Effects of *Helicobacter pylori* on intracellular Ca\(^{2+}\) signaling in normal human gastric mucous epithelial cells

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Marlink, Katie L., Kathy D. Bacon, Brett C. Sheppard, Hassan Ashktorab, Duane T. Smoot, Timothy L. Cover, Clifford W. Deveney, and Michael J. Rutten. Effects of *Helicobacter pylori* on intracellular Ca\(^{2+}\) signaling in normal human gastric mucous epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 285: G163–G176, 2003. First published February 26, 2003; 10.1152/ajpgi.00257.2002.—In stomach, *Helicobacter pylori* (Hp) adheres to gastric mucous epithelial cells (GMEC) and initiates several different signal transduction events. Alteration of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)) is an important signaling mechanism in numerous bacteria-host model systems. Changes in [Ca\(^{2+}\)]\(i\), induced by Hp in normal human GMEC have not yet been described; therefore, we examined effects of Hp on [Ca\(^{2+}\)]\(i\) in normal human GMEC and a nontransformed GMEC line (HFE-145). Cultured cells were grown on glass slides, porous filters, or 96-well plates and loaded with fura 2 or fluo 4. Hp wild-type strain 60190 and vacA\(^-\), cagA\(^-\), and picB\(^-\)/cagE\(^-\) isogenic mutants were incubated with cells. Changes in [Ca\(^{2+}\)]\(i\) were recorded with a fluorimeter or fluorescence plate reader. Wild-type Hp produced dose-dependent biphasic transient [Ca\(^{2+}\)]\(i\) peak and plateau changes in both cell lines. Hp vacA\(^-\) isogenic mutant produced changes in [Ca\(^{2+}\)]\(i\), similar to those produced by wild type. Compared with wild type, cagA\(^-\) and picB\(^-\)/cagE\(^-\) isogenic mutants produced lower peak changes and did not generate a plateau change. Preloading cultures with intracellular Ca\(^{2+}\) chelator BAPTA blocked all Hp-induced [Ca\(^{2+}\)]\(i\) changes. Thapsigargin pretreatment of cultures to release Ca\(^{2+}\) from internal stores reduced peak change. Extracellular Ca\(^{2+}\) removal reduced plateau response. Hp-induced peak response was sensitive to G proteins and PLC inhibitors. Hp-induced plateau change was sensitive to G protein inhibitors, src kinases, and PLAs. These findings are the first to show that *H. pylori* alters [Ca\(^{2+}\)]\(i\) in normal GMEC through a Ca\(^{2+}\) release/influx mechanism that depends on expression of cagA and picB/cagE genes.

\(\text{vacA}/\text{cagA}, \text{picB/cagE} \): bacteria; signal transduction; fura 2; fluo 4; cell culture; immunofluorescence; thapsigargin; genistein; herbimycin; G protein; stomach

\textit{Helicobacter pylori} is associated with the induction of gastric inflammation and is a risk factor for peptic ulcer disease and distal gastric cancer (38). Two important virulence factors produced by *H. pylori* strains are a vacuolating cytotoxin (VacA) and products of a 40-kb genetic locus of \(\sim 31\) genes termed the cag pathogenicity island (PAI) (8). VacA is a secreted toxin that produced multiple functional and morphological changes within gastric epithelial cells (36). The cag PAI encodes proteins that act to form a type IV secretion system that is responsible for translocation of the *H. pylori* CagA protein into gastric cells (2, 6, 12, 33, 51, 56). The picB/cagE gene, which encodes a homolog of the Agrobacterium VirB4 ATPase, is also necessary for the translocation of the *H. pylori* CagA protein into the gastric cell (6, 14, 17, 33). Also, products of the cag PAI act to induce synthesis and secretion of IL-8 (60), increase MAP kinase activity (20), induce gastric epithelial cell proliferation and apoptosis (18, 39), and induce transactivation of the epidermal growth factor receptor (EGFR) (21) and cyclooxygenase-2 mRNA expression (45). Other mutagenesis studies involving the cag PAI have found that disruption of the cagA gene resulted in a reduction in gastric cell arachidonic acid release and prostaglandin \(\text{E}_2\) synthesis (40), in a decreased stress kinase gene expression (30), and in reduced expression of the protooncogenes \(\text{c-fos}\) and \(\text{c-jun}\) (26). The presence of the cagA gene and its expressed product, the CagA protein, have also been reported as necessary for the induction of specific gastric cell morphological changes such as cell elongation, spreading, and scattering, which has been designated as the “hummingbird” phenotype (5, 16, 49–51). Of interest is a recent study suggesting that just the presence of the CagA protein (an not necessarily phosphorylated CagA protein) can induce this hummingbird phenotype (27). Other *H. pylori*-induced morphological changes, such as gastric cell stress fiber formation, were shown to be cagA and vacA independent (49).

Despite the recent advances in *H. pylori*-host cell mutagenesis and transcriptional profiling (14), little is known about certain aspects of *H. pylori* signaling in...
normal gastric cells such as the regulation of intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\)). In other model systems, bacterial adherence to the host cell has been shown to result in specific [Ca\(^{2+}\)]\(_i\) changes (10). For example, the adherence of certain Escherichia coli strains to intestinal epithelial cells results in increased [Ca\(^{2+}\)]\(_i\) and inositol trisphosphate levels (11). It has also been shown that Salmonella induces intracellular Ca\(^{2+}\) changes that were linked to the activation of an NF-κB-dependent inflammatory pathway (13). In this regard, a study using the intracellular Ca\(^{2+}\) chelator BAPTA along with calmodulin inhibitors found that H. pylori activation of NF-κB and IL-8 signaling in MKN45 human gastric cancer cells was Ca\(^{2+}\)-calmodulin dependent (32). The reported H. pylori-induced hummingbird phenotype in gastric cells is similar to the morphological events seen with hepatocyte growth factor or scatter factor (HGF/SF) on MDCK cells or hepatocytes (44). It has also been reported that HGF/SF can induce changes in [Ca\(^{2+}\)]\(_i\), which have been shown to be linked to changes in cell morphology and proliferation (1, 19, 31, 34).

However, despite the extensive work on various aspects of H. pylori-induced signaling in gastric cancer cells, the specific mechanism(s) of intracellular Ca\(^{2+}\) mobilization by H. pylori in normal human gastric mucous epithelial cells has not yet been thoroughly examined. Also, many H. pylori signaling studies have used either nongastric or gastric cancer cell lines as a model system, which always introduces a degree of uncertainty as to whether the events observed are applicable to normal gastric cells. As an alternative to nongastric epithelial cells, several in vitro model systems of normal human gastric mucous epithelial cells have been established (7, 48, 54, 61). The use of nontransformed cell culture models provides a more accurate representation of the environment that H. pylori may encounter in the normal human gastric mucosa. The aim of the present study, therefore, was to examine the effects of H. pylori on intracellular [Ca\(^{2+}\)]\(_i\) signaling in normal human gastric epithelial cells. We found for the first time that H. pylori produces specific transient [Ca\(^{2+}\)]\(_i\) changes in normal human gastric mucous epithelial cells and that these H. pylori-induced [Ca\(^{2+}\)]\(_i\) changes could also be replicated in a nontransformed gastric mucous epithelial cell line (HFE-145 cells). We also found that a G protein-dependent/PLC pathway primarily regulated H. pylori-induced intracellular Ca\(^{2+}\) release, whereas H. pylori-induced Ca\(^{2+}\) influx was primarily regulated by components of a G protein-, src kinase-, and PL\(_{2}\)-dependent pathway. Finally, we report that mutagenesis of \(\text{picB/cagE}\) and \(\text{cagA}\) genes (located within the cag path), but not the vacA gene, alters the capacity of H. pylori to produce a full [Ca\(^{2+}\)]\(_i\) response.

