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Interaction of *Helicobacter pylori* and its fatty acids with parietal cells and gastric H^+/K^+-ATPase

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Abstract

***Helicobacter pylori* and the fatty acids produced by this organism were compared for their acid inhibitory activity in isolated parietal cells and their interaction with gastric H^+/K^+-ATPase. *H pylori* (intact organisms, sonicates, methanolic extracts, and extracts from culture medium) and the fatty acids cis 9,10-methyleneoctadecanoic acid and tetradecanoic acid inhibited at fairly high concentrations histamine- and dibutyryl cyclic adenosine monophosphate stimulated acid production in isolated parietal cells, dissipated (with a slow onset) the H^+/K^+-ATPase created H^+ gradient in gastric membrane vesicles, and inhibited H^+/K^+-ATPase activity in a concentration dependent manner. The inhibitory potency of *H pylori* and the fatty acids in relation to H^+/K^+-ATPase depended on the amount of membrane protein. Bovine serum albumin prevented enzyme inhibition and proton dissipation from gastric vesicles. The data indicate that *H pylori* establishes its antisecretory action in parietal cells by blocking H^+/K^+-ATPase activity and also by a detergent action at the apical parietal cell membrane. The fatty acids cis 9,10-methyleneoctadecanoic acid and tetradecanoic acid are probably the acid inhibitory factors secreted by *H pylori*.**

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Acute infection with *Helicobacter pylori* is associated with reduced gastric acidity. This has been observed during serial experiments in man and after self infection.[1 2] Possible mechanisms by which *H pylori* causes hypochlorhydria include: (i) local immune response with release of acid inhibitory cytokines[3] and (ii) release of a bacterial protein which inhibits parietal cell activity.[4]

In addition to hexadecanoic acid (C 16:0) and octadecanoic acid (C 19:0), *H pylori* produces unusual fatty acids such as tetradecanoic acid (C 14:0) and cis 9,10-methyleneoctadeconoic acid (C 19:0Δ).[5] Fatty acids are known to inhibit the gastric proton pump, H^+/K^+-ATPase.[6] In this report we have studied the effects of *H pylori* and different fatty acids produced by this organism on acid production in isolated guinea pig parietal cells and their reaction pathway with purified pig H^+/K^+-ATPase.

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Methods

H PYLORI CULTURE

The three isolates of *H pylori* used in this study were obtained from antral mucosal biopsy specimens. Isolates were grown on *H pylori*-selective agar plates (Skirrow's medium) or in 20 ml brucella broth medium supplemented with 2·5% heat inactivated fetal calf serum (FCS) in glass Erlenmeyer flasks at 37°C for three days in a microaerophilic atmosphere.

Intact bacteria, sonicates, methanolic bacterial extracts, and extracts from bacterial culture medium were used. Intact bacteria were harvested in phosphate buffered saline solution, standardised to an OD_{600} of 1·0, which is equivalent to about $1{\cdot}5\times10^8$ colony forming units/ml, and then concentrated 100 fold by centrifugation. For sonicates, bacterial suspensions (in 20 mM Tris/HCl buffer, pH 7·0) were sonicated on ice (power: 50 W) in six consecutive treatments lasting 30 seconds. Bacterial extracts were obtained by stirring 10^{10} bacteria in 1 ml methanol for one hour at 4°C. Remaining cell debris was separated from the extract by centrifugation (10 000 *g* for five minutes). Bacterial culture medium was extracted with an equal volume of a chloroform/methanol solution, dried under a stream of N_2, and then redissolved in 1 ml methanol.

PARIETAL CELL ISOLATION AND ENRICHMENT AND MEASUREMENT OF ACID PRODUCTION

Guinea pig parietal cells were isolated by collagenase and pronase digestion and enriched to about 80% purity by the elutriation technique.[7] Acid production in parietal cells was monitored by the [^{14}C]-aminopyrine uptake technique. Parietal cells (5×10^5 cells) were preincubated for 20 minutes at 22°C in 1 ml buffer medium (pH 7·4) containing 8·3 μM aminopyrine and the agents to be tested. The buffer composition was (mM): NaCl (70), $NaHCO_3$ (20), NaH_2PO_4 (0·5), Na_2HPO_4 (1·0), HEPES (50), $CaCl_2$ (1·0), $MgCl_2$ (1·5) and glucose (11). Acid production was initiated by histamine (100 μM) or by the intracellular transmitter analogue dibutyryl cyclic adenosine monophosphate (AMP) (dbcAMP, 1 mM). After 40 minutes incubation at 37°C, the cells were separated from the medium by centrifugation through silicon oil and the radioactivity of intracellularly trapped aminopyrine was counted.

