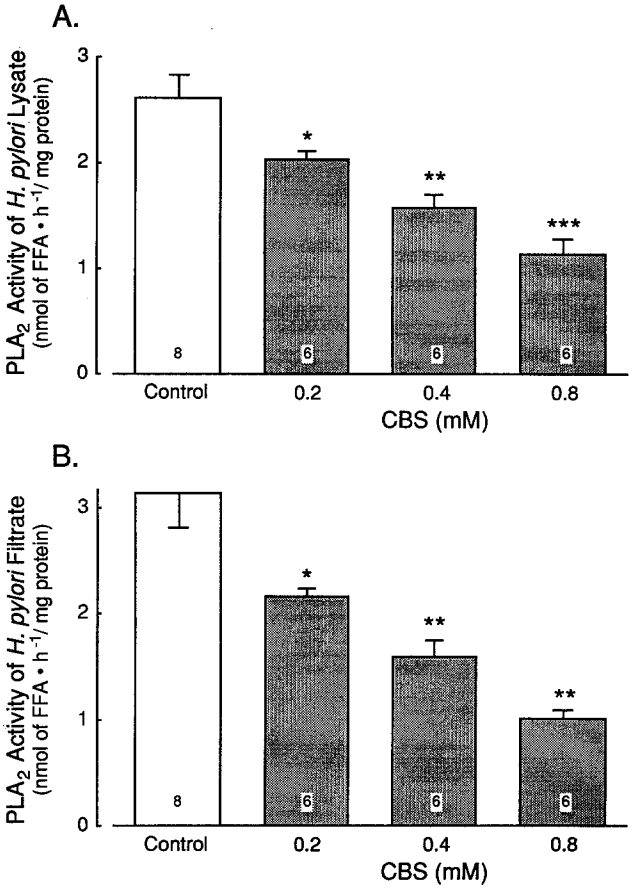

Fig 3. Effect of colloidal bismuth subcitrate (CBS) on the phospholipase A_2 activity of *H. pylori* lysate (A) or filtrate (B) after 24-hr incubation with 2[1-^{14}]DPPC at pH 7.0. Open bar = control, shaded bars = CBS. (A) Vertical lines represents SE of the mean for eight separate determinations. Significant differences versus control: $*P < 0.04$; $**P < 0.002$; $***P < 0.0001$. (B) Vertical lines represent SE of the mean for six separate determinations. Significant differences versus control: $*P < 0.002$; $**P < 0.0001$.

CBS was recorded when the commercially available enzyme, purified from *Naja naja* venom was added to the incubation mixture (adjusted to its pH optimum of 9.0). It should be noted that due to the higher specific activity of the enzyme, the PLA_2 was diluted to an activity of 500–250 ng/test tube.

Addition of $CaCl_2$ to the normal incubation mixture containing either *H. pylori* lysate or the purified *Naja naja* PLA_2 in the presence of 0.8 mM CBS induced a dose-dependent recovery of PLA activity, so that enzyme activity was almost completely restored (>90% of control) at a $CaCl_2$ concentration of 4 mM (Figure 5A and B).

Effect of CBS on PLC Activity. Preincubation of *H. pylori* lysates with different doses of CBS (2–12 mM) for 10 min prior to the addition of PNP-PC substrate for 10 min similarly induced a dose-

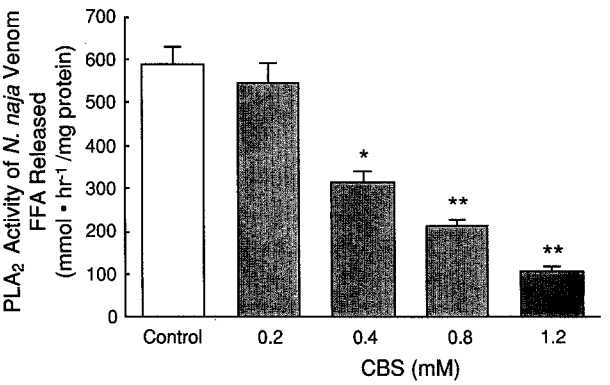

Fig 4. Effect of CBS on the phospholipase A_2 activity of *Naja naja* venom (250 ng) after 40-min incubation with 2[1-^{14}C]DPPC at pH 9.0. Open bar-control, shaded bars = CBS. Vertical lines represent SE of the mean for eight separate determinations. $*P <$ 02.01; $**P < 0.0001$.