**MATERIALS AND METHODS**

**Chemicals and peptides.** The Ca\(^{2+}\) probes fura 2-AM and fluo 4-AM (special packaging) as well as BAPTA-AM were purchased from Molecular Probes (Eugene, OR) and stored at −20°C until needed. All Ca\(^{2+}\) probes were freshly prepared as 5-mM stock solutions on the day of the experiment by dissolving preweighed aliquots in cultured-grade DMSO (Sigma, St. Louis, MO). The compounds methyl arachidonoyl fluorophosphatase (MAFP), thapsigargin, 4-amino-5-(4-chlorophenyl)-7-(t-buty1)pyrazolo[3,4-d] pyridine (PP2), pertussis toxin (PTX), U-73122, and U-73343 were purchased from Calbiochem (San Diego, CA). Type I collagenase, RIA grade BSA powder, Triton X-100, digitonin, and genistein were purchased from Sigma. Cell culture media were from GIBCO (San Diego, CA), and FBS was from Hyclone (Logan, UT). Unless noted otherwise, all Falcon cell culture plasticware was purchased from Fisher Scientific (San Francisco, CA).

**Gastric epithelial cell culture.** Human gastric mucous cells were isolated and cultured as previously described (48). The Oregon Health Sciences University (OHSU) Human Studies Subcommittee approved all procedures and handling of human tissue. Briefly, H. pylori-free gastric tissues were obtained from patients undergoing surgical gastrectomy. The surgical specimens were washed twice in serum-free media and pinned down on polymerized Sylgard, and the epithelium was removed by scraping the surface with a glass slide. The filter paper was placed into a 50-ml syringe with an attached 15-gauge luer-stub adapter, and the contents were pushed through a 200-μm nylon mesh screen. The mesh-filtered suspension was washed twice in serum-free media and centrifuged at 100 g for 3 min, then the pellet was resuspended in 15 ml of serum-free culture media and a 200-μl aliquot was taken for cell counts in a Coulter Counter. The 15-ml suspension was divided into three 5-ml aliquots in 16 × 125-cm Falcon round-bottom tubes, then 5 ml of isosmotic Percoll was added to each tube. The tubes were centrifuged for 15 min at 100 g for 3 min, then the pellet was resuspended in 15 ml of serum-free culture media and a 200-μl aliquot was taken for each cell culture. The pellets were then transferred to siliconized 125-ml screw-cap Erlenmeyer flasks containing 20 ml of serum-free culture media with 20 mg/ml of type I collagenase and 0.1% bovine serum albumin. The flasks were then gassed with 95% O\(_2\)-5% CO\(_2\), put into a 37°C shaking water bath, and gyrated at 120 oscillations/min for 45 min. At the end of the incubation period, the collagenase-digested mixture was put into a 50-ml syringe with an attached 15-gauge luer-stub adapter, and the contents were pushed through a 200-μm nylon mesh screen. The mesh-filtered suspension was washed twice in serum-free media and centrifuged at 100 g for 3 min at 24°C, and the bottom pellet, containing the gastric mucous epithelial cells, was removed. The pellet was washed three times and centrifuged at 20 g for 3 min in serum-free cell culture media, then the cells were plated on 0.45-μm Falcon porous filters (catalog no. 353180; 12 mm, 0.45-μm pore size), 25 mm round glass coverslips, or 96-well plastic dishes.

The HFE-145 human gastric mucous epithelial cell line (provided by D. Smoot) were plated and grown under the same experimental conditions as the above human primary gastric mucous epithelial cells. The HFE-145 cells were originally developed from normal human gastric epithelial cells by the transfection of normal cells with SV40 Large T-antigen and human telomerase vectors (53). The cells have a doubling time of ~24 h and are strongly positive for cytokeratin-10,11,18 and weakly positive for cytokeratin-13,16,20, which is almost identical to cytokeratin staining of the parental cell line. These cells also stain positive for neutral mucin using periodic acid-Schiff and negative for alcian blue (acidic mucin), which is consistent with normal gastric epithelial cells. Growth of these cells was inhibited when cells were placed in soft agar, suggesting that these cells are not tumorigenic. The cells constitutively express mRNAs for Muc-5ac, Muc-5b, and Muc-6 genes, which is consistent with normal gastric epithelial cells. Electron microscopy shows that these cells form tight junctions when grown as monolayers on plastic tissue culture dishes and on glass slides (53).
**H. pylori culture.** The *H. pylori* bacteria used in this study were the wild-type vacA\\(^+\), cagA\\(^+\) 60190 (ATCC 49503), an isogenic vacA\\(^-\) mutant, isogenic cagA\\(^-\) mutant, and an isogenic picB/cagE\\(^-\) mutant. *H. pylori* 60190 contains a type s1a/m1 vacA allele (59). The vacA\\(^+\), cagA\\(^+\), and picB/cagE\\(^-\) mutants have been previously described (39, 52, 59). The bacteria were grown on blood agar plates (trypticase soy agar with 5% sheep blood; PML Microbiologicals, Tulatine, OR) under microaerobic conditions using a CampyPak jar (Fisher Scientific) at 36°C. Unless noted otherwise, all bacteria were harvested at 24 h by using a sterile cotton swab and 3 ml of PBS (pH 7.1). The bacterial suspensions were put into 12-ml Falcon round-bottom tubes, and the *H. pylori* was resuspended by gentle inversion. A 1-ml aliquot of the suspension was put into a cuvette, and the *H. pylori* concentration was determined by using optical density 600 nm (OD\(_{600}\)) which was adjusted with the appropriate mammalian Ringer solution. The mammalian Ringer solution consisted of (in mM): 137 NaCl, 4 KCl, 25 NaHCO\(_3\), 2 KH\(_2\)PO\(_4\), 15 HEPES, 1 MgSO\(_4\), 25 glucose, pH 7.4. The bacteria were grown on blood agar plates (trypticase soy agar) or 25-mm round glass coverslips. After 2 days of incubation, the bacteria and cellular autofluorescence did not exceed 5% of the total fura 2 fluorescence.

In some experiments, the rates of *H. pylori*-induced intracellular Ca\(^{2+}\) release and influx were estimated by using the Ca\(^{2+}\)-add-back technique (43). For these experiments, fura 2-loaded gastric cells are initially incubated in the absence of extracellular Ca\(^{2+}\), then *H. pylori* is added and [Ca\(^{2+}\)]\(_i\) is recorded as intracellular Ca\(^{2+}\) release, then extracellular Ca\(^{2+}\) (2.0 mM) is "added back" and the [Ca\(^{2+}\)] change is measured as Ca\(^{2+}\) influx.

