Helicobacter pylori

ORIGINAL ARTICLE

Protein kinase C isozymes regulate matrix metalloproteinase-1 expression and cell invasion in Helicobacter pylori infection

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ABSTRACT

Background Protein kinase C (PKC) signalling is often dysregulated in gastric cancer and therefore represents a potential target in cancer therapy. The Gram-negative bacterium Helicobacter pylori, which colonises the human stomach, plays a major role in the development of gastritis, peptic ulcer and gastric adenocarcinoma.

Objective To analyse the role of PKC isozymes as mediators of H pylori-induced pathogenesis.

Methods PKC phosphorylation was evaluated by immunoblotting and immunohistochemistry. Gene reporter assays, RT-PCR and invasion assays were performed to assess the role of PKC in the regulation of activator protein-1 (AP-1), matrix metalloproteinase-1 (MMP-1) and the invasion of H pylori-infected epithelial cells.

Results H pylori induced phosphorylation of PKC isozymes α, δ, θ in AGS cells, which was accompanied by the phosphorylation of PKC substrates, including PKCα and myristoylated alanine-rich C kinase substrate (MARCKS), in a CagA-independent manner. Phospholipase C, phosphatidylinositol 3-kinase and Ca2+ were crucial for PKC activation on infection; inhibition of PKC diminished AP-1 induction and, subsequently, MMP-1 expression. Invasion assays confirmed PKC involvement in H pylori-induced MMP-1 secretion. In addition, analysis of biopsies from human gastric mucosa showed increased phosphorylation of PKC in active H pylori gastritis and gastric adenocarcinoma.

Conclusion The targeting of certain PKC isozymes might represent a suitable strategy to interfere with the MMP-1-dependent remodelling of infected tissue and to overcome the invasive behaviour of gastric cancer cells.

INTRODUCTION

Serine/threonine kinases of the protein kinase C (PKC) family are important molecules in the regulation of cellular differentiation, proliferation, apoptosis, adhesion and migration.1 PKC signalling participates in the regulation of gastric acid production2 and is often dysregulated in gastric cancer.3 4 Several PKC isoforms have been implicated in invasion and metastasis; however, the molecular mechanisms are still not well understood.

The PKC family consists of at least 10 isozymes classified into three main groups (figure 1A). Conventional PKC (cPKC) α, βI, βII and γ bind Ca2+ and phosphatidylycerine and require diacylglycerol (DAG) for further activation. The novel PKC (nPKC) δ, ε, θ, η possess a functional C1 domain, but their C2-like domains do not contain Ca2+-binding residues. Therefore, nPKC isozymes are regulated by DAG and phosphatidylycerine, but not by Ca2+. The atypical PKCs (PKCξ and PKCη)

Significance of this study

What is already known on this subject?

▸ Protein kinase C (PKC) isozymes regulate a number of cellular functions including processes related to a polarised epithelial layer formation, and exert a crucial role in carcinogenesis.

▸ The differences in mode of activation, intracellular distribution, and expression in normal and pathological tissue suggest that there are unique and mostly not investigated roles for each particular PKC isozyme in gastrointestinal signal transduction.

▸ H pylori-induced matrix metalloproteinase-1 (MMP-1) expression in stomach epithelium involves mitogen-activated protein kinases (MAPK).

What are the new findings?

▸ PKCα, PKCδ, PKCζ and a number of PKC substrates are phosphorylated in H pylori-infected gastric cells independently of H pylori’s virulence factor cytotoxin A associated antigen (CagA).

▸ H pylori induces PKC through phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ (PLCγ) and Ca2+-.

▸ PKCα, PKCδ and PKCζ contribute to c-Fos up-regulation and activator protein-1 (AP-1) activation in a MAPK-independent manner, leading to an increase of matrix metalloproteinase-1 expression in H pylori-infected cells.

▸ PKC are involved in cell invasion and, therefore, could play a causative role in gastric mucosa destruction following H pylori infection.

▸ Phosphorylated PKC is increased in gastric tissue specimens from patients with H pylori-associated gastritis and gastric adenocarcinoma.

How might it impact on clinical practice in the foreseeable future?