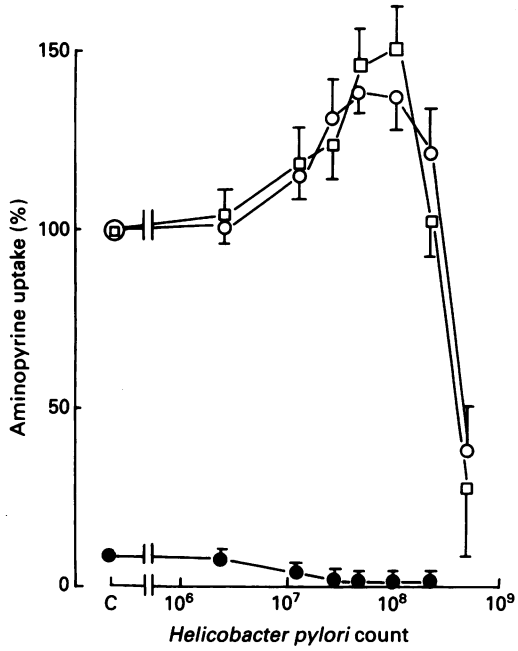

Figure 1: The effect of increasing Helicobacter pylori *concentrations on basal (●), histamine (○), and dbcAMP (□) stimulated aminopyrine uptake in isolated and enriched guinea pig parietal cells. Parietal cells were treated for 20 minutes at 22°C with the indicated amounts of* H pylori. *Basal, histamine (100 μM), and dbcAMP (1 mM) stimulated aminopyrine uptake were then determined after 40 minutes incubation at 37°C. Histamine and dbcAMP stimulated aminopyrine uptakes were 93 (19) and 73 (14) pmol [^{14}C]-aminopyrine/10^5 cells, respectively. These values were set to 100%. Values are the means (SEM) from three cell experiments with three strains of* H pylori.

PREPARATION OF GASTRIC MEMBRANE VESICLES

Gastric membrane vesicles containing H^+/K^+-ATPase were prepared from pig gastric mucosa as described.[8] ATPase activity in the material used was 16 (0·6) μmol P_i/mg protein per h in the presence of $MgCl_2$ alone and 62 (4) μmol P_i/mg protein per h in the presence of $MgCl_2$ plus 100 mM KCl and the H^+/K^+ ionophore gramicidin (25 μg/ml).

PROTON TRANSPORT

Proton transport in membrane vesicles was monitored by the fluorescence quenching of the weak base acridine orange as described.[9] Membrane protein (120 μg) was suspended in 2 ml samples containing 2 mM $MgCl_2$, 150 mM KCl, 10 mM Pipes/Tris buffer (pH 7·0), and 5 μM acridine orange (AO). The test agents were added to the suspension before proton uptake was initiated by ATP (final concentration: 2 mM) and valinomycin (50 μM). The K^+ ionophore valinomycin allows entry of K^+ along with Cl^- into the interior of the vesicles where the K^+ stimulatory site of the enzyme is located. Activation of the ATPase in the presence of Mg^{2+}, ATP, KCl, and valinomycin results in proton accumulation inside the vesicles.

TABLE I *Comparison of inhibitory potency (IC_{50}) of* Helicobacter pylori *and fatty acids on histamine-stimulated acid production in isolated and enriched guinea pig parietal cells*

Compound	*IC_{50}*
H pylori	3·2 (0·07)×10^8 bacteria/ml
Cis 9,10-methyleneoctadecanoic acid	46 (13) μM
Tetradecanoic acid	308 (69) μM
Hexadecanoic acid	No inhibition
Octadecanoic acid	No inhibition

Parietal cells (5×10^5/ml) were preincubated for 20 minutes at 22°C with at least five different concentrations of the compounds to be tested. Subsequently, histamine was added and aminopyrine uptake was determined after 40 minute's incubation at 37°C. IC_{50} values were obtained from concentration-response curves. Values are means (SEM) from three cell experiments with three different strains of *H pylori*.