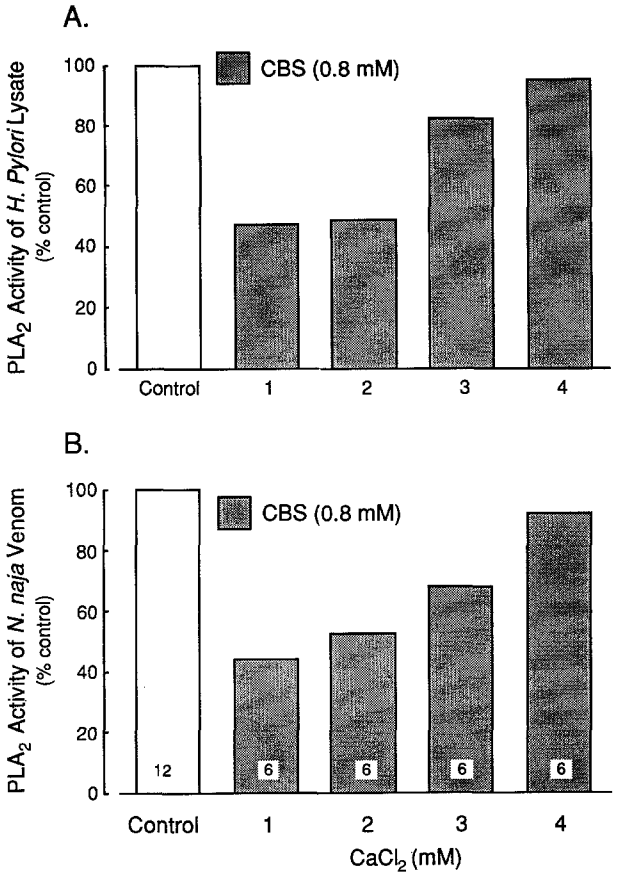

Fig 5. $CaCl_2$ antagonizes the inhibitory effect of CBS (0.8 mM) on the phospholipase A_2 activity of (A) *H. pylori* lysate incubated for 24 hr at pH 7.0, and (B) *Naja naja* venom (250 ng) incubated for 40 min at pH 9.0 with 2[1-^{14}C]DPPC. Open bar = 0.5 mU PLA_2 enzyme, shaded bars = CBS 0.8 mM CBS at different doses of $CaCl_2$ (1–4 × 10^{-3} M). Changes are expressed in the percent of the control.

dependent inhibition of PLC activity (Figure 6). In contrast to the former situation with PLA_2, further

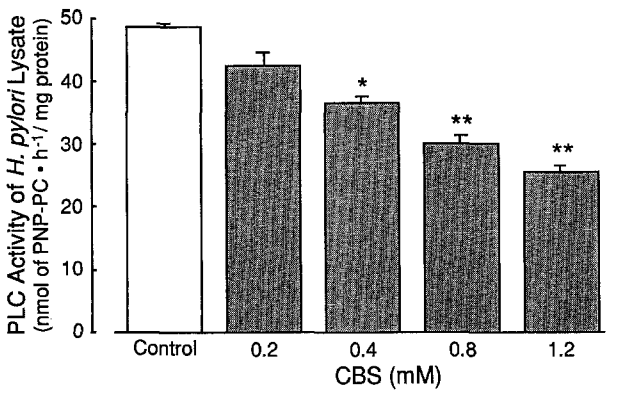

Fig 6. Effect of CBS on the phospholipase C activity of *H. pylori* lysate after 1-hr incubation at pH 7.2. Vertical lines represent SE of the mean for six separate determinations. *$P < 0.002$; **$P < 0.0001$ versus control.

