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

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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 ~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 *picBcagE* 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 E\(_2\) synthesis (40), in a decreased stress kinase gene expression (30), and in reduced expression of the protooncogenes *c-fos* and *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\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]). In other model systems, bacterial adherence to the host cell has been shown to result in specific [Ca\textsuperscript{2+}] changes (10). For example, the adherence of certain Escherichia coli strains to intestinal epithelial cells results in increased [Ca\textsuperscript{2+}] and inositol trisphosphate levels (11). It has also been shown that Salmonella induces intracellular Ca\textsuperscript{2+} changes that were linked to the activation of an NF-kB-dependent inflammatory pathway (13). In this regard, a study using the intracellular Ca\textsuperscript{2+} chelator BAPTA along with calmodulin inhibitors found that H. pylori activation of NF-kB and IL-8 signaling in MK45 human gastric cancer cells was Ca\textsuperscript{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\textsuperscript{2+}], 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\textsuperscript{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 gastric cancer cell lines, 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\textsuperscript{2+} signaling in normal human gastric epithelial cells. We found for the first time that H. pylori produces specific transient [Ca\textsuperscript{2+}] changes in normal human gastric mucous epithelial cells and that these H. pylori-induced [Ca\textsuperscript{2+}] 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\textsuperscript{2+} release, whereas H. pylori-induced Ca\textsuperscript{2+} influx was primarily regulated by components of a G protein-, src kinase-, and PL\textsubscript{A2}-dependent pathway. Finally, we report that mutagenesis of vacA gene, alters the capacity of H. pylori to produce a full [Ca\textsuperscript{2+}] response.

**MATERIALS AND METHODS**

**Chemicals and peptides.** The Ca\textsuperscript{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\textsuperscript{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 arachidonyl fluorophosphatase (MAFP), thapsigargin, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d] pyrimidine (PP2), pertussis toxin (PTX), U-73122, and U-73343 were purchased from Calbiochem (San Diego, CA). Type I collagenase, RIA grade TSA powders, Triton X-100, digitonin, and genistein were purchased from Sigma. Cell culture media 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 Sygard, and the epithelium was removed by scraping the surface with a glass slide. The scraped tissue pieces were enzymatically digested by mRNAs slides then washed three times at 100 g for 3 min in serum-free media. 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\textsubscript{2}-5% CO\textsubscript{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, 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 at 24°C, and the bottom pellet, containing the gastric mucous epithelial cells, were 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 *picBl-cagE* mutant. *H. pylori* 60190 contains a type 1α/1a vacA allele (59). The vacA°, cagA°, and *picBl-cagE* mutants have been previously described (39, 52, 59). The bacteria were grown on blood agar plates (tryptose soy agar with 5% sheep blood; PML Microbiologicals, Duluth, 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 (OD600) where an OD of 1 = 1.2 x 10⁸ colony-forming units (CFU)/ml. All final bacterial suspensions (1 x 10⁸–1 x 10⁹ CFU/ml) were adjusted with the appropriate mammalian Ringer solution. The mammalian Ringer solution consisted of (in mM): 137 NaCl, 4 KCl, 25 NaHCO₃, 2 KH₂PO₄, 15 HEPES, 1 MgSO₄, 2 CaCl₂, 25 glucose, pH 7.4. Periodically, the bacteria were plated in serial dilutions on agar plates and H. pylori concentrations were checked by counting the bacterial colonies after 3 days of incubation.

*H. pylori* sonicates were made by growing the bacteria on agar plates for 24 h and then harvesting the bacteria in PBS as indicated above. The bacteria were washed twice in PBS by centrifugation at 10,000 g for 15 min, and then the pellet was resuspended in mammalian Ringer (pH 7.4). The bacterial suspensions were disrupted by sonication (10 30-s pulses), the sonicates were filtered through a 0.2-μm filter, and the protein content was determined by using a Bio-Rad protein assay. Aliquots were frozen and stored at -80°C until needed. For control studies, both live bacteria and bacterial sonicates were heated to 70°C for 30 min to generate heat-inactivated bacteria and sonicates.