The final calibration of the fura 2 signal was done at the end of each experiment by adding 5 \(\mu\)M ionomycin for 10 min to saturate fura 2 with Ca\(^{2+}\) to obtain maximal fluorescence (F\(_{\text{max}}\)) then 10 mM EGTA and 10 mM EDTA plus 60 mM Tris-HCl pH 10.5, was added to chelate the Ca\(^{2+}\) from fura 2 to determine the minimal fluorescence (F\(_{\text{min}}\)). Additional points on the calibration curve were determined by using a series of defined Ca\(^{2+}\)-calibration solutions (Kit #1, C-3008; Molecular Probes), and [Ca\(^{2+}\)]\(_i\) was calculated (58). The fluorescence tracing analysis and data smoothing were done with software provided by the manufacturer (SLM-Aminco). *H. pylori* and gastric cell [Ca\(^{2+}\)]\(_i\) were measured using a fluo 4 96-well assay. In some experiments we wanted a quicker throughput assay to measure the relative change in [Ca\(^{2+}\)]\(_i\) produced by *H. pylori* with different kinase inhibitors. For these experiments, measurement of [Ca\(^{2+}\)]\(_i\) was done using a modification of a 96-well fluorescence assay (25). Cultures of normal human gastric epithelial cells were grown to confluence in 96-well plates and loaded with 2.5 \(\mu\)M fluo 4-AM for 45 min at 37°C. After being loaded, the cultures were washed twice with mammalian Ringer and then incubated for another 30 min with fresh 37°C Ringer solution. Fluor 4 fluorescence was recorded using a 96-well fluorescence plate reader (FluoStar; BMG Technologies, Durham, NC) equipped with excitation (485 ± 20 nm) and emission (530 ± 20 nm) filters. After the addition of *H. pylori* or drugs, fluorescence measurements were made every 2 s. The F\(_{\text{max}}\) measurement for the human gastric epithelial cells was obtained by adding a solution of 5 \(\mu\)M ionomycin and 100 \(\mu\)M digitonin to the gastric cell cultures after 5 min. After this, the F\(_{\text{min}}\) measurement was obtained by adding a solution of 10 mM EGTA-10 mM EDTA in 60 mM Tris-HCl pH 10.5, for 15 min. The values for F\(_{\text{max}}\) and F\(_{\text{min}}\) were graphed, and individual fluorescence values were obtained by using a single wavelength equation for fluo 4 (58).

**Statistics.** All data are expressed as means ± SE. The differences between means were considered significant when the P value calculated from Students’ t-test for paired cultures was <0.05. Multiple cell culture comparisons were analyzed by using ANOVA and Duncan’s multiple-range tests. Unless stated otherwise, n represents the total number of different “individual” cell preparations isolated from different surgical specimens. All statistical calculations were made using SigmaStat statistical software (SPSS, San Rafael, CA).

**RESULTS**

*H. pylori* mobilizes intracellular Ca\(^{2+}\) in normal gastric mucous epithelial cells. For the first series of experiments, we used a modified horizontal chamber on an inverted microscope connected to a spectrophotom-
eter. This particular setup has the advantage that smaller volumes can be used, allowing a quicker rate of *H. pylori* settling and adherence to the gastric cells. Before the addition of *H. pylori*, a baseline fluorescence measurement was taken of the fura 2-loaded gastric mucous cell cultures, then varying doses of *H. pylori* (1 × 10^5–1 × 10^6 CFU/ml) were added and [Ca^{2+}]_i was measured. As shown in the composite tracings in Fig. 1, we found that wild-type *H. pylori* (strain 60190) induced a dose-dependent change in [Ca^{2+}]_i, in primary cultures of human gastric mucous epithelial cells. Over the time course of 60 min, the wild-type strain produced a characteristic biphasic [Ca^{2+}]_i increase (transient “peak”) that was followed by a decline and then a return to above [Ca^{2+}]_i baseline values (“plateau” phase) (Fig. 1). At the highest *H. pylori* concentration used (1 × 10^9 CFU/ml), there was a change in [Ca^{2+}]_i from a basal level of 104 ± 2 nM to a peak of 184 ± 4 nM, followed by a decline and then a rise again to a plateau level of 145 ± 4 nM (n = 14; Table 1).

To independently validate the previous results, we tested the effects of *H. pylori* on [Ca^{2+}]_i in the nontumorigenic HFE-145 human gastric mucous epithelial cell line. The HFE-145 cell line has morphological and phenotypic properties similar to those seen in primary gastric mucous epithelial cells (53). Compared with the primary gastric mucous epithelial cell cultures, we found that *H. pylori* produced similar [Ca^{2+}]_i changes in the HFE-145 cell line (Fig. 1B). That is, *H. pylori* dose-dependently produced the characteristic biphasic peak and plateau changes in [Ca^{2+}]_i (Fig. 1B; Table 1). For example, *H. pylori* (1 × 10^9 CFU/ml) produced a change in baseline [Ca^{2+}]_i from 106 ± 2 nM to a peak of 177 ± 4 nM, which was followed by a decline and then an increase to plateau levels of 140 ± 4 nM (n = 14; Table 1). Although some quantitative differences in *H. pylori*-induced intracellular Ca^{2+} signaling existed between the primary human gastric mucous epithelial cell cultures and the HFE-145 cell line, overall the qualitative patterns of *H. pylori*-induced [Ca^{2+}]_i changes were similar in both cell types. It should be noted that after several passages (>25) of the HFE-145 cell line, we began to lose the characteristic *H. pylori-

### Table 1. *Helicobacter pylori*-induced [Ca^{2+}]_i changes in gastric mucous epithelial cells

<table>
<thead>
<tr>
<th>H. pylori concentration, cfu/ml</th>
<th>Human Gastric Mucous Epithelial Cells</th>
<th>HFE-145 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak</td>
<td>Plateau</td>
</tr>
<tr>
<td>1 × 10^6</td>
<td>184 ± 4*</td>
<td>145 ± 4*</td>
</tr>
<tr>
<td>1 × 10^7</td>
<td>170 ± 4*</td>
<td>136 ± 3*</td>
</tr>
<tr>
<td>1 × 10^8</td>
<td>144 ± 3*</td>
<td>125 ± 3*</td>
</tr>
<tr>
<td>1 × 10^9</td>
<td>131 ± 3*</td>
<td>119 ± 4*</td>
</tr>
</tbody>
</table>

Comparative dose-response changes in *H. pylori*-induced intracellular Ca^{2+} concentration ([Ca^{2+}]_i) peak and plateau responses between primary cultures of human gastric mucous epithelial cells and the HFE-145 human gastric mucous epithelial cell line. Data are means ± SE (in nM) of 14 individual experiments done in duplicate. *P < 0.05 vs. baseline [Ca^{2+}]_i, control (104–107 nM) values.