ATPASE ACTIVITY

H^+/K^+-ATPase activity was assayed at 37°C in 1 ml samples (maintained isosmotic with sucrose) containing 2 mM $MgCl_2$, 2 mM ATP, 10 mM Pipes/Tris buffer (pH 7·2), and 20 or 60 μg membrane protein in the absence and presence of 100 mM KCl and 25 μg gramicidin. Gramicidin is a K^+/H^+ ionophore which allows K^+ access to the interior of the vesicles but, in contrast to valinomycin, uncouples ATPase activity from the formation of a proton gradient.

Membrane protein was preincubated at 22°C with the test agents. After 20 minutes the enzyme reaction was initiated with ATP and it was stopped after 20 minutes (20 μg protein) or five minutes (60 μg protein) incubation by the addition of 1 ml 1 N HCl. The amount of inorganic phosphate released from ATP was determined according to Carter and Karl.[10]

PROTEIN DETERMINATION

Protein was determined according to Lowry *et al.*[11]

COMPOUNDS

All chemicals were purchased from Sigma (München, Germany) except pronase E, acridine orange (Merck Darmstadt, Germany) and dibutyryl cyclic AMP (Boehringer Mannheim, Germany). Cis 9,10-methyleneoctadecanoic acid (purity 99%) was synthesised by J Holzkamp (Department of Organic Chemistry, University of Hannover, Hannover, Germany).

Results

EFFECT OF H PYLORI AND FATTY ACIDS ON ACID PRODUCTION IN PARIETAL CELLS

As shown in Figure 1, *H pylori* affected secretagogue stimulated acid production in parietal cells in a biphasic manner. At 10^7-10^8 bacteria/ml histamine- and dbcAMP-stimulated acid secretion were enhanced; higher concentrations (>2×10^8 bacteria/ml) progressively inhibited histamine- and dbcAMP-stimulated aminopyrine accumulation. In contrast, basal aminopyrine uptake was progressively reduced by the bacteria. Studies with *H pylori* sonicates and extracts from bacterial culture supernatant gave identical results (data not shown). The fatty acids, cis 9,10-methyleneoctadecanoic acid and tetradecanoic acid inhibited basal, histamine-, and dbcAMP-stimulated aminopyrine uptake in a concentration dependent manner. The IC_{50}

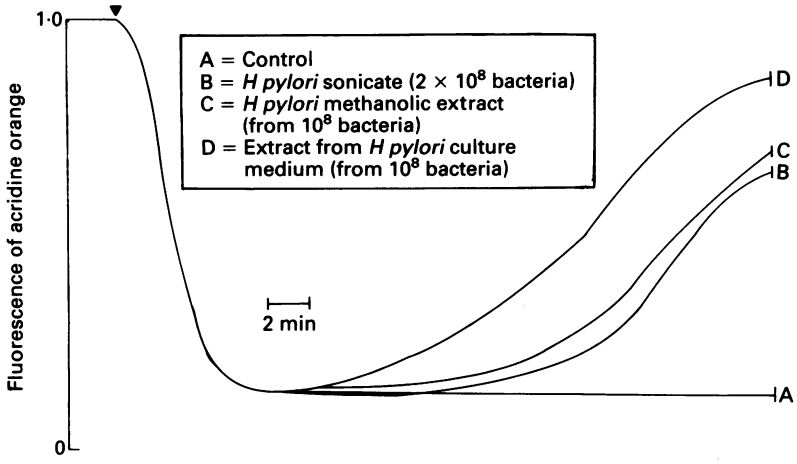

Figure 2: The effect of Helicobacter pylori *(sonicate, methanolic extract, extract from culture medium) on H^+/K^+-ATPase mediated H^+ uptake in gastric membrane vesicles.* H pylori *was added to the vesicles immediately before the pump reaction was started by ATP and valinomycin (▼).*

values for inhibition of histamine stimulated aminopyrine uptake are given in Table I. Hexadecanoic acid and octadecanoic acid up to 500 μM showed no inhibitory action on parietal cell aminopyrine uptake.

EFFECT OF H PYLORI AND FATTY ACIDS ON H^+/K^+-ATPASE MEDIATED H^+ TRANSPORT AND H^+/K^+-ATPASE ACTIVITY

Effects on H^+ transport

Addition of ATP and the K^+ ionophore valinomycin to gastric membrane vesicles resulted in an accumulation of H^+ inside the vesicles as demonstrated by quenching of acridine orange fluorescence. The maximal acid-interior H^+ concentration was reached after seven minutes and was maintained for at least 30 minutes.