▸ Post-translational modifications (eg, phosphorylation) of PKC represent a potential biomarker for diagnostics and a molecular target for treatment of H pylori-induced gastric diseases.
lack both functional C1 and C2 domains and are neither Ca\(^{2+}\)-
or DAG-dependent.\(^5\)

In addition to their regulation by lipid second messengers, phosphorylation of conserved Ser/Thr sites within the C3 domain plays an important role in stabilisation and catalytic competence of PKC. Phosphorylation allows for the binding of the kinase domain to pseudosubstrate (within their own regulatory domain) to keep the enzyme in a latent conformation or promotes PKC binding to real substrates for full activation.\(^6\)

*Helicobacter pylori* colonises the stomach in at least 30\(^\sim\)50\% of the world’s population and increases the risk of peptic ulcers and gastric cancer. *H pylori* secretes effector molecules (lipo-polysaccharide, VacA) into the extracellular space or injects them (CagA, muropeptides) directly into the cytoplasm of the host cell via the type IV secretion system (T4SS).\(^7\)\(^8\) Thereby *H pylori* controls the inflammatory, proliferative, pro- and anti-apoptotic cellular statuses.\(^8\) Other bacterial factors, including adhesins, urease, flagellae and components of the outer membrane, also contribute to the colonisation of the gastric mucosa.\(^9\) Bacteria–gastric epithelial cell interactions lead to induction of a range of matrix metalloproteinases (MMPs),\(^10\)\(^11\) MMPs participate in extracellular matrix (ECM) remodelling, the cleavage of cell adhesion molecules (eg, E-cadherin) and the processing and activation of chemoattractants and ligands for growth factor receptors,\(^12\) which leads to an increase in epithelial permeability and promotes leucocyte infiltration into the gastric mucosa.

The involvement of PKC in many cellular functions and in pathophysiology, for example, carcinogenesis, suggests that PKC may play a role in *H pylori* infection. However, very few studies have addressed the activation of PKC during *H pylori* infection. Obst *et al*\(^13\) have demonstrated the translocation of PKCA to the plasma membrane in *H pylori*-infected AGS cells, and Brandt *et al*\(^14\) have shown the *H pylori*-induced phosphorylation of PKC\(\alpha\) and PKC\(\beta\) in these cells. There is only limited knowledge about the functional role of PKC in *H pylori* infection. By using a number of inhibitors, PKC has been demonstrated to participate in *H pylori*-induced alteration of the barrier properties of the

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**Figure 1** *H pylori* activates protein kinase C (PKC). (A) The protein domains of the PKC family members, showing the pseudosubstrate (dark blue rectangle), the C1 domain that binds DAG, phosphatidylserine and phorbol esters, the C2 domain that binds Ca\(^{2+}\) or PIP2 (in the case of nPKC), and the C3 kinase domain. Also shown in orange are the conserved Ser/Thr residues phosphorylated during *H pylori* infection. (B) AGS cells were infected with *H pylori* P1 wt, cagA or virB7 mutants for different periods of time or were stimulated with PMA for 1 h. Cell lysates were analysed by immunoblotting using antibodies as indicated. Unphosphorylated PKC\(\alpha\) and PKC\(\beta\) served as loading controls. (C) Analysis of phosphorylation of PKC substrates in cells treated as described in (B). GAPDH was immunodetected to show equal protein amounts in the cell samples.
epithelium and NF-kB-dependent cyclooxygenase-2 expression in gastric epithelial cells. Contradictory data exist concerning PKC involvement in IL-8 regulation in the gastric epithelium on infection.

The aim of this study was to investigate the mechanisms and the functional consequences of H. pylori-induced PKC activation. We show here that H. pylori induces PKC in gastric epithelial cells, which involves the classical upstream PKC regulators P13K, phospholipase C (PLC) and Ca²⁺. Our data demonstrate for the first time that PKC contributes to C-fos expression and activator protein-1 (AP-1) induction, which leads to matrix metalloproteinase-1 (MMP-1) up-regulation on H. pylori infection. In addition, we show the induction of PKC phosphorylation in gastric mucosa tissue from patients with active H. pylori gastritis and gastric adenocarcinoma.