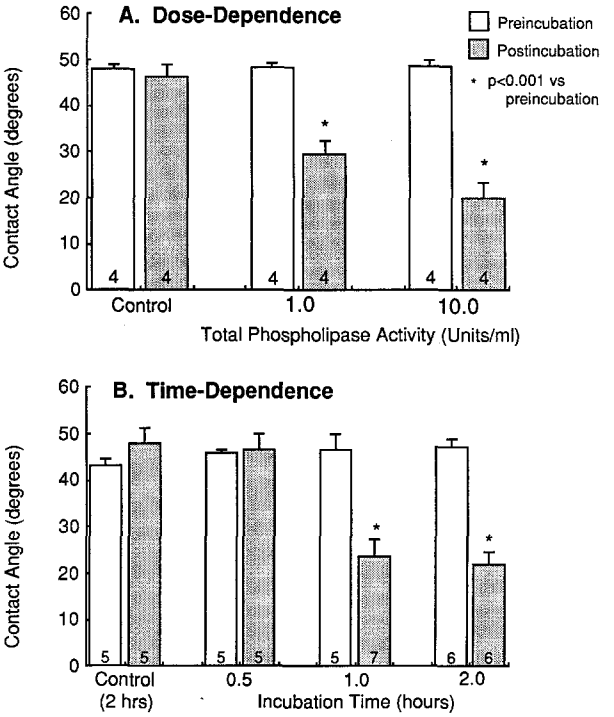

Fig 7. Dose- and time-dependent ability of purified phospholipase enzymes to remove surface DPPC monolayer from glass slides as determined by contact angle analysis. In the experiments described in panel A, the DPPC-coated slides were incubated in 0.1 M Tris buffer containing 2–5 mM $CaCl_2$ in the presence and absence of equivalent units of purified phospholipase A_2 (*Naja naja*)-C (*Clostridium perfringens*) activity at 1 unit:1 unit ratio for a 2-hr incubation period at 37° C. In panel B, DPPC-coated slides were incubated in calcium-containing Tris buffer containing 10 units/ml of total phospholipase enzymes (5 units of phospholipase A_2 and 5 units of phospholipase C) for 0.5–2 hr. Control slides were incubated in buffer in the absence of the purified enzymes. In this and the subsequent figure, numbers at the base of the bars represent the numbers of slides tested. *Statistically significant reduction of the hydrophobicity of the glass slide in comparison to the preincubation values.

supplementation of the incubation mixture with $CaCl_2$ failed to antagonize the inhibitory action of the bismuth salt. This inhibitory effect of CBS on PLC activity was only observed when the *H. pylori* enzyme was assayed in the absence of $ZnCl_2$.

Ability of Purified Phospholipase Enzymes and *H. pylori* Lysates to Remove DPPC Monolayers from Inert Surfaces. Figure 7A and B demonstrate that a synthetic mixture of purified phospholipase A_2 and C had the capacity to remove a DPPC monolayer from a glass surface in both a time- and dose-dependent manner, as the contact angles decreased from >50° to <25° after an incubation period of 2 hr or longer. It is important to note that the DPPC monolayer remained firmly adsorbed to the glass slide when incubated in the buffer in the absence of

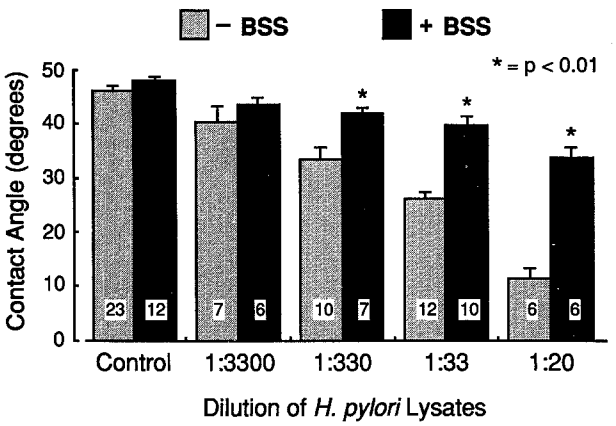

Fig 8. Dose-dependent ability of *H. pylori* French Press lysates (protein concentration 5–6 mg/ml) to remove DPPC monolayer from glass slides generated in the absence and presence of bismuth salts, during a 2-hr incubation period in Tris buffer. *The presence of a statistically significant intergroup difference (−BSS vs +BSS) at the various lysate concentrations tested. Incubation of the artificial DPPC monolayers in buffer containing *H. pylori* lysates (at all titers tested ±BSS) induced a statistically significant reduction in the hydrophobicity of the lipid-coated glass slides in comparison to the contact angle values of monolayers incubated for 2 hr in control buffer.