*H. pylori* and gastric cell [Ca²⁺], using fura 2. Primary cultures of gastric mucous epithelial cells, the HFE-145 cell line, and AGS gastric cancer cells were grown on either permeable Falcon filters or 25-mm round glass coverslips. After 24 h in serum-free media, the cells were loaded with fura 2-AM according to modifications of previously described techniques (47). Briefly, the cells were loaded with 2.5 μM fura 2-AM in fresh serum-free media for 45 min at 37°C. After fura 2-AM loading, the cells were washed twice with fresh serum-free media, then twice with mammalian Ringer. When extracellular Ca²⁺-free Ringer solutions were used, the CaCl₂ was replaced with NaCl and the solution was characterized as nominally Ca²⁺-free Ringer. In preliminary experiments, we found that the use of our nominally Ca²⁺-free Ringer solution did not affect *H. pylori* adherence. However, the addition of 1 mM EDTA and 1 mM EGTA to the nominally Ca²⁺-free Ringer solution decreased *H. pylori* adherence and disrupted monolayer integrity over the experimental time period and therefore could not be used (data not shown). Solutions were oxygenated with 5% CO₂–95% O₂, kept warm at 37°C in a heated water bath, and perfused into the chamber by using a variable Millipore pump.

After fura 2 loading, the gastric cultures were transferred to a horizontal open perfusion chamber that had been modified to hold permeable Falcon filters or a glass coverslip (48). The chamber was then placed on the stage of a Nikon Diaphot TMD inverted microscope equipped with a fluorescence objective (Nikon Fluor-phase-3DM, numerical aperture 1.00/0.7, 160-mm working distance). [Ca²⁺], measurements were made at 340/380 nm excitation and 510 nm emission wavelengths from an SLM-Aminco spectrophotometer (Rochester, NY). The effects of nonspecific *H. pylori* fluorescence scatter and cell autofluorescence were determined by placing an unloaded gastric cell monolayer with varying doses of *H. pylori* (1 x 10⁵–1 x 10⁶ CFU/ml) on the microscope stage, then emission ratios were recorded and subtracted from the final fura 2 tracings. In preliminary experiments, we found light scattering by 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 Ca²⁺ release and influx were estimated by using the “Ca²⁺ add-back technique” (43). For these experiments, fura 2-loaded gastric cells are initially incubated in the absence of extracellular Ca²⁺, then *H. pylori* is added and [Ca²⁺]i is recorded as intracellular Ca²⁺ release, then extracellular Ca²⁺ (2.0 mM) is “added back" and the [Ca²⁺]i change is measured as Ca²⁺ influx.

The final calibration of the fura 2 signal was done at the end of each experiment by adding 5 μM ionomycin for 10 min to saturate fura 2 with Ca²⁺ to obtain maximal fluorescence (FMAX), then 10 mM EGTA and 10 mM EDTA plus 60 mM Tris-HCl Tris-HCl pH 10.5 was added to chelate the Ca²⁺ from fura 2 to determine the minimal fluorescence (FMIN). Additional points on the calibration curve were determined by using a series of defined Ca²⁺-calibration solutions (Kit #1, C-3008; Molecular Probes), and [Ca²⁺]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 [Ca²⁺], using a fluo 4 96-well assay. In some experiments we wanted a quicker throughput assay to measure the relative change in [Ca²⁺]i produced by *H. pylori* with different kinase inhibitors. For these experiments, measurement of [Ca²⁺]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 μM 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. Fluo 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 FMAX measurement for the human gastric epithelial cells was obtained by adding a solution of 5 μM ionomycin and 100 μM digitonin to the gastric cells for 15 min. After this, the FMIN 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 FMAX and FMIN 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 Sigmasoft statistical software (SPSS, San Rafael, CA).

**RESULTS**

*H. pylori* mobilizes intracellular Ca²⁺ 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 × 10^9 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*-induced intracellular Ca^{2+} response that was observed in early cultures (data not shown).