MATERIALS AND METHODS

The antibodies and the chemicals used in this work are described in supplementary tables 1 and 2. The descriptions of the procedures for preparation of cell lysates, immunoblotting, immunofluorescence, immunohistochemistry, RNA isolation, RT-PCR, transfection, the reporter gene assay, the invasion chamber by a polycarbonate membrane (0.4 μM pore size). The antibodies and the chemicals used in this work are described in supplementary tables 1 and 2. The descriptions of the procedures for preparation of cell lysates, immunoblotting, immunofluorescence, immunohistochemistry, RNA isolation, RT-PCR, transfection, the reporter gene assay, the invasion chamber by a polycarbonate membrane (0.4 μM pore size).

RESULTS

H. pylori induces phosphorylation of PKCα, PKCδ, PKCθ and PKCd

While studying the effect of H. pylori on PKC, a transient increase in phosphorylation was observed within 30 min for PKCα (Ser657), within 30–60 min for PKCδ (Thr505) and within 5–6 h for PKCθ (Thr558) following infection with P1 wt strain (figure 1A,B). Phorbol myristoyl acetate (PMA), a membrane-permeable substitute for DAG, was used as a positive control. To investigate the involvement of H. pylori virulence factors in PKC phosphorylation, AGS cells were infected with H. pylori mutants deficient in either CagA or VirB7 protein, which is required for the integrity of the T4SS. Both mutants adhered equally to AGS cells (data not shown). The cagA, but not the virB7, mutant induced PKCα phosphorylation. No differences between the wt, cagA or virB7 mutants were observed for PKCδ or PKCθ phosphorylation (figure 1B).

Intracellular localisation plays an important role in PKC function. To study PKC activity in vivo, human gastric biopsies were obtained from 160 patients (age range 19–96 years) according to the recommendations of the updated Sydney System and were examined by the same experienced gastrointestinal pathologist who was blinded to the clinical and endoscopic data. Biopsies were stained with H&E, experienced gastrointestinal pathologist who was blinded to the clinical and endoscopic data. Biopsies were stained with H&E, experienced gastrointestinal pathologist who was blinded to the clinical and endoscopic data. Biopsies were stained with H&E.

Patients and tissue samples

Stomach biopsy specimens were obtained from 160 patients (age range 19–96 years) according to the recommendations of the updated Sydney System and were examined by the same experienced gastrointestinal pathologist who was blinded to the clinical and endoscopic data. Biopsies were stained with H&E, and also with Warthin-Starry—silver stain for detection of H. pylori. Histological features of the gastric mucosa, including inflammation and atrophy were scored according to the updated Sydney System. Diagnosis of neoplasia was made according to the WHO classification 2010.

Statistical analyses

Statistical analyses of the results were performed using the Student t test. The data are expressed as the mean fold changes from at least three separate experiments ± SEM with the value of the control arbitrarily normalised to 1; p<0.05 was considered significant. The immunohistochemical data were analysed using analysis of variance (IBM SPSS 18). The statistical decisions were two-tailed with a critical probability of α=5% using a post-hoc t-test.
H pylori-induced activation of PKC involves PLCγ1, Ca2+,
tyrosine kinases and PI3K

The PKC activator DAG is mainly produced from phosphati-
dylinositol 4,5-biphosphate (PIP2) or phosphatidylcholine (PC)
through direct cleavage with phosphatidylinositol-specific PLC
(PI-PLC) or PC-specific PLC (PC-PLC), respectively.25 Pretreat-
ment of AGS cells with U73122 or D609, selective inhibitors of
PI-PLC or PC-PLC, respectively, reduced the phosphorylation of
PKC substrates and MARCKS following infection (figure 2A).
Thus, the H pylori-induced activation of PKC involves PC-PLC
and PI-PLC.

PI-PLC comprises a group of Ca2+-dependent enzymes,
including PLCβ, γ, δ, ε, θ and PLCη families.26 PLCβ and PLCγ
are the most studied isoforms. PLCβ (four isoforms) is induced
in response to the activation of G protein-coupled trans-
membrane receptors. PLCβ3 is ubiquitous, whereas PLCβ1 is
not expressed in the stomach; PLCβ2 and PLCβ4 are highly
expressed in cells of haematopoietic origin as well as in the
cerebellum and retina.27 PLCγ (two isoforms) is stimulated on
activation of receptor and non-receptor tyrosine kinases.28
PLCγ1 is widely distributed, whereas PLCγ2 is expressed
primarily in cells of haematopoietic origin. To determine the role
of particular isoforms in infected cells, transient transfections
with siRNAs targeting either PLCγ1 or PLCβ3 were performed.
In contrast to PLCβ3, PLCγ1 depletion suppressed H pylori-
induced phosphorylation of PKC substrates, including MARCKS
(figure 2B,C). Therefore, PI-PLCγ1 contributes to PKC regulation
on H pylori infection.