In addition to live bacteria, we also tested the capacity of *H. pylori* sonicates (0–5 µg/ml) to induce changes in [Ca^{2+}]_i in normal gastric mucous epithelial cells. However, we found that the *H. pylori* sonicates, even at the highest concentration used (5 µg/ml), produced only a small, slow, steady change in [Ca^{2+}]_i, from a baseline level of 103 ± 3 nM to 119 ± 4 nM over a 60-min time course (n = 9). Because the bacterial sonicates did not reproduce the [Ca^{2+}]_i changes observed with live intact *H. pylori*, they were not used for the remainder of our experiments. Compared with the untreated control cultures, we also found that heat-killed *H. pylori* produced no significant change (P > 0.05) in [Ca^{2+}]_i over the 60-min time period (control = 106 ± 3 nM; heat-killed bacteria = 108 ± 4 nM; n = 9; Fig. 1). We also found that the pretreatment of the gastric mucous epithelial cell cultures with the Ca^{2+} chelator BAPTA completely abolished the wild-type *H. pylori*-induced [Ca^{2+}]_i changes over 60 min (control = 106 ± 3 nM; *H. pylori*-treated = 107 ± 3 nM; n = 7). These data confirm that intact live *H. pylori* are able to induce a specific [Ca^{2+}]_i change in normal gastric mucous epithelial cells. In addition, the *H. pylori*-induced [Ca^{2+}]_i change is characterized by an initial transient

Fig. 1. Graphs showing representative tracings of intracellular Ca^{2+} concentration ([Ca^{2+}]_i) changes with varying concentrations (1 × 10^5–1 × 10^6 colony-forming units (CFU/ml) of wild-type (60190) *Helicobacter pylori* (Hp) on fura 2-loaded primary cultures of human gastric mucous epithelial cells (A) and the HFE-145 gastric mucous epithelial cell line (B). Note that the addition of *H. pylori* (thick arrow) produced a concentration-dependent biphasic change in [Ca^{2+}]_iNa, which was followed by a return to baseline [Ca^{2+}]_i levels in both cell types. Heat-killed wild-type *H. pylori* produced no detectable [Ca^{2+}]_i change in either the primary gastric cultures or the HFE-145 gastric cell line. For all tracings, n = 14 independent experiments.
peak \([\text{Ca}^{2+}]_i\), increase followed by a sustained plateau \([\text{Ca}^{2+}]_i\), phase that can be specifically blocked by the \(\text{Ca}^{2+}\) chelator BAPTA.

The role of \(\text{vacA}, \text{cagA}, \text{and picB/cagE}\) genes in \(H.\ pylori\)-induced \([\text{Ca}^{2+}]_i\) changes. It is well established that the \(\text{VacA}\) toxin secreted by \(H.\ pylori\) is an important virulence factor in the pathogenesis of peptic ulcer disease (36). In addition, the \(\text{CagA}\) protein encoded by the \(\text{cagA}\) gene within the \(H.\ pylori\) \(\text{cag PAI}\) is involved in gastric host cell cytotoxic responses (5, 16, 49–51). Also, residing within the \(\text{cag PAI}\) is the \(\text{picB/cagE}\) gene, which encodes a structural component of the type IV secretion system that is important in translocating the \(\text{CagA}\) protein as well as activation of signaling mechanisms involved in immune responses and cell growth and apoptosis (37). We therefore were interested in testing the role of the \(H.\ pylori\) \(\text{vacA}, \text{cagA}, \text{and picB/cagE}\) genes on intracellular \(\text{Ca}^{2+}\) signaling. Using a \(\text{vacA}^-\) isogenic mutant strain on primary cultures of human gastric mucous epithelial cells, we found that the \(\text{vacA}^-\) isogenic mutant produced peak and plateau \([\text{Ca}^{2+}]_i\) changes that were nearly identical to those seen in the wild-type 60190 strain (Fig. 2A; Table 2). The heat-killed \(H.\ pylori\) \(\text{vacA}^-\) isogenic mutant strain produced no change in \([\text{Ca}^{2+}]_i\); in the gastric cells (Fig. 2A). In contrast to the wild-type and \(\text{vacA}^-\) isogenic mutant strains, the \(\text{cagA}^-\) and \(\text{picB}^-/\text{cagE}^-\) isogenic strains produced markedly attenuated \([\text{Ca}^{2+}]_i\) changes (Fig. 2B). That is, the wild-type strain (1 \(\times\) 10^9 CFU/ml) produced a peak \([\text{Ca}^{2+}]_i\); response of 157 \pm 5 nM compared with the significantly \((P < 0.05)\) smaller peak responses of 157 \pm 4 and 143 \pm 5 nM, respectively, from the \(\text{cagA}^-\) and \(\text{picB}^-/\text{cagE}^-\) isogenic mutants (Table 2). Of the two mutant strains, the \(\text{picB}^-/\text{cagE}^-\)-induced \([\text{Ca}^{2+}]_i\); peak response of 143 \pm 5 nM was also found to be significantly \((P < 0.05)\) lower than the \(\text{cagA}^-\)-induced \([\text{Ca}^{2+}]_i\); peak response of 157 \pm 4 nM (Table 2). We also found that after treating the gastric cells for 15 min with either the \(\text{cagA}^-\) or \(\text{picB}^-/\text{cagE}^-\) mutant strains we could no longer detect a \([\text{Ca}^{2+}]_i\); change over the remainder of the 60-min time course (Fig. 2B; Table 2). That is, the \(\text{cagA}^-\) and \(\text{picB}^-/\text{cagE}^-\) isogenic mutants did not generate the typical prolonged plateau phase as seen with the \(H.\ pylori\) wild-type strain or \(\text{vacA}^-\) isogenic mutant (compare Fig. 2, B to A; Table 2). We have also found this same attenuated \([\text{Ca}^{2+}]_i\); response using other independent \(\text{picB}^-/\text{cagE}^-\) and \(\text{cagA}^-\) mutant strains derived from wild-type 60190 strain (M. J. Rutten and T. L. Cover, unpublished data). These data suggest that the expressed products of the \(\text{cagA}\) and \(\text{picB/cagE}\) genes (but not \(\text{vacA}\)) contribute to \(H.\ pylori\)-induced \([\text{Ca}^{2+}]_i\) changes.

\(H.\ pylori\)-induced \([\text{Ca}^{2+}]_i\) intracellular \(\text{Ca}^{2+}\) release vs. \(\text{Ca}^{2+}\) influx. One of the main intracellular \(\text{Ca}^{2+}\) stores within most cells is the thapsigargin-sensitive sarcoendoplasmic reticular (SERCA) \(\text{Ca}^{2+}\) store (43, 58). Thapsigargin inhibits the \(\text{Ca}^{2+}\)-ATPase on the SERCA membrane, which then leads to the release of \(\text{Ca}^{2+}\) and depletion of the intracellular \(\text{Ca}^{2+}\) store (43).