phospholipase enzymes (see posttreatment controls). Figure 8 clearly demonstrates that the addition of *H. pylori* lysates to the incubation buffer similarly induced a dose-dependent reduction in the contact angle of the DPPC-coated slides. These studies, therefore, provide biophysical evidence that *H. pylori* lysates contain phospholipase activity that is capable of eroding a phospholipid monolayer. It also can be appreciated that the ability of purified and *H. pylori* phospholipase activity to remove a DPPC monolayer from a glass slide was clearly reduced if the monolayer was generated and transferred in the presence of bismuth salts (Figure 8, black bars).

DISCUSSION

It is now well established that >90% of duodenal ulcer patients and ~60% of gastric ulcer patients are infected with the bacterium *H. pylori,* with the highest incidence of colonization occurring in the gastric antrum, where the inflammatory changes are most severe (2, 25). More direct evidence on the role of the bacteria in the etiology of peptic ulcer disease comes from several clinical reports on the onset of gastritis and the development of symptoms in individuals who either intentionally infected themselves with *H. pylori* or in documented cases of accidental infection of subjects due to the improper sterilization of endoscopic equipment (16). Further, inoculation of *H. pylori* into gnotobiotic pigs has been reported to induce gastritis several weeks after infection (26). It has also been reported that placement of patients with active duodenal ulcer disease and *H. pylori* infection on bismuth and antibiotic therapy results in both an eradication of the infection and a reduction in the incidence of recurrence (27–30).

Although the evidence is building that *H. pylori* may be a major etiological factor in the development of antral gastritis and peptic ulcer disease, the pathogenic mechanism involved is only beginning to be investigated. Hazell and Lee (17) proposed that the urease enzyme, elaborated by *H. pylori,* is required to provide the bacteria with urea nitrogen and may influence the development of ulcers. Evidence also supports the possibility that ammonia, the major product formed by the urease-catalyzed hydrolysis of urea, may induce gastric mucosal injury directly, as has been demonstrated in rats in response to either the acute exposure to combinations of urease and urea or chronic administration of ammonium salts (31, 32). Alternatively ammonia may promote the colonization of *H. pylori* in the mucus gel layer, where the bacteria lives, by raising the pH of its microenvironment towards neutrality. This concept has been supported by recent *in vitro* findings of Marshall et al (33) that, unlike bacterial strains that are urease-negative, *H. pylori* is protected from low pH (<2.0) if urea is added to the culture medium.

In addition to the possible injurious role of NH_3, generated by bacterial urease, evidence has also been obtained that *H. pylori* elaborates several other factors that may be cytotoxic to the gastric mucosa. In this regard Leunk et al (4) have identified a protein released by *H. pylori* into culture medium that they have called cytotoxin, which induces the vacuolization of eukaryotic culture cells. Slomiany and coworkers have also characterized protease, lipase, and phospholipase activity in culture broth of *H. pylori* that may have pathogenic potential (9, 10, 13). The characteristics and cofactor requirements of these enzymes, however, were not described in detail by these workers, although they made the interesting observation that CBS had an inhibitory effect on both lipolytic enzymes.

In the present study we focused our attention on characterizing the phospholipase enzymes of *H. pylori* and the effect of pH and stimulatory and inhibitory cofactors on this family of lipolytic enzymes. This interest was sparked by our earlier

observations that extracellular phospholipids associated with the mucus gel layer may generate a hydrophobic barrier to protect the gastric epithelium from luminal acid (14–16, 34). Furthermore, it has been reported that the hydrophobic surface characteristics of human gastric biopsy tissue are attenuated in individuals infected with *H. pylori* and restored after these subjects are treated with CBS to eradicate the infection (35, 36). These findings, therefore, suggest that *H. pylori* elaborates one or more phospholipase enzymes that may induce gastritis by compromising either this putative extracellular hydrophobic lining of the stomach or the plasma membrane of gastric epithelial cells.