H pylori has been shown to provoke a CagA-independent
increase of (Ca2+), in gastric epithelial cells.29 Investigating the
role of Ca2+ in PKC activation, we found that the phosphory-
lolation of PKC substrates was dramatically reduced on treatment
with either PLCγ1 or PLCβ3 or PMA (51.5-fold induction;
figure 2D). Additionally, the tyrosine kinase inhibitor genistein
and PI3K inhibitor LY294002 diminished the phosphorylation of
PKC substrates, especially MARCKS (figure 2E). Taken together,
these data indicate that Ca2+, tyrosine kinases and PI3K
are involved in PKC regulation during infection of epithelial cells
with H pylori.

To substantiate that the phosphorylation of PKC substrates
reflects PKC catalytic activity, bisindolylmaleimide I (BIS 1),
a selective inhibitor of conventional and novel PKC, was used.
The phosphorylation of PKC substrates and MARCKS in response
to H pylori or PMA was completely abolished in BIS 1-
treated cells (figure 2F). Importantly, BIS 1 demonstrated no
toxicity towards H pylori, in contrast to many other PKC
inhibitors, including rottlerin (supplementary figure 3) and
calphostin C (data not shown).

Inhibition of PKC reduces MMP-1 expression in H pylori-infected
cells

While studying the role of PKC activation, we observed that BIS
1 significantly inhibited MMP-1 gene expression (figure 3A)
and protein accumulation (figure 3B) both in H pylori-infected
and in PMA-treated AGS cells. MMP-1 expression following infection
with P12 wt reached a maximum at 6 h post-infection (figure 3C),
and MMP-1 accumulated in the membranes and nuclei of infected
cells (figure 3D). MMP-1 gene up-regulation depended
on the strain used for infection, and the P1 strain was less potent
in inducing MMP-1 than the P12 strain (figure 3A,E). The cagA H
pylori mutant was as effective as the wt, but the virB7 mutant
up-regulated MMP-1 to a lesser extent (figure 3E,F).

Importantly, BIS 1 suppressed the MMP-1 expression induced
by P12 wt in both the human HCA-7 colon cancer cell line
and in primary stomach cells (supplementary figure 4A). Impor-
tantly, in both cell systems H pylori P12 wt induces PKC, which
leads to phosphorylation of PKC substrates (supplementary
figure 4B). HSC constitutively express mRNA from Muc-Sac and
Muc-6 genes and stain positive for H+,K +-ATPase and pan-
cytokeratins (supplementary figure 4C), which is consistent
with normal gastric epithelial cells.

PKC regulates MMP-1 by activating AP-1 transcription factor

The MMP-1 promoter is predominantly regulate by AP-1 (figure
4A).30 Infection with H pylori led to a 4.4-fold increase in AP-1
activity (figure 4B). A more pronounced effect on AP-1 activity
was achieved by treatment with PMA (51.5-fold induction;
figure 4B). Pretreatment of the cells with BIS 1 completely
abolished PMA-induced and diminished H pylori-induced AP-1
transactivation (figure 4B). AP-1 is a dimer that consists of
Fos (c-Fos, FosB, Fra-1, Fra-2) and Jun (c-Jun, JunD, JunB) proteins
and is positively regulated by mitogen-activated protein ki-

Table 1 Protein kinase C (PKC) phosphorylation in human gastric mucosa tissue

<table>
<thead>
<tr>
<th>Gastric mucosa biopsies</th>
<th>Number of specimens</th>
<th>Age</th>
<th>Gender</th>
<th>Mean ± SEM, cells/hpf</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infected</td>
<td>36</td>
<td>19–72</td>
<td>M</td>
<td>20 ± 6</td>
<td>1.96 ± 0.28</td>
</tr>
<tr>
<td>H pylori gastritis</td>
<td>38</td>
<td>32–82</td>
<td>F</td>
<td>24 ± 14</td>
<td>25.32 ± 5.18</td>
</tr>
<tr>
<td>Adenoma</td>
<td>21</td>
<td>31–82</td>
<td></td>
<td>11 ± 10</td>
<td>11.10 ± 2.15</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>65</td>
<td>34–96</td>
<td></td>
<td>29 ± 36</td>
<td>61.37 ± 7.56</td>
</tr>
</tbody>
</table>

*p < 0.05 versus ‘non-infected’ group, as determined by the Dunnett t-test.