As a first step in identifying the source(s) of the \(H.\ pylori\)-induced \([\text{Ca}^{2+}]_i\); change, fura 2-loaded gastric mucous epithelial cells grown on glass slides were pretreated with 500 nM thapsigargin, and then \(H.\ pylori\) (1 \(\times\) 10^9 CFU/ml) was added and \([\text{Ca}^{2+}]_i\) was measured. In thapsigargin-pretreated gastric cells, \(H.\ pylori\) wild-type and \(\text{vacA}^-, \text{cagA}^-, \text{and picB}^-/\text{cagE}^-\) mutant strains all had reduced \([\text{Ca}^{2+}]_i\); peak levels from 184 \pm 4, 177 \pm 5, 157 \pm 4, and 143 \pm 3 nM to 117 \pm 4, 115 \pm 3, 112 \pm 4, and 110 \pm 3 nM, respectively \((n = 9;\) Fig. 3A). The \(H.\ pylori\)-induced wild-type and \(\text{vacA}^-\) \([\text{Ca}^{2+}]_i\); plateau change was only slightly diminished by the thapsigargin pretreatment (Fig. 3A).

Because the \(\text{cagA}^-\) and \(\text{picB}^-/\text{cagE}^-\) \(H.\ pylori\) strains were previously found to produce no \([\text{Ca}^{2+}]_i\); plateau change (see Fig. 2), the thapsigargin pretreatment of the gastric cells was without effect on this portion of the \([\text{Ca}^{2+}]_i\); response (Fig. 3B).

In addition to the release of intracellular \(\text{Ca}^{2+}\) stores, the opening of \(\text{Ca}^{2+}\) channels in the plasma cell membrane can also contribute to the change in \([\text{Ca}^{2+}]_i\); (43). A general experimental approach in examining the contribution of \(\text{Ca}^{2+}\); channels to the total agonist-induced \([\text{Ca}^{2+}]_i\); change is to use the \(\text{Ca}^{2+}\); add-back technique (see MATERIALS AND METHODS; Ref. 43). For these experiments, fura 2-loaded primary gastric mucous epithelial cells from wild-type 60190 and the \(\text{vacA}^-, \text{cagA}^-, \text{and picB}^-/\text{cagE}^-\) isogenic strains on \([\text{Ca}^{2+}]_i\); changes in fura 2-loaded primary cultures of normal human gastric mucous epithelial cells. The addition of the \(\text{vacA}^-\) strain (1 \(\times\) 10^9 CFU/ml) produced nearly identical \([\text{Ca}^{2+}]_i\); changes compared with the wild-type strain (A). Compared with the wild-type strain, the addition of the \(\text{cagA}^-, \text{and picB}^-/\text{cagE}^-\) isogenic strains (1 \(\times\) 10^9 CFU/ml) to the cultures produced only small \([\text{Ca}^{2+}]_i\); peak changes with no \([\text{Ca}^{2+}]_i\); plateau change (B). Heat-killed \(\text{vacA}^-, \text{cagA}^-, \text{and picB}^-/\text{cagE}^-\) isogenic strains produced no change in \([\text{Ca}^{2+}]_i\). For all tracings, \(n = 14\) independent experiments.
cous epithelial cells were first pretreated with extracellular Ca\(^{2+}\)-free Ringer solution. *H. pylori* was added, and [Ca\(^{2+}\)]\(_i\) was recorded, followed 30 min later by the readdition of extracellular Ca\(^{2+}\) to the Ringer. We found that there was only a small, nonsignificant (P > 0.05) reduction in the wild-type and vacA\(^-\) *H. pylori*-induced [Ca\(^{2+}\)]\(_i\) peak responses using extracellular Ca\(^{2+}\)-free buffer (Fig. 4A). However, the extracellular Ca\(^{2+}\) treatment greatly reduced the wild-type and vacA\(^-\) *H. pylori*-induced [Ca\(^{2+}\)]\(_i\), plateau response from control levels of 145 ± 4 and 141 ± 3 nM to 103 ± 4 and 100 ± 4 nM, respectively (n = 10; Fig. 4A). When extracellular Ca\(^{2+}\) was added back to the Ringer solution, we found that the [Ca\(^{2+}\)]\(_i\) in the *H. pylori* wild-type and vacA\(^-\)–treated gastric cultures rose to near control plateau levels (Fig. 4A). The extracellular Ca\(^{2+}\) treatment also did not significantly (P > 0.05) reduce the cagA\(^-\) and picB\(^-\)/cagE\(^-\)–induced [Ca\(^{2+}\)]\(_i\), peak response from control levels of 157 ± 4 and 106 ± 4 nM to 143 ± 5 and 105 ± 4 nM, respectively (n = 10; Fig. 4B). Because the cagA\(^-\) or picB\(^-\)/cagE\(^-\) mutant strains do not produce an [Ca\(^{2+}\)]\(_i\) plateau change (see Fig. 2), the effect of readdition of extracellular Ca\(^{2+}\) to the Ringer solution to detect Ca\(^{2+}\) influx was without effect on this portion of the [Ca\(^{2+}\)]\(_i\) response (Fig. 4B).

Fig. 3. Effects of intracellular Ca\(^{2+}\) depletion on *H. pylori*-induced [Ca\(^{2+}\)]\(_i\), changes in primary cultures of human gastric mucous epithelial cells. Fura 2-loaded human gastric mucous epithelial cell cultures were pretreated for 30 min with 500 nM thapsigargin to release intracellular Ca\(^{2+}\). The appropriate *H. pylori* strains (1 × 10\(^9\) CFU/ml) were then added, and [Ca\(^{2+}\)]\(_i\) changes were recorded. Compared with untreated control cultures, thapsigargin pretreatment of the gastric cells primarily affected the wild-type and vacA\(^-\) *H. pylori*-induced [Ca\(^{2+}\)]\(_i\), peak response with only small reductions in the [Ca\(^{2+}\)]\(_i\), plateau change (A). Thapsigargin pretreatment also reduced the cagA\(^-\) and picB\(^-\)/cagE\(^-\) *H. pylori*-induced [Ca\(^{2+}\)]\(_i\), peak changes (B). Because the cagA\(^-\) and picB\(^-\)/cagE\(^-\) *H. pylori* strains were found to produce no [Ca\(^{2+}\)]\(_i\), plateau change, the thapsigargin pretreatment of the gastric cells was without effect on this portion of the cagA\(^-\) and picB\(^-\)/cagE\(^-\) [Ca\(^{2+}\)]\(_i\), response (B). For all tracings, n = 10 independent experiments.

### Table 2. Effects of *H. pylori* wild-type and isogenic strains on [Ca\(^{2+}\)]\(_i\), changes in primary cultures of normal human gastric mucous epithelial cells

<table>
<thead>
<tr>
<th>Wild-type Strain 60190</th>
<th>vacA(^-)</th>
<th>cagA(^-)</th>
<th>picB(^-)/cagE(^-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak</td>
<td>Plateau</td>
<td>Peak</td>
<td>Plateau</td>
</tr>
<tr>
<td>184 ± 4(^a)</td>
<td>145 ± 4(^a)</td>
<td>177 ± 5(^a)</td>
<td>141 ± 3(^a)</td>
</tr>
</tbody>
</table>

Comparative effects of *H. pylori* wild-type and isogenic mutant strains on [Ca\(^{2+}\)]\(_i\), peak and plateau changes in primary cultures of normal human gastric mucous epithelial cells. Data are means ± SE (in nM) of 14 individual experiments done in duplicate. *P < 0.05 vs. baseline [Ca\(^{2+}\)], (104–106 nM) values. †P < 0.05 for [Ca\(^{2+}\)], peak value vs. cagA\(^-\) mutant [Ca\(^{2+}\)], peak value.