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

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**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>Peak</td>
<td>Plateau</td>
<td>Peak</td>
</tr>
<tr>
<td>1 × 10^9</td>
<td>184 ± 4*</td>
<td>145 ± 4*</td>
</tr>
<tr>
<td>1 × 10^8</td>
<td>170 ± 4*</td>
<td>136 ± 3*</td>
</tr>
<tr>
<td>1 × 10^7</td>
<td>144 ± 3*</td>
<td>125 ± 3*</td>
</tr>
<tr>
<td>1 × 10^6</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.

**Fig. 1.** Graphs showing representative tracings of intracellular Ca^{2+} concentration ([Ca^{2+}]_i) changes with varying concentrations [1 × 10^5–1 × 10^9 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+}]_i, 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.
The role of vacA, cagA, and picB/cagE genes in H. pylori-induced [Ca\(^{2+}\)]\(_i\) changes. It is well established that the VacA toxin secreted by H. pylori is an important virulence factor in the pathogenesis of peptic ulcer disease (36). In addition, the CagA protein encoded by the cagA gene within the H. pylori cag PAI is involved in gastric host cell cytoskeletal responses (5, 16, 49–51). Also, residing within the cag PAI is the picB/cagE gene, which encodes a structural component of the type IV secretion system that is important in translocating the 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 vacA, cagA, and picB/cagE genes on intracellular Ca\(^{2+}\) signaling. Using a vacA\(^{-}\) isogenic mutant strain on primary cultures of human gastric mucous epithelial cells, we found that the vacA\(^{-}\) isogenic mutant produced peak and plateau [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 vacA\(^{-}\) isogenic mutant strain produced no change in [Ca\(^{2+}\)]; in the gastric cells (Fig. 2A). In contrast to the wild-type and vacA\(^{-}\) isogenic mutant strains, the cagA\(^{-}\) and picB\(^{-}\)/cagE\(^{-}\) isogenic strains produced markedly attenuated [Ca\(^{2+}\)]\(_i\) changes (Fig. 2B). That is, the wild-type strain (1 \(\times\) 10\(^9\) CFU/ml) produced a peak [Ca\(^{2+}\)]; response of 184 ± 4 nM compared with the significantly (P < 0.05) smaller peak responses of 157 ± 4 and 143 ± 5 nM, respectively, from the cagA\(^{-}\) and picB\(^{-}\)/cagE\(^{-}\} isogenic mutants (Table 2). Of the two mutant strains, the picB\(^{-}\)/cagE\(^{-}\} induced [Ca\(^{2+}\)]\(_i\); peak response of 143 ± 5 nM was also found to be significantly (P < 0.05) lower than the cagA\(^{-}\} induced [Ca\(^{2+}\)]\(_i\); peak response of 157 ± 4 nM (Table 2). We also found that after treating the gastric cells for 15 min with either the cagA\(^{-}\) or picB\(^{-}\)/cagE\(^{-}\} mutant strains we could no longer detect a [Ca\(^{2+}\}]\(_i\) change over the remainder of the 60-min time course (Fig. 2B; Table 2). That is, the cagA\(^{-}\} and picB\(^{-}\)/cagE\(^{-}\} isogenic mutants did not generate the typical prolonged plateau phase as seen with the H. pylori wild-type strain or vacA\(^{-}\} isogenic mutant (compare Fig. 2, B to A; Table 2). We have also found this same attenuated [Ca\(^{2+}\)]\(_i\) response using other independent picB\(^{-}\)/cagE\(^{-}\} and 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 cagA and picB/cagE genes (but not vacA) contribute to H. pylori-induced [Ca\(^{2+}\)]\(_i\) changes.