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T4SS-dependent (supplementary figure 5). These results indicate that in H pylori-treated cells, PKC is involved in up-regulation of the AP-1 members c-Fos and c-Jun, but the exact integrative mechanism and bacterial factors involved remain elusive.

Additionally, we found no accumulation of the AP-1 co-activator polyomavirus enhancer activator-3 (PEA3), which might promote MMP-1 expression in infected cells (data not shown).34

PKCα, PKCδ and PKCζ control H pylori-induced MMP-1 expression through c-Fos

Given our results, which demonstrate that H pylori induces phosphorylation of PKCα, PKCδ and PKCζ, the involvement of these isoforms in MMP-1 regulation was subsequently tested. Specific PKC-targeting siRNAs reduced MMP-1 mRNA (figure 5A) and protein expression (figure 5B) in the infected cells. Depletion of PKCζ and PKCθ, but not PKCα, partially inhibited induction of MMP-1 by PMA (figure 5A,B).

A prominent decrease of c-Fos expression in PKCα, PKCθ or PKCζ siRNA-treated cells was observed when studying the signalling molecules involved in the activation of AP-1 in response to H pylori (figure 5C). c-Jun expression and phosphorylation of c-Jun, ERK, JNK and MKK4 were not affected (figure 5C). These results suggest that PKCα, PKCθ and PKCζ contribute to c-Fos up-regulation during infection with H pylori.

In PMA-exposed cells, PKCθ depletion slightly affected the expression of c-Fos and c-Jun (figure 5C).

To confirm the crucial role of c-Fos in MMP-1 up-regulation, AGS cells were transfected with a c-Fos-targeting siRNA. On c-Fos knockdown, MMP-1 synthesis was diminished in both H pylori- and PMA-treated cells (figure 5D). Thus, c-Fos represents an important mediator in PKC-regulated MMP-1 expression.

Overexpression of PKC leads to AP-1 activation

To substantiate that PKCα, PKCθ and PKCζ regulate AP-1 in AGS cells, gene reporter assays were performed (figure 5E). Overexpression of constitutively active PKCα, δ and θ led to the transactivation of the AP-1 reporter gene 48 h post-transfection (1.8-, 1.3- and 3.5-fold, respectively) and 72 h post-transfection (2.8-, 4- and 2.1-fold, respectively). Immunoblotting revealed an accumulation of c-Fos in cells overexpressing PKC, which correlated with AP-1 activity and MMP-1 accumulation, and was most prominent in PKCζ-overexpressing cells (figure 5F). PKC overexpression did not induce the accumulation or phosphorylation of c-Jun (figure 5F). Thus, the PKC isoforms α, δ and θ regulate c-Fos leading to AP-1 activation in gastric cells.

PKC promote invasion of AGS cells in H pylori infection

To further analyse the role of PKC in MMP-1 secretion, invasion assays using collagen I-coated filters were performed. Figure 6A

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**Figure 2**  Protein kinase C (PKC) activation implicates PLC, Ca²⁺, tyrosine kinases and PI3K. AGS cells were pre-incubated with U73122 or D609 (A), were transiently transfected with siRNAs targeting PLCγ1 (B) or PLCβ3 (C), or were pretreated with BAPTA-AM (D), genistein and LY294002 (E) or with the indicated concentrations of BIS I (in μM; (F)) and infected with H pylori P12 for 45 min or for the indicated periods of time. Cell lysates were analysed by immunoblotting using antibodies as indicated. GAPDH was immunodetected to show equal protein amounts in the cell samples.
shows that co-culturing of AGS cells with \textit{H. pylori} led to enhanced cellular invasion, which was less prominent on infection with the \textit{virB7} mutant strain in comparison to the wt and \textit{cagA} strains. To assess the role of MMP-1 in \textit{H. pylori}-induced invasiveness, AGS cells were transfected with siRNA targeting MMP-1. Depletion of MMP-1 inhibited both basal and \textit{H. pylori}-induced MMP-1 expression in AGS cells (figure 6B) and suppressed invasion in response to \textit{H. pylori} (figure 6A). Treatment of the cells with the PKC inhibitor BIS I prior to infection markedly reduced the number of invading cells (figure 6C). Similar results were obtained for PMA. Depletion of PKC\textalpha, PKC\textbeta, and PKC\textgamma, which is crucial for MMP-1 production in response to \textit{H. pylori}, abolished transmigration of infected cells, indicating a functional role for these