Overall, these experiments suggest that the *H. pylori*-induced peak [Ca\(^{2+}\)]\(_i\) change is dependent primarily on release of intracellular thapsigargin-sensitive Ca\(^{2+}\) stores, whereas the *H. pylori*-induced plateau [Ca\(^{2+}\)]\(_i\) change is primarily dependent on extracellular Ca\(^{2+}\) influx.

*H. pylori*-induced Ca\(^{2+}\) release is regulated by a PLC-dependent mechanism. Several agonists as well as certain bacterial pathogens have been shown to induce Ca\(^{2+}\) release from intracellular stores through a PLC-mediated process (42). In the next series of experiments, we used the PLC inhibitor U-73122 to test whether PLC activation is involved in *H. pylori*-induced [Ca\(^{2+}\)]\(_i\) changes. In the wild-type and vacA\(^-\) isogenic strains, the U-73122 pretreatment considerably reduced the [Ca\(^{2+}\)]\(_i\), peak increase with less of an effect on the [Ca\(^{2+}\)]\(_i\), plateau change (Fig. 5, A and B). Compared with untreated controls, the U-73122 also significantly reduced the cagA\(^-\) or picB\(^-\)/cagE\(^-\) peak change in [Ca\(^{2+}\)]\(_i\), (Fig. 5, C and D). Because the cagA\(^-\) and picB\(^-\)/cagE\(^-\) isogenic strains do not generate a plateau [Ca\(^{2+}\)]\(_i\) change, U-73122 was without effect on this portion of the [Ca\(^{2+}\)]\(_i\), response (Fig. 5, C and D). As a control, cultures were pretreated with the structurally related but ineffective PLC drug U-73343, and it...
did not alter any of the [Ca\textsuperscript{2+}]i changes produced by the wild-type, vacA\textsuperscript{-}, cagA\textsuperscript{-}, and picB\textsuperscript{-}cagE\textsuperscript{-} strains on the gastric cells (data not shown). However, it should be noted that the control compound U-73343 used at concentrations >2.5 \mu M actually inhibited H. pylori-induced Ca\textsuperscript{2+} signaling, indicating that at >2.5 \mu M both U-73122 and U-73343 exhibit nonspecific inhibitory effects (data not shown). These data suggest that H. pylori-induced intracellular Ca\textsuperscript{2+} release (but not Ca\textsuperscript{2+} influx) is under the control of a PLC-dependent mechanism.

H. pylori-induced [Ca\textsuperscript{2+}]i changes are regulated by src kinases. It has now been well documented that the src kinases play an important signaling role in the phosphorylation of the translocated CagA protein as well as participation of H. pylori-induced cytoskeletal changes (2, 16, 50, 55). It has also been shown in other cell types that src kinases play a modulatory role in agonist-induced [Ca\textsuperscript{2+}]i changes (9). We therefore wanted to examine the role of src kinases in H. pylori-induced intracellular Ca\textsuperscript{2+} signaling in gastric cells where src kinase activity was inhibited by using PP2. Pretreatment of the primary human gastric mucus epithelial cell cultures with PP2 produced a dose-dependent differential decrease in H. pylori wild-type and vacA\textsuperscript{-} isogenic [Ca\textsuperscript{2+}]i peak and plateau changes (Fig. 6). That is, PP2 pretreatment (0.5–5.0 \mu M) of the gastric cells was most effective in reducing the H. pylori wild-type and vacA\textsuperscript{-} mutant [Ca\textsuperscript{2+}]i plateau response (Fig. 6, A and B). Only at higher concentrations of PP2 (15 \mu M) did we observe a reduction in both the H. pylori wild-type strain and vacA\textsuperscript{-} mutant [Ca\textsuperscript{2+}]i peak and plateau changes to baseline levels (Fig. 6, A and B). We also found that only at the highest PP2 concentration used (15 \mu M) was the cagA\textsuperscript{-} and picB\textsuperscript{-}cagE\textsuperscript{-} [Ca\textsuperscript{2+}]i peak change reduced to baseline levels (Fig. 6, C and D). Because the cagA\textsuperscript{-} and picB\textsuperscript{-}cagE\textsuperscript{-} isogenic strains do not generate an [Ca\textsuperscript{2+}]i plateau change (see Fig. 2), the PP2 was without effect on this portion of the [Ca\textsuperscript{2+}]i response (Fig. 6, C and D). Overall, these results with the various concentrations of PP2 suggest that the src kinases are more likely to have a regulatory role in controlling the H. pylori wild-type and vacA\textsuperscript{-} mutant-induced [Ca\textsuperscript{2+}]i “plateau” (Ca\textsuperscript{2+} release) than the [Ca\textsuperscript{2+}]i “influx” change.

H. pylori-induced Ca\textsuperscript{2+} influx is regulated by G proteins and a PLA\textsubscript{2} dependent mechanism. In several cell types, agonist-induced changes in [Ca\textsuperscript{2+}]i, can be altered by PTX-sensitive G proteins as well as PLA\textsubscript{2} activity (23, 28). In addition, it has been reported that H. pylori-induced arachidonic acid release from the human cervical adenocarcinoma HeLa cell line could be abolished by PTX and the PLA\textsubscript{2} inhibitor MAFP (40). For the next series of experiments, we were therefore interested in determining the effects of PTX treatment on H. pylori-induced Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} influx in cultures of normal human gastric mucus epithelial cells. We found that PTX pretreatment of the gastric cells caused a reduction in both the wild-type and vacA\textsuperscript{-} H. pylori-[Ca\textsuperscript{2+}]i peak and plateau changes to baseline [Ca\textsuperscript{2+}]i levels (Fig. 7, A and B). The effect of PTX pretreatment also caused a reduction of the cagA\textsuperscript{-} and picB\textsuperscript{-}cagE\textsuperscript{-} mutant [Ca\textsuperscript{2+}]i peak change to near baseline levels (Fig. 7, C and D). Because the cagA\textsuperscript{-}
and picB⁻/cagE⁻ isogenic strains do not generate a [Ca²⁺]ₜ plateau change, the PTX pretreatment had no effect on this portion of the [Ca²⁺]ₜ response (Fig. 7, C and D).

