Helicobacter pylori

ORIGINAL ARTICLE

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

Olga Sokolova,1 Michael Vieth,2 Michael Naumann1

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 isoforms 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 isoforms 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 isoforms 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 isoforms classified into three main groups (figure 1A). Conventional PKC (cPKC) α, βI, βII and γ bind Ca2+ and phosphatidyserine 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 isoforms are regulated by DAG and phosphatidyserine, but not by Ca2+. The atypical PKCs (PKCζ and PKCδ)

Significance of this study

What is already known on this subject?

- Protein kinase C (PKC) isoforms 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 isoform 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 and PKCζ play a number of PKC isoforms are phosphorylated in H pylori-infected gastric cells independently of H pylori’s virulence factor cytoxin A associated antigen (CagA).
- H pylori induces PKC through phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ (PLCγ) and Ca2+.
- PKCζ, 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\(^{2+}\)-nor 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\% 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.\(^6\) 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 PKC\(\alpha\) 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

![Image](http://gut.bmj.com/)

**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\(\beta\) and PKC\(\gamma\) 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\textsuperscript{15} and NF-kB-dependent cyclooxygenase-2 expression in gastric epithelial cells.\textsuperscript{16} Contradictory data exist concerning PKC involvement in IL-8 regulation in the gastric epithelium on infection.\textsuperscript{17, 18}

The aim of this study was to investigate the mechanisms and the functional consequences of $H. \textit{pylori}$-induced PKC activation. We show here that $H. \textit{pylori}$ induces PKC in gastric epithelial cells, which involves the classical upstream PKC regulators PI3K, phospholipase C\textgamma (PLC\gamma) and Ca\textsuperscript{2+}.\textsuperscript{9} 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 $H. \textit{pylori}$ infection. In addition, we show the induction of PKC phosphorylation in gastric mucosa tissue from patients with active $H. \textit{pylori}$ 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 and wound healing assays are provided in the online data supplement.

**Cell culture and bacteria**

AGS (ATCC) and HCA-7 (European Collection of Cell Cultures, Salisbury, UK) cells were grown in RPMI 1640 medium (PAA Laboratories, Pasching, Austria) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. Cells derived from human prenatal stomach tissue (HSC, Innopriv, Derio, Spain) were cultured as described previously.\textsuperscript{19} Sixteen hours before infection, the cell medium was replaced with fresh RPMI 1640 supplemented with 0.5% FCS.

The wild-type (wt) $H. \textit{pylori}$ P1 strain and isogenic mutants cagA and virB7 or P12 wt and its VacA deficient mutant were cultured for 48–72 h, as described previously,\textsuperscript{20} and added to AGS cells at a multiplicity of infection of 100. In a set of experiments, the bacteria were loaded into the upper inserts of a 100 mm Transwell plate (Costar, Corning, New York, USA), and thereby separated from AGS cells cultured in the bottom chamber by a polycarbonate membrane (0.4 $\mu$m pore size).

**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\textsuperscript{21} 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 $H. \textit{pylori}$. Histological features of the gastric mucosa, including inflammation and atrophy were scored according to the updated Sydney System.\textsuperscript{21} 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 $\pm$ 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 $\alpha$=5% using a post-hoc t-test.

**RESULTS**

$H. \textit{pylori}$ induces phosphorylation of PKC\alpha, PKC\beta, PKC\delta and PKC\zeta

While studying the effect of $H. \textit{pylori}$ on PKC, a transient increase in phosphorylation was observed within 30 min for PKC\alpha (Ser657), within 30–60 min for PKC\beta (Thr505) and within 5–6 h for PKC\delta (Thr558) following infection with P1 wt strain (figure 1A,B). Phorbol myristate acetate (PMA), a membrane-permeable substitute for DAG, was used as a positive control. To investigate the involvement of $H. \textit{pylori}$ virulence factors in PKC phosphorylation, AGS cells were infected with $H. \textit{pylori}$ 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 PKC\alpha phosphorylation. No differences between the wt, cagA or virB7 mutants were observed for PKC\delta or PKC\beta phosphorylation (figure 1B). Thus, $H. \textit{pylori}$ induced transient phosphorylation of cPKC\alpha in a CagA-independent, but T4SS-dependent manner; however, nPKC\delta and nPKC\beta were induced in a CagA- and T4SS-independent manner. No changes were detected in the Ser497, Ser729 and Thr410/Thr403 phosphorylation of PKC\alpha, PKC\beta and PKC\zeta, respectively (figure 1B).

Further, infection of AGS cells with $H. \textit{pylori}$ induced phosphorylation of PKC\zeta, a nPKC target,\textsuperscript{22} at the sites that correlate closely with kinase activity (figure 1B).

Intracellular localisation plays an important role in PKC function.\textsuperscript{23}–6 Treatment with $H. \textit{pylori}$ (or PMA) led to an accumulation of phosphorylated PKC\alpha in the membranes and nuclei of AGS cells (supplementary figure 1A–C). In contrast to PMA, $H. \textit{pylori}$ promoted no translocation of PKC isoforms $\delta$ and $\theta$ from the cytosol to membranes and nuclei (supplementary figure 1B).