**Figure 3** \textit{H. pylori} up-regulates MMP-1 in a protein kinase C (PKC)-dependent manner. BIS I-treated or non-treated AGS cells were incubated with \textit{H. pylori} P12 wt, PMA (A–D) or \textit{H. pylori} P1 wt or the \textit{cagA} and \textit{virB7} mutants (E, F) for 3 h or for the indicated periods of time. MMP-1 expression was analysed by qRT-PCR (A, C, E) or immunoblotting (B, D, F). The graphs in (B) summarise the densitometric analysis of three independent immunoblots (experiments). GAPDH, occludin and histone H3 were immunodetected to show the appropriate fractionation and equal protein amounts in the cell samples. Bacterial lysate was used as a negative control. *p<0.05, **p<0.01 versus non-stimulated cells; #p<0.05, ##p<0.01 versus BIS I-free stimulated cells.

**Figure 4** \textit{H. pylori} up-regulates AP-1 in a protein kinase C (PKC)-dependent manner. (A) The composition of MMP-1 promoter.\textsuperscript{30} The AP-1 element binds members of the c-Fos and c-Jun family of transcription factors. c/EBP\textbeta, CCATT/enhancer binding protein-\textbeta, SBE, STAT binding element; TIE, TGF\beta inhibitory element. (B) A reporter gene assay was performed using an inducible reporter construct encoding the firefly luciferase gene under the control of the AP-1 binding element. Firefly luciferase activity was normalised relative to Renilla’s one. BIS I-treated/non-treated AGS cells were incubated with \textit{H. pylori} P12 wt or PMA for 3 h. *p<0.05, **p<0.01 vs non-stimulated cells; #p<0.05, ##p<0.01 vs BIS I-free stimulated cells. (C) Regulation of \textit{c-jun} and \textit{c-fos} expression by MAPK. (D) BIS I-treated cells were incubated with P12 wt or PMA for 1 h. The cell lysates were analysed by immunoblotting using antibodies as indicated.
isozymes in invasion (figure 6D). Depletion of PKCa, PKCb or PKCq had a less prominent effect in PMA-treated cells (figure 6D). To confirm the regulatory role of PKCa, δ and θ in invasion, constitutively active isozymes were overexpressed. Figure 6E shows that PKC overexpression increased the number of invading cells. Taken together, these results indicate that MMP-1-dependent collagen I digestion involves PKC in *H pylori*-infected cells.

Invasion is an integrative process that depends on the adhesive and migratory behaviours of cells, in addition to their proteolytic activity towards the ECM. Therefore, involvement of PKC in regulation of cellular motility was investigated using a wound healing assay. In contrast to PMA, the P1 and P12 strains of *H pylori* did not stimulate wound healing (supplementary figure 6A,B). Treatment with *H pylori* or PMA for 24 h slightly decreased the total number of AGS cells (data not shown). Thus, *H pylori*-induced invasion depends mainly on the increased proteolytic activity of AGS cells. Bis I, but not siRNAs against PKCa, PKCb or PKCq, inhibited PMA-induced cell migration (supplementary figure 6A,C). Therefore, several PKC isozymes are engaged to stimulate both the proteolytic activity and migration of PMA-treated cells, leading to increased cell invasiveness.

In addition to the wound healing assay, the involvement of PKC in *H pylori*-induced scattered phenotype was studied, and no effects of Bis I or PKC-specific siRNAs on cell morphology were found (supplementary figure 7). However, PMA-induced AGS cell spreading was completely blocked by Bis I but not by siRNAs against PKCa, PKCb or PKCq (supplementary figure 7).