We next investigated the effects of inhibiting cPLA₂ on H. pylori-induced intracellular Ca²⁺ signaling in primary cultures of normal human gastric mucous epithelial cells. We found no significant effect (P > 0.05) of MAFP on the wild-type and vacA⁺ H. pylori-induced [Ca²⁺]ₜ peak change, whereas MAFP had a significant effect (P < 0.05) on the wild-type and vacA⁻ H. pylori-induced [Ca²⁺]ₜ plateau change (Fig. 8, A and B). Also, MAFP had no significant effect (P > 0.05) on the cagA⁻ or picB⁻/cagE⁻-induced [Ca²⁺]ₜ peak change (Fig. 8, C and D). Because the cagA⁻ and picB⁻/cagE⁻ isogenic strains do not generate a [Ca²⁺]ₜ plateau change, MAFP was without effect on this portion of the [Ca²⁺]ₜ response (Fig. 8, C and D). Overall, these results suggest that the H. pylori-induced plateau phase ([Ca²⁺]ₜ influx) is under the regulation of a G protein/cPLA₂-dependent pathway.

**DISCUSSION**

Pathogenic bacteria have developed a variety of mechanisms to survive and interact with their respective host cells, where they exploit their respective host cell signaling pathways (11). H. pylori has also been shown to activate or use several different signaling pathways within the host gastric cell that eventually lead to the development of ulcers or gastric cancer (38, 57). However, the effect of H. pylori on intracellular Ca²⁺ signaling in normal human gastric epithelial cells has not been investigated. The present study is the first to describe the effects of H. pylori on the regulation of [Ca²⁺]ₜ changes in this cell type. We found that wild-type H. pylori produced a dose-dependent biphasic [Ca²⁺]ₜ change within primary human gastric mucous epithelial cells and in a nontransformed gastric mucous epithelial cell line (HFE-145 cells). The changes in [Ca²⁺]ₜ by H. pylori were dependent on the presence of intact live bacteria, since bacterial sonicates or heat-killed bacteria produced no change in [Ca²⁺]ₜ. The wild-type H. pylori-induced [Ca²⁺]ₜ response was also found to consist of two phases, the first being the release of Ca²⁺ from intracellular Ca²⁺ stores (peak phase) followed by the activation of a Ca²⁺-influx mechanism (plateau phase).

In the present study, we also examined a role for the H. pylori vacA, cagA, and picB/cagE genes on intracellular Ca²⁺ signaling. The H. pylori VacA toxin has been shown to produce several membrane permeability events in gastric cells, and it is also an important virulence factor in the pathogenesis of peptic ulcer disease (36). However, from our studies we conclude that the H. pylori VacA toxin has no direct role in mediating H. pylori-induced [Ca²⁺]ₜ changes. That is,
we found no difference between our *H. pylori* wild-type strain and a *vacA*− isogenic mutant in their abilities to produce an [Ca\(^{2+}\)]\(_i\) change. This finding is in contrast to other pathogens in which extracellular toxins have been shown to have a role in host cell Ca\(^{2+}\) signaling. For example, the pore-forming toxin aerolysin, from *Aeromonas hydrophila*, has been shown to activate G protein-dependent intracellular Ca\(^{2+}\) release in human granulocytes (24). In contrast to the *vacA* mutant, we found that the [Ca\(^{2+}\)]\(_i\) response was greatly reduced when the gastric cells were treated with either a *cagA*− or *picB*−/*cagE*− isogenic mutant strain. Specifically, we found that the [Ca\(^{2+}\)]\(_i\) peak change was markedly reduced with the *cagA*− and *picB*−/*cagE*− isogenic mutants, and these mutant stains did not generate the typical prolonged plateau phase as seen with the *H. pylori* wild-type strain or *vacA*− mutant. In addition, the *picB*−/*cagE*−-induced [Ca\(^{2+}\)]\(_i\) peak response was found to be significantly lower than the *cagA*−-induced [Ca\(^{2+}\)]\(_i\) peak response.

**Role of signaling intermediates on *H. pylori*-induced [Ca\(^{2+}\)]\(_i\) changes.** After identifying the initial *H. pylori*-induced [Ca\(^{2+}\)]\(_i\) response, the mechanistic components for each of the *H. pylori*-induced [Ca\(^{2+}\)]\(_i\) phases were examined by using different kinase or drug inhibitors. We found, for example, that the pretreatment of the gastric cells with the PLC inhibitor U-73122, and not the structural control U-73343 analog, attenuated the wild-type *H. pylori*-induced [Ca\(^{2+}\)]\(_i\) peak phase to near control levels. Even more effective was the G\(_\alpha\) protein inhibitor PTX, which completely reduced the *H. pylori*-induced [Ca\(^{2+}\)]\(_i\) peak phase to baseline control levels in all of the *H. pylori* wild-type and mutant strains tested. PTX pretreatment was also effective in reducing the wild-type and *vacA*− mutant *H. pylori*-induced [Ca\(^{2+}\)]\(_i\) plateau (Ca\(^{2+}\) influx) phase. However, because the *cagA*− and *picB*−/*cagE*− isogenic strains do not generate an [Ca\(^{2+}\)]\(_i\) plateau response, PTX was without effect on this portion of the [Ca\(^{2+}\)]\(_i\) response (C and D). Data are from 10 independent experiments and expressed as means ± SE; *p < 0.05 vs. control peak and plateau levels.

![Diagram](http://ajpgi.physiology.org/)

Fig. 6. Dose-response effects of src kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(4-butylopyrazolo[3,4-d] pyrimidine (PP2) on *H. pylori*-induced [Ca\(^{2+}\)]\(_i\) changes in cultures of human gastric mucous epithelial cells. Fluor-loaded human gastric mucous epithelial cell cultures were pretreated for 30 min with varying doses of PP2; then the appropriate *H. pylori* strains (1 × 10\(^6\) CFU/ml) were added and [Ca\(^{2+}\)]\(_i\), was recorded. In the wild-type (A) and *vacA*− isogenic (B) strains, low to intermediate doses of PP2 (0.5–5.0 μM) primarily attenuated the [Ca\(^{2+}\)]\(_i\) plateau change, with higher doses (15 μM) of PP2 completely reducing both the peak and plateau changes to control levels. Compared with untreated controls, PP2 also dose-dependently reduced the *cagA*− (C) and *picB*−/*cagE*− (D) [Ca\(^{2+}\)]\(_i\) peak change. Because the *cagA*− and *picB*−/*cagE*− isogenic strains do not generate an [Ca\(^{2+}\)]\(_i\) plateau response, PP2 was without effect on this portion of the [Ca\(^{2+}\)]\(_i\) response (C and D).
cPLA₂ activity will increase arachidonic acid, which can modulate Ca²⁺ influx (23). It is possible that the first \textit{H. pylori}-induced phase of intracellular Ca²⁺ mobilization will secondarily activate cPLA₂, which in turn could generate arachidonic acid and modulate Ca²⁺ influx (Fig. 9).