H pylori sonicates (2×10^8 bacteria), extracts (from 1×10^8 bacteria), and extracts from *H pylori* culture medium (from 1×10^8 bacteria) had no effect on the initial rate of acidification in gastric membrane vesicles, but induced a time dependent dissipation of the pH gradient (Fig 2). Dissipation of the pH gradient was prevented by bovine serum albumin (BSA 2 mg) (data not shown). Heat treatment of the *H pylori* sonicates at 95°C for 30 minutes or treatment with trypsin did not eliminate the inhibitory action on H^+ uptake (data not shown). The addition of an *H pylori* sonicate (2×10^8 bacteria) to vesicles in which H^+/K^+-ATPase reaction had been stopped by EDTA at the steady state level of ATP and valinomycin-induced H^+ uptake, resulted in an enhanced proton dissipation rate (Fig 3). To study the action of intact bacteria on vesicular H^+ uptake, membrane vesicles (120 μg protein) were coincubated with 1 and 4×10^8 bacteria for two hours at 37°C in the proton transport buffer medium. Subsequently, *H pylori* was separated by centrifugation (8000 *g* for five minutes) and H^+ uptake was initiated. As shown in Figure 4, pretreatment of the gastric membrane vesicles with *H pylori* resulted in inhibition of H^+ uptake in response to ATP and ATP plus valinomycin.

Figure 5 shows the effect of different fatty acids produced by *H pylori* on H^+ transport in gastric membrane vesicles. Similar to the action of *H pylori*, tetradecanoic acid (25 μM) and cis 9,10-methyleneoctadeconoic acid (12·5 and 25 μM) failed to affect the initial rate of acidification but then induced a time dependent dissipation of the established pH gradient. Proton dissipation was not observed when BSA (2 mg) was present in the medium (data not shown). Hexadecanoic acid and octadecanoic acid (25 μM) did not dissipate the acid-interior pH gradient.

Effects on H^+/K^+-ATPase activity

H pylori extracts and the four fatty acids inhibited H^+/K^+-ATPase activity in gastric membrane vesicles, in a concentration dependent

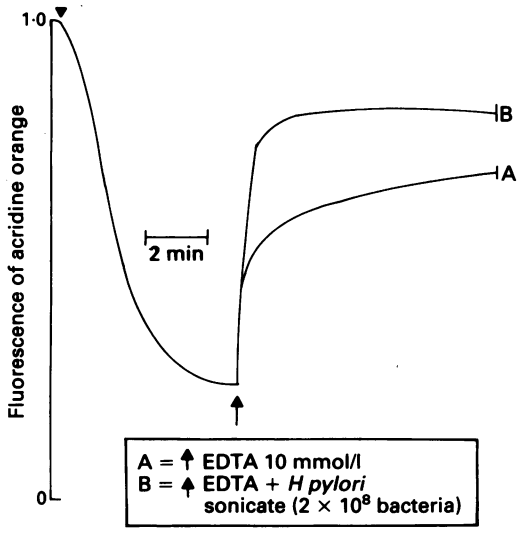

Figure 3: The effect of an Helicobacter pylori *sonicate on a preformed H^+ gradient in vesicles in which the ATPase reaction was stopped by the magnesium chelating agent EDTA. EDTA alone (final concentration: 10 mM) and EDTA together with the Helicobacter sonicate were added as indicated by the arrows.*