H. pylori-induced [Ca\(^{2+}\)]\(_i\): intracellular Ca\(^{2+}\) release vs. Ca\(^{2+}\) influx. One of the main intracellular Ca\(^{2+}\) stores within most cells is the thapsigargin-sensitive sarcoplasmic reticular (SERCA) Ca\(^{2+}\) store (43, 58). Thapsigargin inhibits the Ca\(^{2+}\)-ATPase on the SERCA membrane, which then leads to the release of the intracellular Ca\(^{2+}\) store (43). As a first step in identifying the source(s) of the H. pylori-induced [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 [Ca\(^{2+}\)]\(_i\) was measured. In thapsigargin-pretreated gastric cells, H. pylori wild-type and vacA\(^{-}\}, cagA\(^{-}\}, and picB\(^{-}\}/cagE\(^{-}\} mutant strains all had reduced [Ca\(^{2+}\)]\(_i\) compared with the wild-type strain, the vacA\(^{-}\} isogenic mutant (com-}
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 (\pm) 4(^a)</td>
<td>145 (\pm) 4(^a)</td>
<td>177 (\pm) 5(^a)</td>
<td>141 (\pm) 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 \(\pm\) 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 cagA\(^{-}\) mutant [Ca\(^{2+}\)], peak value.

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 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 \(\pm\) 4 and 141 \(\pm\) 3 nM to 103 \(\pm\) 4 and 100 \(\pm\) 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 \(\pm\) 4 and 151 \(\pm\) 4 nM to 143 \(\pm\) 5 and 138 \(\pm\) 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).

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

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 \(\times\) 10\(^6\) 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.
and vacA mechanism. We found that H. pylori-induced [Ca\(^{2+}\)]\(_{i}\) was an immediate rise in extracellular Ca\(^{2+}\) and vacA--H. pylori-induced [Ca\(^{2+}\)]\(_{i}\) plateau response with only small reductions in the [Ca\(^{2+}\)]\(_{i}\), peak change (A). When extracellular Ca\(^{2+}\) was “added back” to the Ringer solution (double arrows), there was an immediate rise in [Ca\(^{2+}\)]\(_{i}\), to plateau levels like that seen H. pylori-treated gastric cells in Ringer with Ca\(^{2+}\) (A; compare with Fig. 1A). In contrast, the Ca\(^{2+}\)-free Ringer pretreatment of the gastric cells only slightly reduced the cagA- and picB-/[cagE] [Ca\(^{2+}\)]\(_{i}\) peak changes (B). Because there is no H. pylori-induced [Ca\(^{2+}\)]\(_{i}\) plateau change produced by either the cagA- or picB-/[cagE] mutant strains, the effect of the removal and readdition of extracellular Ca\(^{2+}\) to the Ringer solution (double arrows) was without effect on this portion of the [Ca\(^{2+}\)]\(_{i}\), response (B). For all tracings, n = 10 independent experiments.

Fig. 4. Effects of extracellular Ca\(^{2+}\)-free Ringer 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 first exposed to extracellular Ca\(^{2+}\)-free Ringer for 15 min to minimize Ca\(^{2+}\) influx; then the appropriate H. pylori strains (1 × 10\(^6\) CFU/ml) were introduced and [Ca\(^{2+}\)]\(_{i}\) was recorded. Later, 2.0 mM Ca\(^{2+}\) was returned to the extracellular Ringer and [Ca\(^{2+}\)]\(_{i}\) was measured again. We found that Ca\(^{2+}\)-free Ringer pretreatment of the gastric cells primarily affected the wild-type and vacA--H. pylori-induced [Ca\(^{2+}\)]\(_{i}\), plateau response with only small reductions in the [Ca\(^{2+}\)]\(_{i}\), peak change (A). When extracellular Ca\(^{2+}\) was “added back” to the Ringer solution (double arrows), there was an immediate rise in [Ca\(^{2+}\)]\(_{i}\), to plateau levels like that seen H. pylori-treated gastric cells in Ringer with Ca\(^{2+}\) (A; compare with Fig. 1A). In contrast, the Ca\(^{2+}\)-free Ringer pretreatment of the gastric cells only slightly reduced the cagA- and picB-/[cagE] [Ca\(^{2+}\)]\(_{i}\) peak changes (B). Because there is no H. pylori-induced [Ca\(^{2+}\)]\(_{i}\) plateau change produced by either the cagA- or picB-/[cagE] mutant strains, the effect of the removal and readdition of extracellular Ca\(^{2+}\) to the Ringer solution (double arrows) was without effect on this portion of the [Ca\(^{2+}\)]\(_{i}\), response (B). For all tracings, n = 10 independent experiments.