\textbf{H. pylori, Ca²⁺ signaling, and host cell pathogenesis.}

It is now known that \textit{H. pylori} can initiate multiple signaling pathways within the host gastric cell by using a variety of effector stimuli, ranging from small extracellular molecules, such as urease-generated ammonia, to the VacA toxin or the use of specialized injected molecules such as the CagA protein (57). As a focus for our own study, we chose to look at what role the VacA toxin, the CagA protein, and a functional type IV injection system may have on intracellular Ca²⁺ changes in normal human gastric mucous epithelial cells. We also chose to use a buffered urea-free Ringer solution (pH 7.4) to help minimize the potential effects solution (pH 7.4) to help minimize the potential effects of the bacteria, we believe that soon after bacteria adherence, certain signaling intermediates are immediately activated, such as G proteins and PLC, which were both found to be important in the \textit{H. pylori}-induced [Ca²⁺]ᵢ peak change (Fig. 9). In other cell types, it is well established that agonists or pathogens can quickly increase PLC activity and the formation of inositol trisphosphate, which releases Ca²⁺ from intracellular Ca²⁺ stores (3). Our time-course and inhibitor studies suggest that \textit{H. pylori} is likely to involve a similar PLC pathway that produced the characteristic rise and fall of [Ca²⁺]ᵢ observed within the first 15 min after the addition of bacteria. It should be emphasized again that our cagA⁻ isogenic mutant produced only a small [Ca²⁺]ᵢ peak response (and no [Ca²⁺]ᵢ plateau change) and that this cagA⁻-induced peak [Ca²⁺]ᵢ change was further reduced to baseline [Ca²⁺]ᵢ levels by the PLC inhibitor U-73122. These data indicate that there may be CagA-dependent and CagA-independent pathways for PLC activation, but overall, increases in PLC activity along with the physical translocation of the CagA protein are likely to be the major contributing factors to the initial \textit{H. pylori}-induced [Ca²⁺]ᵢ change. It is noteworthy that other studies (2) have shown that phosphorylated CagA protein can be detected as early as 15 min after the addition of \textit{H. pylori} to gastric cells, which is well within the time course of our [Ca²⁺]ᵢ peak response.

Another facet of intracellular Ca²⁺ signaling is the potential for cross-talk between different receptor systems (9). For example, it has been reported that \textit{H. pylori}, Ca²⁺ signaling in human gastric mucous cells.
H. pylori can transactivate the EGFR (21, 62). Although the role of H. pylori transactivation of the EGFR and Ca\(^{2+}\) signaling was not examined in our study, it has been reported that Salmonella can transactivate the EGFR to produce an increase in \([\text{Ca}^{2+}]_i\) that was important for bacterial entry (35). H. pylori can also activate adenylate cyclase and increase intracellular cAMP within AGS gastric cancer cells, and the H. pylori-induced cAMP increase is independent of the vacA, cagA, and cag PAI genes (63). In this regard, intracellular Ca\(^{2+}\) release has also been shown to be regulated by cAMP and PKA (3), suggesting that H. pylori is capable of activating multiple receptor systems that are in turn capable of coordinating host cell Ca\(^{2+}\) signaling.

Our study also suggests that the translocated CagA protein may be necessary for the continuation of the Ca\(^{2+}\) signal for the induction of \([\text{Ca}^{2+}]_i\) plateau (Ca\(^{2+}\) influx) change. That is, in addition to the inability of our cagA\(^{-}\) mutant to produce the \([\text{Ca}^{2+}]_i\) peak phase, we also found that the src kinase inhibitor PP2 (which has been shown to block CagA protein phosphorylation; Ref. 50) was also capable of inhibiting the H. pylori-induced \([\text{Ca}^{2+}]_i\) peak phase. It has been suggested that the ability of the CagA protein to perturb host cell functions is dependent on the number and sequences of tyrosine sites that are phosphorylated (15, 41). At this time we do not know to what degree the src kinase is responsible for CagA protein phosphorylation.
CagA protein has to be phosphorylated to induce the \textit{H. pylori} [Ca\textsuperscript{2+}] changes.

Several studies have also implicated a link between an \textit{H. pylori}-induced [Ca\textsuperscript{2+}] change and a biological response. That is, pretreatment of MKN45 gastric cancer cells with the intracellular Ca\textsuperscript{2+} chelator BAPTA was shown to completely block wild-type \textit{H. pylori}-induced IL-8 secretion (32). It has also been reported that BAPTA was able to block \textit{H. pylori}-induced arachidonic acid release that is involved in the production of prostaglandin E\textsubscript{2} (40). Of interest, however, was the fact that the expression of the CagA protein was not important for the above-mentioned Ca\textsuperscript{2+}-dependent \textit{H. pylori}-induced IL-8 release (32), whereas the CagA protein was necessary for Ca\textsuperscript{2+}-dependent \textit{H. pylori}-induced arachidonic acid synthesis (40). One might propose that, depending on the final \textit{H. pylori}-induced [Ca\textsuperscript{2+}] change, different signaling pathways could be activated based on a specific [Ca\textsuperscript{2+}]\textsuperscript{−} mutant, which we have shown generates only a small [Ca\textsuperscript{2+}]\textsuperscript{−} peak change, this small [Ca\textsuperscript{2+}]\textsuperscript{−} response may be sufficient enough to release IL-8, but a larger [Ca\textsuperscript{2+}] threshold and the CagA protein are both needed for activation of the arachidonic acid/prostaglandin signaling pathway. In this context, it is also possible that the various components of the \textit{H. pylori}-induced biphasic [Ca\textsuperscript{2+}] signal, i.e., the peak and plateau phases, may be utilized differently depending on the nature of the Ca\textsuperscript{2+}-dependent signaling molecule within the host gastric cell. It also appears that differential signaling by \textit{H. pylori} may hold true for other gastric host cell responses. That is, \textit{H. pylori}-induced MAP kinase activity has also been reported to be induced in a “biphasic” manner over several hours (27). In addition, recent microarray transcriptional studies of \textit{H. pylori}-treated AGS gastric cancer cells found that many \textit{H. pylori}-induced signaling genes are transiently expressed within 1 h (14). It is also highly likely that there are other genes inside or outside the \textit{H. pylori} cag PAI, as well certain structural components from \textit{H. pylori} itself, that could participate in the \textit{H. pylori}-induced [Ca\textsuperscript{2+}] response. For example, it has been suggested that the direct binding of the defective picB/cagE type IV injection apparatus to the plasma cell membrane itself is enough to activate other receptors, as well as translocate other unknown molecules through the type IV injection apparatus that can participate in host cell responses (51).

In summary, we found that \textit{H. pylori} produces specific transient [Ca\textsuperscript{2+}] changes in normal human gastric mucous epithelial cells and that these \textit{H. pylori}-induced [Ca\textsuperscript{2+}] changes could also be replicated in a nontransformed gastric mucous epithelial cell line (HFE-145 cells). A G protein/PLC-dependent pathway primarily regulated the \textit{H. pylori}-induced intracellular Ca\textsuperscript{2+} release, whereas \textit{H. pylori}-induced Ca\textsuperscript{2+} influx was under the control of a G protein-, src kinase-, and PLA\textsubscript{2} dependent pathway (Fig. 9). Finally, we report that mutagenesis of picB/cagE and cagA genes (located within the cag PAI), but not the vacA gene, alters the capacity of \textit{H. pylori} to produce a full [Ca\textsuperscript{2+}] response.

For future studies, it will be important to look at other genes inside and outside the cag PAI to determine their effects on \textit{H. pylori}-induced [Ca\textsuperscript{2+}] changes.

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