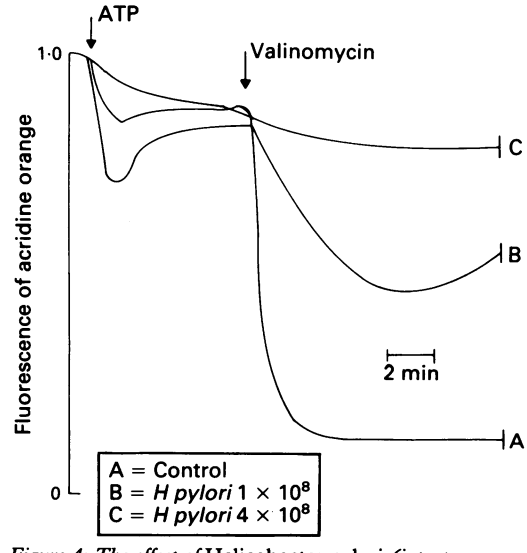

Figure 4: The effect of Helicobacter pylori *(intact organisms) on H^+/K^+-ATPase mediated H^+ uptake in gastric membrane vesicles. The membrane vesicles were preincubated with* H pylori *for two hours at 37°C. Afterwards the bacteria were separated by centrifugation (8000 g, 5 minutes) and H^+/K^+-ATPase mediated H^+ uptake was initiated by consecutive addition of ATP and valinomycin.*

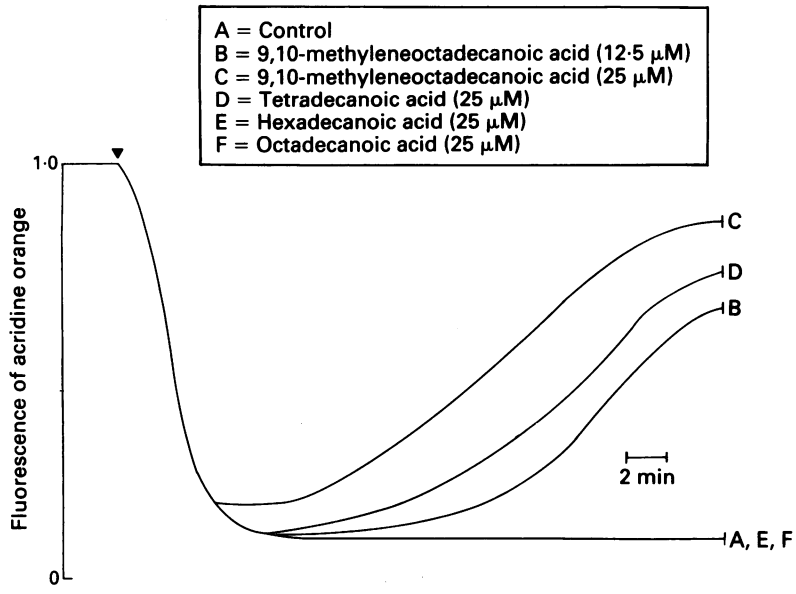

Figure 5: Effect of cis 9,10-methyleneoctadecanoic, tetradecanoic, hexadecanoic and octadecanoic acid on H^+/K^+-ATPase-mediated H^+ uptake in gastric membrane vesicles. The fatty acids were added to the membrane vesicles before the pump reaction was started by ATP and valinomycin (▼).

manner. The inhibitory potency was dependent on the amount of membrane protein in the assay (Table II). At a protein concentration of 60 μg/ml (the proton concentration used in H^+ transport studies), the effectiveness of the fatty acids in inhibiting K^+-stimulated ATPase activity was cis 9,10-methyleneoctadecanoic acid > tetradecanoic acid >>> hexadecanoic acid and octadecanoic acid. The inhibitory action of *H pylori* extract and the fatty acids on ATPase activity was prevented by BSA (Table II).

Discussion

We have shown that *H pylori* and its two major fatty acids, tetradecanoic acid and cis 9,10-methyleneoctadecanoic acid,[5] inhibit: (i) acid production in isolated parietal cells, (ii) H^+/K^+-ATPase activity, and (iii) dissipated H^+ transport in gastric membrane vesicles. This finding strongly supports the view that *H pylori* establishes its antisecretory activity in vitro – that is, in isolated parietal cells – at the H^+ transporting enzyme system.