did not alter any of the [Ca\(^{2+}\)]\(_{i}\) changes produced by the wild-type, vacA--, cagA--, and picB-/[cagE] strains on the gastric cells (data not shown). However, it should be noted that the control compound U-73343 used at concentrations >2.5 μM actually inhibited H. pylori-induced Ca\(^{2+}\) signaling, indicating that at >2.5 μM both U-73122 and U-73343 exhibit nonspecific inhibitory effects (data not shown). These data suggest that H. pylori-induced intracellular Ca\(^{2+}\) release (but not Ca\(^{2+}\) influx) is under the control of a PLC-dependent mechanism.

H. pylori-induced [Ca\(^{2+}\)]\(_{i}\) 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\(^{2+}\)]\(_{i}\) changes (9). We therefore wanted to examine the role of src kinases in H. pylori-induced intracellular Ca\(^{2+}\) signaling in gastric cells where src kinase activity was inhibited by using PP2. Pretreatment of the primary human gastric mucous epithelial cell cultures with PP2 produced a dose-dependent differential decrease in H. pylori wild-type and vacA- isogenic [Ca\(^{2+}\)]\(_{i}\) peak and plateau changes (Fig. 6). That is, PP2 pretreatment (0.5–5.0 μM) of the gastric cells was most effective in reducing the H. pylori wild-type and vacA- mutant [Ca\(^{2+}\)]\(_{i}\) plateau response (Fig. 6, A and B). Only at higher concentrations of PP2 (15 μM) did we observe a reduction in both the H. pylori wild-type strain and vacA- mutant [Ca\(^{2+}\)]\(_{i}\) peak and plateau changes to baseline levels (Fig. 6, A and B). We also found that only at the highest PP2 concentration used (15 μM) was the cagA- and picB-/[cagE] [Ca\(^{2+}\)]\(_{i}\) peak change reduced to baseline levels (Fig. 6, C and D). Because the cagA- and picB-/[cagE] isogenic strains do not generate an [Ca\(^{2+}\)]\(_{i}\) plateau change (see Fig. 2), the PP2 was without effect on this portion of the [Ca\(^{2+}\)]\(_{i}\) 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- mutant-induced [Ca\(^{2+}\)]\(_{i}\) “plateau” (Ca\(^{2+}\) release) than the [Ca\(^{2+}\)]\(_{i}\) “influx” change.

H. pylori-induced Ca\(^{2+}\) influx is regulated by G proteins and a PLA\(_{2}\)-dependent mechanism. In several cell types, agonist-induced changes in [Ca\(^{2+}\)]\(_{i}\), can be altered by PTX-sensitive G proteins as well as PLA\(_{2}\) 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\(_{2}\) 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\(^{2+}\) release and Ca\(^{2+}\) influx in cultures of normal human gastric mucous epithelial cells. We found that PTX pretreatment of the gastric cells caused a reduction in both the wild-type and vacA- H. pylori-[Ca\(^{2+}\)]\(_{i}\), peak and plateau changes to baseline [Ca\(^{2+}\)]\(_{i}\), levels (Fig. 7, A and B). The effect of PTX pretreatment also caused a reduction of the cagA- and picB-/[cagE] mutant [Ca\(^{2+}\)]\(_{i}\), peak change to baseline levels (Fig. 7, C and D). Because the cagA-
and picB−/cagE− isogenic strains do not generate a \([\text{Ca}^{2+}]\); plateau change, the PTX pretreatment had no effect on this portion of the \([\text{Ca}^{2+}]\); response (Fig. 7, C and D).