Results outlined in this paper both confirmed and extended the earlier findings of Slomiany et al (13) by demonstrating that *H. pylori* lysates and filtrates had detectable PLA_1, PLA_2, and PLC activity. We also observed that the latter enzyme had the greatest activity in the bacterial lysates, was found in trace amounts in the media filtrates, and, contrary to other PLC enzymes, appeared to be inhibited by Zn^{2+}. Because of this unexpected Zn^{2+}-sensitivity, we considered the possibility that the recorded activity may be attributable to a ubiquitous nucleotide phosphodiesterase enzyme that may nonspecifically hydrolyze our water-soluble substrate. Contrary to this possibility, the activity of *H. pylori* lysates to hydrolyze PNP-PC was unaffected in the presence of isomethylbutylxanthine (1 mM), a well-established inhibitor of this type of phosphodiesterase enzyme (unpublished findings). We also found it of interest that the specific activities of both PLA_1 and PLA_2 in the media filtrate were comparable or greater than that measured in the lysate. This finding, which suggests an extracellular role for the enzyme, was not surprising since extracellular phospholipases have been previously demonstrated to be involved in microbial pathogenicity (37, 38). We also confirmed earlier reports that bismuth salts, commonly prescribed in the treatment of *H. pylori*-induced gastritis, inhibits PLA_2 activity (13), and our evidence suggests that it does so by competing with Ca^{2+} for a stimulatory site on the enzyme. CBS also inhibited PLC activity by a different mechanism, as the inhibitory activity was not antagonized by calcium.

Another potential source of PLA enzymes are the polymorphonuclear leukocytes (PMNs) that are recruited to sites of gastric injury during the inflammatory process and occasionally appear in the luminal space. Indeed, it is well documented that neutrophils and PMNs have both a membrane-bound and soluble/lysosomal PLA_2, the latter of which has an absolute requirement for divalent cations (39, 40). However, it is most likely that if the PLA_2 and proteolytic enzymes of PMNs play a role in ulcer formation, it would be by aggravating the primary *H. pylori*-induced mucosal injury as the inflammatory process progresses.

These observations therefore suggest that *H. pylori* both directly and indirectly (via PMNs) release phospholipase enzymes, which may result in cell injury by either catalyzing the hydrolysis of the plasma membrane of gastric epithelial cells or the breakdown of hydrophobic elements within or on the surface of the mucus gel layer. We have earlier proposed and provided morphological and biophysical evidence consistent with the possibility that these hydrophobic elements may be oriented as a phospholipid monolayer at the interface of the gastric mucus gel layer and the bulk luminal solution to provide a nonwettable high-energy barrier against the penetration of luminal acid (14–16). It is conceivable that one of the targets of the lipolytic enzymes elaborated by *H. pylori* is this putative phospholipid lining. It is also possible that extracellular phospholipids may be present throughout the mucus gel layer, as suggested by Slomiany and Slomiany (41) to provide mucus with acid-resistant hydrophobic properties. Hydrolysis of these extracellular hydrophobic phospholipids, as appears to occur with *H. pylori* infection (35, 36), would make the mucus gel and the underlying tissue more sensitive to luminal acid, which may be the initiating event in the development of gastritis. In support of the concept that a phospholipid monolayer may be the site of phospholipase attack we have applied a biophysical assay system employing contact angle analysis to demonstrate that *H. pylori* lysates have the capability of removing a DPPC monolayer from a glass slide. Further, consistent with our biochemical findings, this ability of the lysates to catalyze the transition of a phospholipid-coated surface from a nonwettable to a wettable state was clearly attenuated if bismuth salts had been incorporated into the artificial phospholipid lining at the time of monolayer transfer. Future studies are planned to purify the phospholipase enzymes of *H. pylori* and to compare their avidity for cellular and extracellular phospholipids.

ACKNOWLEDGMENTS

We would like to thank Ms. Grace Belcher for typing the manuscript, and Dr. D.G. Evans, VAMC, and Baylor College of Medicine, Houston for providing *H. pylori* strain 8826 which was used to inoculate our culture dishes. This work was supported by NIH grants DK 33239 and DK 39919.