As shown in the H^+ transport studies, *H pylori* (sonicates, methanolic extracts, and extracts from bacterial culture medium) caused release of H^+ from the vesicles and showed a slow onset of action. An identical action was seen with the fatty acids, tetradecanoic acid and cis 9,10-methyleneoctadecanoic acid. Proton dissipation was not observed when BSA was present in the media. Albumin binds fatty acids.[12] The identical mode of action of *H pylori* and both fatty acids in proton transport studies (onset of action, protective effect of BSA) favours the hypothesis that the acid inhibitory factors produced and released by the bacterium are cis 9,10-methyleneoctadecanoic acid and tetradecanoic acid. Fatty acids contain both charged (carboxyl group) and uncharged (hydrocarbon tail) groups – that is, the molecule has hydrophilic and hydrophobic properties. They could affect H^+/K^+-ATPase mediated H^+ transport by disordering the surrounding membrane lipids (detergent action), or by hydrophobic interaction with the H^+/K^+-ATPase.[6] K^+-stimulated ATPase activity in membrane vesicles was inhibited in a concentration dependent manner by *H pylori* extracts and the fatty acids tested. Inhibition was prevented by BSA and was dependent on the amount of vesicular protein. This observation suggests that fatty acids and *H pylori* extracts interfere with hydrophobic sites of the H^+/K^+-ATPase and thereby block the catalytic function of the enzyme. The observation that *H pylori* sonicates enhanced the rate of proton dissipation from membrane vesicles in which the ATPase activity had been stopped by the magnesium chelating agent EDTA, show that *H pylori* affects H^+/K^+-ATPase mediated H^+ transport not only by blockade of H^+/K^+-ATPase activity but also by a detergent action.

Recently, Cave *et al*[13] postulated that *H pylori* produces two acid inhibitory factors (AIF 1 and AIF 2). AIF 1 was characterised as a heat labile, pronase-sensitive protein with a molecular weight of approximately 50·000, and AIF 2 as a probably small and pronase-resistant factor. From the observations made in our study we suggest that AIF 2 are the fatty acids cis 9,10-methyleneoctadecanoic acid and tetradecanoic acid.

An important question is whether the observed mechanism of action of *H pylori* on acid secretion shown in this in vitro study accounts for its antisecretory action in vivo. The experiments with extracts of bacterial culture medium indicate that sufficient amounts of the acid inhibitor are secreted into the media and coincubation of *H pylori* (intact organisms) with gastric membrane vesicles resulted in inhibition of ATPase mediated H^+ transport. Once in the stomach, *H pylori* is attached to the mucus layer, to gastric epithelial cells, and is found even within the secretory canaliculi of parietal cells.[14] The presence of *H pylori* in this subcellular compartment of parietal cells implies that large quantities of the acid inhibitory factor do not need to be secreted to abolish H^+ transport at the secretory parietal cell membrane.

Clinical studies, however, have shown that most humans infected with *H pylori* are not hypochlorhydric and that bacteria induced hypochlorhydria is reversible.[15] Possible explanations for this phenomenon include

TABLE II *Comparison of inhibitory potency (IC_{50}) of* Helicobacter pylori *extracts and fatty acids on H^+/K^+-ATPase activity at different vesicular protein concentrations*

	Membrane protein concentration		
Compound	*20 μg*	*60 μg*	*60 μg+2 mg BSA*
H pylori (methanolic extract) (bacteria/ml)	6×10^6	8×10^7	No inhibition at: 2×10^8
Cis 9,10-methyleneoctadecanoic acid	1·1 μM	19 μM	100 μM
Tetradecanoic acid	3·6 μM	46 μM	100 μM
Hexcadecanoic acid	3·6 μM	>>100 μM	100 μM
Octadecanoic acid	7·2 μM	>>100 μM	100 μM

Gastric membrane vesicles (20 or 60 μg protein) were preincubated for 20 minutes at 22°C with at least four different concentrations of the compounds tested in the absence and presence of 2 mg bovine serum albumin (BSA). The reaction was initiated by adding ATP and lasted for 20 minutes (20 μg protein) or five minutes (60 μg protein) at 37°C. IC_{50} values were obtained from concentration-response curves. Values are means from three different membrane proteins.

change in numbers of bacteria or a genetic 'switch off' (down regulation) in the bacterial organism itself.[4] Our parietal cell experiments showed that *H pylori* has a dual effect on acid production. At bacterial concentrations ranging from 10^7–10^8, secretagogue stimulated aminopyrine uptake was enhanced; higher concentrations inhibited acid production. How *H pylori* stimulates acid production in parietal cells is not yet known. We have observed no stimulatory effect of an *H pylori* extract (at low concentrations) on K^+- (0·5–100 mM) or ATP- (0·05–2 mM) induced ATPase activity (W Beil, unpublished results). Recently, König *et al*[16] showed that *Escherichia coli* can activate platelets at an early stimulus transducing event (Ca^{2+}-mobilisation, translocation of protein kinase C). Whether *H pylori* stimulates parietal cell function at this signal transduction level is under investigation.

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