We next investigated the effects of inhibiting ePLA2 on H. pylori-induced intracellular \(\text{Ca}^{2+}\) 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 \([\text{Ca}^{2+}]\); peak change, whereas MAFP had a significant effect \((P < 0.05)\) on the wild-type and vacA− H. pylori-induced \([\text{Ca}^{2+}]\); plateau change (Fig. 8 A and B). Also, MAFP had no significant effect \((P > 0.05)\) on the cagA− or picB−/cagE−-induced \([\text{Ca}^{2+}]\); peak change (Fig. 8, C and D). Because the cagA− and picB−/cagE− isogenic strains do not generate a \([\text{Ca}^{2+}]\); plateau change, MAFP was without effect on this portion of the \([\text{Ca}^{2+}]\); response (Fig. 8, C and D). Overall, these results suggest that the H. pylori-induced plateau phase \([\text{Ca}^{2+}]\); influx is under the regulation of a G protein/ePLA2-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 \(\text{Ca}^{2+}\) 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 \([\text{Ca}^{2+}]\); changes in this cell type. We found that wild-type H. pylori produced a dose-dependent biphasic \([\text{Ca}^{2+}]\); change within primary human gastric mucous epithelial cells and in a nontransformed gastric mucous epithelial cell line (HFE-145 cells). The changes in \([\text{Ca}^{2+}]\); by H. pylori were dependent on the presence of intact live bacteria, since bacterial sonicates or heat-killed bacteria produced no change in \([\text{Ca}^{2+}]\). The wild-type H. pylori-induced \([\text{Ca}^{2+}]\); response was also found to consist of two phases, the first being the release of \(\text{Ca}^{2+}\); from intracellular \(\text{Ca}^{2+}\); stores (peak phase) followed by the activation of a \(\text{Ca}^{2+}\);-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 \(\text{Ca}^{2+}\) 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 \([\text{Ca}^{2+}]\); 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 [Ca²⁺]ᵢ change. This finding is in contrast to other pathogens in which extracellular toxins have been shown to have a role in host cell Ca²⁺ signaling. For example, the pore-forming toxin aerolysin, from *Aeromonas hydrophila*, has been shown to activate G protein-dependent intracellular Ca²⁺ release in human granulocytes (24). In contrast to the *vacA* mutant, we found that the [Ca²⁺]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 [Ca²⁺]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 [Ca²⁺]ᵢ peak response was found to be significantly lower than the *cagA*⁻-induced [Ca²⁺]ᵢ peak response.

**Role of signaling intermediates on *H. pylori*-induced [Ca²⁺]ᵢ changes.** After identifying the initial *H. pylori*-induced [Ca²⁺]ᵢ response, the mechanistic components for each of the *H. pylori*-induced [Ca²⁺]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 [Ca²⁺]ᵢ peak phase to near control levels. Even more effective was the G₁₀ protein inhibitor PTX, which completely reduced the *H. pylori*-induced [Ca²⁺]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 [Ca²⁺]ᵢ, plateau (Ca²⁺ influx) phase. However, because the *cagA*⁻ and *picB*⁻/*cagE*⁻ isogenic strains do not generate an [Ca²⁺]ᵢ plateau response, PP2 was without effect on this portion of the [Ca²⁺]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.

**Fig. 6.** Dose-response effects of src kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(butyl)pyrazolo[3,4-d] pyrimidine (PP2) on *H. pylori*-induced [Ca²⁺]ᵢ changes in cultures of human gastric mucus epithelial cells. Fluor 4-loaded gastric mucus epithelial cell cultures were pretreated for 30 min with varying doses of PP2; then the appropriate *H. pylori* strains (1 × 10⁶ CFU/ml) were added and [Ca²⁺]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 [Ca²⁺]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) [Ca²⁺]ᵢ peak change. Because the *cagA*⁻ and *picB*⁻/*cagE*⁻ isogenic strains do not generate an [Ca²⁺]ᵢ plateau response, PP2 was without effect on this portion of the [Ca²⁺]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.
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).