**DISCUSSION**

The aim of this work was to investigate the activity and role of PKC isozymes in infected gastric epithelial cells. We show here that, on *H pylori* infection, cPKCa is phosphorylated within its hydrophobic motif and accumulates in both membranes and nuclei, which might represent sources of DAG35 and PKCα-interacting proteins. Autophosphorylation of the hydrophobic motif of PKCa has been reported to stabilise the enzyme and to be triggered by the mammalian target of rapamycin complex 2 and HSP90. Further, our results demonstrate that nPKCs δ and θ are transiently phosphorylated within their activation loops in a T4SS-independent manner. This finding is consistent...
with reports that both *H. pylori* cagPAI and the outer membrane protein OipA activate phosphatidylinositol kinase 1 (PDK-1),\(^3\) which phosphorylates the activation loop of PKC, leading to enzyme maturation and activation\(^4\) (figure 6F). In contrast to the study by Brandt et al.,\(^5\) we did not detect any CagA-dependent PKC\(_b\) phosphorylation at 6–9 h post-infection.

Activated PKC regulate their substrates, including MARCKS, vinculin and adducin.\(^6\) Our experiments demonstrate that *H. pylori* causes phosphorylation of downstream targets of PKC, including MARCKS in a CagA- and VacA-independent manner. The phosphorylation of PKC substrates is less prominent during infection with the *virB7* mutant strain. The most plausible model is that the phosphorylation of PKC substrates implicates a range of PKC isoforms that are activated independently of T4SS (eg, PKC\_b and PKC\_o) and via T4SS (eg, PKC\_a) (figure 6F).

For full activation, conventional and novel PKC require DAG, generated following PI\(_{3,4,5}\)-tripliphosphatase hydrolysis by PLCs. Here, we show that both PI-PLC and PC-PLC inhibitors reduce the phosphorylation of PKC substrates in *H. pylori*-infected cells, with the PI-PLC inhibitor being more efficient. Indeed, PI-PLC-dependent hydrolysis of PI\(_{3,4,5}\)-tripliphosphate (IP\(_3\))\(^7\) which provokes an increase of intracellular Ca\(^{2+}\), thus, PI-PLC promotes activation of both DAG- and Ca\(^{2+}\)-dependent PKC isoforms (figure 6F).

Within PI-PLCs, PLC\_y1 plays an important role in PKC activation, as shown here using PLC\_y1-targeting siRNA. Additionally, PLC\_y1 activation in *H. pylori*-infected gastric epithelial cells has been reported previously.\(^8\)

Our experiments using BAPTA-AM further confirm a contributory role of intracellular Ca\(^{2+}\) in PKC activation on infection. As functional T4SS (but not CagA) is required for Ca\(^{2+}\) release during *H. pylori* infection,\(^9\) we propose that T4SS is implicated in the regulation of Ca\(^{2+}\)-activated PKC isoforms. Consistently, phosphorylation of Ca\(^{2+}\)-regulated PKC\_a is T4SS-dependent.

It has previously been shown that PI3K signalling is activated by *H. pylori*,\(^8\) which phosphorylates PIP2 and leads to PI3K generation, has been implicated in PDK-1 activation. Here, PI3K inhibition diminished the phosphorylation of PKC substrates and MARCKS in response to *H. pylori*. Moreover, tyrosine kinases, which act up-stream of PLCs and PI3K, play a role in PKC activation during infection with *H. pylori*, as demonstrated using genistein.

While studying PKC in vivo, we detected an increase of phosphorylated PKC in patients with *H. pylori*-induced gastritis or gastric adenocarcinoma, which indicates that post-translational modifications of these enzymes may be crucial for *H. pylori*-induced pathogenesis.