REFERENCES

1. Andersen LP, Holck S, Poulsen CO, Elsborg L, Justensen T: *Campylobacter pyloridis* in peptic ulcer disease. I. Gastric and duodenal infection caused by *C. pyloridis* histopathologic and microbiologic findings. Scand J Gastroenterol 22:219–224, 1987
2. Buck GE, Gourely EG, Lee WK, Subramanyam K, Latimer JM, Di Nuzzo AR: Relation of *Campylobacter pyloridis* to gastritis and peptic ulcer. J Infect Dis 153:664–669, 1986
3. Jones DM, Lessels AM, Eldridge J: *Campylobacter*-like organisms on the gastric mucosa: Culture, histological, and serological studies. Clin Pathol 37:1002–1006, 1984
4. Leunk RD, Johnson PT, David BC, Kraft WG, Morgan DR: Cytotoxic activity in broth-culture, filtrates of *Campylobacter pylori.* J Med Microbiol 26:93–99, 1988
5. Marshall BJ, Warren JR: Unidentified curved bacilli in the stomachs of patients with gastritis and peptic ulceration. Lancet 1:1311–1315, 1984
6. Marshall BJ: The *Campylobacter pylori* story. Scand J Gastroenterol 23(suppl):58–66, 1988
7. Price AB, Levi J, Dolby JM, Dunscombe PL, Smith A, Clark J, Stephenson ML: *Campylobacter pyloridis* in peptic ulcer disease: Microbiology, pathology, and scanning electron microscopy. Gut 26:1183–1188, 1985
8. Warren JR: Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet 1:1273–1275, 1983
9. Sarosiek J, Slomiany BL: Evidence for weakening of gastric mucus integrity by *Campylobacter pylori.* Scand J Gastroenterol 23:585–590, 1988
10. Slomiany BL, Bilski J, Sarosiek J, Murty VLN, Dworkin B, VanHorn K, Zielenski J, Slomiany A: *Campylobacter pyloridis* degrades mucin and undermines gastric mucosal integrity. Biochem Biophys Res Commun 144:397–314, 1987
11. Czinn S, Carr H, Yang P: Demonstration of a soluble phosphodiesterase, phospholipase C (PLC) produced by *Helicobacter pylori:* A potent virulent factor. Gastroenterology 98:A34, 1990
12. O'Morain C, Mathai E, Daw M, Cafferky M, Keane C, O'Moore R: Lipolytic activity of *Helicobacter pylori* (HP). Gastroenterology 98:A101, 1990
13. Slomiany BL, Kasinathan C, Slomiany A: Lipolytic activity of *Campylobacter pylori:* Effect of colloidal bismuth subcitrate (De-Nol). Am J Gastroenterol 84:1273–1277, 1989
14. Butler BD, Lichtenberger LM, Hills BA: Distribution of surfactants in the canine GI tract and their ability to lubricate. Am J Physiol 7:G645–G651, 1983
15. Hills BA, Butler BD, Lichtenberger LM: Gastric mucosal barrier: The hydrophobic lining to the lumen of the stomach. Am J Physiol 7:G561–G568, 1983
16. Kao Y-C, Goddard PJ, Lichtenberger LM: Morphological effects of aspirin and prostaglandins on the canine gastric mucosal surface. Gastroenterology 98:592–606, 1990
17. Hazell SL, Lee A: *Campylobacter pyloridis,* urease, hydrogen ion back diffusion, and gastric ulcers. Lancet 2:15–17, 1986
18. Morgan DR, Freedman R, Depew CE, Kraft WG: Growth of *Campylobacter pylori* in liquid media. J Clin Microbiol 25:2123–2125, 1987
19. Shakir KMM: Phospholipase A_2 activity of post-heparin plasma: Rapid and sensitive assay and partial characterization. Anal Biochem 114:64–70, 1981
20. Kröner EE, Peskar BA, Fischer H, Ferber E: Control of arachidonic acid accumulation in bone marrow-derived macrophages by acyltransferases. J Biol Chem 256:3690–3697, 1981
21. Kurioka S, Matsuda M: Phospholipase C assay using *p*-nitrophenylphosphorylcholine together with sorbitol and its application to studying the metal and detergent requirement of the enzyme. Anal Biochem 75:281–289, 1976
22. Romero JJ, Kao Y-C, Lichtenberger LM: Gastric protective activity of mixtures of polar and neutral lipids in rats. Gastroenterology 99:311–326, 1990
23. Blodgett KB: Films built by depositing successive monomolecular layers on a solid surface. J Am Chem Soc 57:1007–1022, 1935
24. Gaines GL: Transfer of monolayers to solid multilayers. *In* Insoluble Monolayers at Liquid–Gas Interfaces. GL Gaines (ed). New York, Wiley, 1966, pp 326–346
25. Graham DY: *Campylobacter pylori* and peptic ulcer disease. Gastroenterology 96:615–625, 1989
26. Krakowka S, Morgan DR, Kraft WG, Leunk RD: Establishment of gastric *Campylobacter pylori* infection in the neonatal gnotobiotic piglet. Infect Immun 55:2789–2796, 1987
27. Lambert JR, Borromeo M, Korman MG, Hansky J, Eaves ER: Effect of colloidal bismuth (De-Nol) on healing and relapse of duodenal ulcers—role of *Campylobacter pylordis.* Gastroenterology 92:1489, 1987
28. Marshall BJ, Goodwin CS, Warren JR, Murray R, Blincow ED, Blackbourn SJ, Phillips M, Waters TE, Sanderson CR: Prospective double-blind trial of duodenal ulcer relapse after eradication of *Campylobacter pylori.* Lancet 2:1437–1442, 1988
29. McNulty CAM, Greaty JC, Crump B, Davis M, Donovan IA, Melikian V, Lister DM, Wise R: *Campylobacter pyloridis* and associated gastritis: Investigator blind, placebo controlled trial of bismuth salicylate and erythromycin ethylsuccinate. Br Med J 293:645–649, 1986
30. Rauws EA, Mytgat GNJ: Cure of duodenal ulcer associated with eradication of *Helicobacter pylori.* Lancet 2:1233–1235, 1990
31. Murakami M, Yoo JK, Teramura S, Yamamoto K, Saita H, Matuok, Asada T, Kita T: Generation of a mucosa and mucosal lesion formation following hydrolysis of urea by urease in the rat stomach. J Clin Gastroenterol 12(suppl 1):S104–S109, 1990
32. Kawano S, Tsujii M, Fusamoto H, Sato N, Kamada T: Chronic effect of intragastric ammonia on gastric mucosal structures in rats. Dig Dis Sci 36:33–38, 1991
33. Marshall BJ, Barrett LJ, Prakash C, McCallum RW, Guerrant RL: Urea protects *Helicobacter (Campylobacter) pylori* from the bactericidal effect of acid. Gastroenterology 99:697–702, 1990
34. Lichtenberger LM, Graziani LA, Dial EJ, Butler BD, Hills BA: Role of surface-active phospholipids in gastric cytoprotection. Science 219:1327–1329, 1983
35. Spychal RT, Goggin PM, Marrero JM, Saverymuttu SH, Yu