*\textit{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+ signaling 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 that \textit{H. pylori}-generated urease activity or ammonia might have on the overall \textit{H. pylori}-induced [Ca2+] response (4). However, we cannot rule out in our study the contribution of other \textit{H. pylori}-secreted/shed factors that may contribute to the overall [Ca2+]i response (29). In addition, some of the \textit{H. pylori} extracellular shed/secreted proteins, such as HP0305, have been reported to have sequence homology to regulators of G protein signaling, which makes it possible that these proteins could modulate or contribute to the overall \textit{H. pylori} host cell [Ca2+]i response (22). However, because \textit{H. pylori} was able to increase [Ca2+]i within minutes after the addition 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}-induced [Ca2+]i changes to baseline levels. The effect of PTX pre-treatment also caused a reduction of the wild-type (A) and vacA − (B) \textit{H. pylori}-[Ca2+]i, peak and plateau changes to baseline levels. The effect of PTX pre-treatment also caused a reduction of the cagA − (C) and picB −/cagE − (D) [Ca2+]i peak change to near baseline levels. Because the cagA − and picB −/cagE − isogenic strains do not generate a [Ca2+]i plateau change, the PTX pre-treatment had no effect on this portion of the [Ca2+]i response (C and D). Data are from 9 independent experiments and expressed as means ± SE; *P < 0.05 vs. control peak and plateau levels.
*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 CagA protein becomes phosphorylated by src kinases, will directly or indirectly control Ca\(^{2+}\) influx.
CagA protein has to be phosphorylated to induce the *H. pylori* [Ca\(^{2+}\)]\(_i\) changes.

Several studies have also implicated a link between an *H. pylori*-induced [Ca\(^{2+}\)]\(_i\) change and a biological response. That is, pretreatment of MKN45 gastric cancer cells with the intracellular Ca\(^{2+}\) chelator BAPTA was shown to completely block wild-type *H. pylori*-induced IL-8 secretion (32). It has also been reported that BAPTA was able to block *H. pylori*-induced arachidonic acid release that is involved in the production of prostaglandin E\(_2\) (40). Of interest, however, was the fact that the expression of the CagA protein was not important for the above-mentioned Ca\(^{2+}\)-dependent *H. pylori*-induced IL-8 release (32), whereas the CagA protein was necessary for Ca\(^{2+}\)-dependent *H. pylori*-induced arachidonic acid synthesis (40). One might propose that, depending on the final *H. pylori*-induced [Ca\(^{2+}\)]\(_i\) change, different signaling pathways could be activated based on a specific [Ca\(^{2+}\)]\(_i\) threshold. That is, even in the presence of a *cagA*\(^{-}\) mutant, which we have shown generates only a small [Ca\(^{2+}\)]\(_i\) peak change, this small [Ca\(^{2+}\)]\(_i\) response may be sufficient enough to release IL-8, but a larger [Ca\(^{2+}\)]\(_i\) 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 *H. pylori*-induced biphasic [Ca\(^{2+}\)]\(_i\) signal, i.e., the peak and plateau phases, may be utilized differently depending on the nature of the Ca\(^{2+}\)-dependent signaling molecule within the host gastric cell. It also appears that differential signaling by *H. pylori* may hold true for other gastric host cell responses. That is, *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 *H. pylori*-treated AGS gastric cancer cells found that many *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 *H. pylori* cag PAI, as well certain structural components from *H. pylori* itself, that could participate in the *H. pylori*-induced [Ca\(^{2+}\)]\(_i\) 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 *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). A G protein/PLC-dependent pathway primarily regulated the *H. pylori*-induced intracellular Ca\(^{2+}\) release, whereas *H. pylori*-induced Ca\(^{2+}\) influx was under the control of a G protein-, src kinase-, and PL2A-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 *H. pylori* to produce a full [Ca\(^{2+}\)]\(_i\) response. For future studies, it will be important to look at other genes inside and outside the *cag* PAI to determine their effects on *H. pylori*-induced [Ca\(^{2+}\)]\(_i\) changes.

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