Given our results demonstrating that *H. pylori* induces the phosphorylation of PKC\_a, PKC\_o and PKC\_b, we focused on their role in infected gastric epithelial cells. All of these PKC isoforms are involved in regulation of the cytoskeleton, adherence junctions and barrier permeability in the gastrointestinal epithelium.\(^2\) PKC may play a role in the pathogenesis of *H. pylori*-caused diseases by affecting the integrity of the gastric epithelium.\(^10\)
Helicobacter pylori

Gastric mucosa disturbances in response to H pylori imply a range of MMPs, including MMP-1.\(^1\) MMP-1 not only degrades collagens I-III, VII, VIII and X, gelatin, and entactin,\(^2\) but also has functions extending beyond the degradation of the ECM components. For example, MMP-1 was found in the nucleus where it appears to confer resistance to apoptosis.\(^3\) Cytokines, growth factors and LPS induce MMP-1 synthesis via MAPK cascades in different cell types.\(^4\) MMP-1 is often up-regulated in gastric ulcercs and cancer.\(^5\) \(^6\) Our data indicate that H pylori stimulates MMP-1 synthesis in gastric epithelial cells, which is in accordance with published data.\(^7\) \(^8\) \(^9\) Although both P1 and P12 belong to the type I vacA** vacA** katA** flaA** strains, P1 was less potent in inducing MMP-1 than the P12 strain; this finding requires further investigation. We found that MMP-1 accumulates in membranous structures and nuclei of infected cells. Further, our results show that similar to PKC activation, MMP-1 expression requires both functional T4SS and other T4SS-independent bacterial factors, for example, OipA.\(^10\) Using the PKC inhibitor BIS I or PKC-specific siRNAs, we discovered that PKCα, PKCδ and PKCθ up-regulate MMP-1, leading to enhanced invasion by infected AGS cells. Importantly, we observed no significant enhancement of migration on infection. Therefore, it is apparent that H pylori-stimulated invasion depends mainly on the proteolytic, but not the migratory, activity of AGS cells. The inhibitory effect of BIS I on MMP-1 expression was not restricted to AGS cells and was also detected in tumour HCA-7 cells and non-cancerous HSC, which suggests that this represents a common phenomenon.

It is well established that PMA, which induces a sustained activation of almost all of the PKC isoforms, up-regulates MMP-1.\(^11\) In this study, PMA stimulated MMP-1 synthesis, invasion and migration of AGS cells. Depletion of one particular PKC isoform (eg, PKCδ) had a weak effect on these processes, probably because of a contributory role of intact PKC isoforms activated by PMA.

How does PKC regulate MMP-1? PMA has been reported to activate ERK and JNK,\(^12\) leading to AP-1 assembly on the MMP-1 promoter.\(^13\) \(^14\) Consistently, PMA activates MAPK and AP-1 in AGS cells, and BIS I abolishes this effect. H pylori also induces MAPK, c-Jun and c-Fos, and activates AP-1 in AGS cells.\(^15\) \(^16\) We found that BIS I suppresses c-Fos and c-Jun expression and AP-1 activity in infected cells. Surprisingly, BIS I had no effect on the phosphorylation of ERK or JNK, which mediate MMP-1 induction by H pylori.\(^17\) \(^18\) These observations suggest that c-Jun and c-Fos regulation by PKC occurs apart from MAPK. In particular, the serum response factor (SRF) and members of the CREB/ATF family that control (together with Elk-1) c-Fos expression (figure 6F) are regulated by several Ca\(^{2+}\)-dependent kinases, including PKC.\(^19\) Further, depletion of PKCα, PKCδ and PKCθ suppresses H pylori-induced c-Fos accumulation, and c-Fos depletion diminishes MMP-1 expression, indicating an important role of these PKC isoforms in c-Fos-dependent MMP-1 up-regulation. Indeed, in uninfected AGS cells, overexpression of active PKCα, PKCδ and PKCθ increased the amount of c-Fos, AP-1 activity and invasion through collagen I-coated filters.

With respect to the mechanistic role of H pylori virulence factors, pronounced T4SS-dependent and T4SS-independent processes exist.\(^20\) Future work on the identification of the bacterial factor(s) responsible for PKC activation will give additional insights into the mechanisms of gastric mucosa colonisation by H pylori and could provide a comprehensive picture of host–microbial interaction.

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Contributors

OS: experiments, analysis, interpretation of data and manuscript preparation; MV: biopsy collection, immunohistochemistry; MN: interpretation of data, manuscript preparation, and study supervision.

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Competing interests

None.

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Helicobacter pylori

Protein kinase C isozymes regulate matrix metalloproteinase-1 expression and cell invasion in *Helicobacter pylori* infection

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