CW, Corbishley CM, Maxwell JD, Northfield TC: Surface hydrophobicity in gastric mucosa in peptic ulcer disease: Relationship to gastritis and *Campylobacter pylori* infection. Gastroenterology 98:1250–1254, 1990
36. Goggin PM, Marrero JM, Spychal RT, Jackson PA, Corbishley CM, Northfield TC: Surface hydrophobicity of gastric mucosa in *Helicobacter pylori* infection: Effect of clearance and erradication. Gastroenterology 103:1486–1490, 1992
37. Barrett-Bee K, Hayes Y, Wilson RG, Ryley J-F: A comparison of phospholipase activity, cellular adherence and pathogenicity of yeasts. J Gen Microbiol 131:1217–1221, 1985
38. Brockerhoff H, Jensen RG: Lipolytic Enzymes. New York, Academic Press, 1974
39. Traynor JR, Authi KS: Phospholipase A_2 activity of lysosomal origin secreted by polymorphonuclear leukocytes during phagocytosis or in treatment with calcium. Biochim Biophys Acta 665:571–577, 1981
40. Balsinde J, Dez E, Schuller A, Molinedo F: Phospholipase A_2 activity in resting and activated human neutrophils. J Biol Chem 263:1929–1936, 1988
41. Slomiany BL, Slomiany A: Role of mucus in gastric mucosal protection. J Physiol Pharmacol 42:147–161, 1991