To analyse the phosphorylation of PKC substrates, we used an antibody to phosphorylated Ser residues surrounded by Arg or Lys at the $-2$ or $+2$ positions and a hydrophobic residue at the $+1$ position.\textsuperscript{23} Figure 1C shows that wt and CagA-deficient $H. \textit{pylori}$ induced a strong increase in Ser-phosphorylation of PKC\alpha in AGS cells. Infection with the virB7 mutant led to a less prominent phosphorylation of PKC substrates. Actin-binding protein myristoylated alanine-rich C kinase substrate (MARCKS), a downstream target of cPKC and nPKC,\textsuperscript{24} was phosphorylated in cells infected with the wt and cagA mutant of $H. \textit{pylori}$ within 1 h. Again, phosphorylation induced by the virB7 mutant was less prominent (figure 1C).

The P12 wt and vacA mutant of $H. \textit{pylori}$, as well as the P1 wt, induced the phosphorylation of PKC substrates. Heat-inactivated bacteria were not able to move, settle on the surface of the AGS cells (data not shown), or induce the phosphorylation of PKC substrates (supplementary figure 1D). Additionally, experiments using Transwell plates demonstrated that $H. \textit{pylori}$ does not induce any phosphorylation of PKC substrates in the absence of direct contact with AGS cells (supplementary figure 1D). Thus, the adherence of living $H. \textit{pylori}$ to host cells is required for PKC induction.

To study PKC activity in vivo, human gastric biopsies were analysed by immunohistochemistry. A pan-specific antibody, which recognises phosphorylation within the activation loop (Ser497, Ser505 and Ser558 of PKC\alpha, PKC\beta and PKC\delta, respectively), was used (supplementary figure 2). PKC phosphorylation was determined in the gastric tissue of patients with $H. \textit{pylori}$-active
gastritis or gastric adenocarcinoma, but not in the non-infected normal gastric mucosa (table 1).

In the non-infected group, 100% of specimens demonstrated an immunoreactivity less than the median of all variables (3 cells/hpf). In the ‘HP-gastritis’ and ‘adenocarcinoma’ groups, 52.6% and 81.5% of specimens, respectively, were strongly positive for phospho-PKC. In the studied biopsies, no changes in the expression of PKCδ were observed (supplementary table 3).

**H pylori**-induced activation of PKC involves PLCγ1, Ca2+ and tyrosine kinases and PI3K

The PKC activator DAG is mainly produced from phosphatidylinositol 4,5-biphosphate (PIP2) or phosphatidylcholine (PC) through direct cleavage with phosphatidylinositol-specific PLC (PI-PLC) or PC-specific PLC (PC-PLC), respectively.27 Pretreatment 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, ζ and PLCη families.26 PLCβ and PLCγ are the most studied isozymes. PLCβ (four isoforms) is induced in response to the activation of G protein-coupled transmembrane 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.27 PLCγ1 is widely distributed, whereas PLCγ2 is expressed primarily in cells of haematopoietic origin. To determine the role of particular isozymes 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.28 Investigating the role of Ca2+ in PKC activation, we found that the phosphorylation of PKC substrates was dramatically reduced on treatment of the cells with the Ca2+-chelator BAPTA-AM prior to infection (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 1 (BIS I), 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 I-treated cells (figure 2F). Importantly, BIS I 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 I significantly inhibited MMP-1 gene expression (figure 3A) and protein accumulation (figure 3B) in both *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 I 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). Importantly, 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-5a and Muc-6 genes and stain positive for H+, K+-ATPase and mucus-positive cells (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 regulated by AP-1 (figure 4A).29 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 I 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 kinases (MAPK) (figure 4C).31 *H pylori* has been shown to activate a heterodimer composed of c-Fos and c-Jun.32 While exploring the molecular mechanism of AP-1 activation, we observed a strong phosphorylation of ERK1/2, JNK1/2, p38, c-Jun, ATF-2, JNK up-stream kinase MKK4, and accumulation of c-Jun and c-Fos in both *H pylori*-infected AGS cells (figure 4D). BIS I abolished all of the effects of PMA and reduced *H pylori*-induced c-Fos and c-Jun up-regulation.

Surprisingly, in the infected cells, BIS I had no effect on the phosphorylation of ERK, p38 or JNK, which are considered to be up-stream regulators of c-Fos and c-Jun (figure 4C, D). The infection of AGS cells with wt *H pylori* and mutants showed that c-Jun was expressed following infection with the virB7 mutant, but delayed in comparison to the wt, which is in agreement with a report by Ding et al.33 However, phosphorylation of JNK1/2 and p38 was clearly

---

**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</td>
<td>16</td>
</tr>
<tr>
<td>Hp-gastritis</td>
<td>38</td>
<td>32–82</td>
<td>M</td>
<td>24</td>
<td>14</td>
</tr>
<tr>
<td>Adenoma</td>
<td>21</td>
<td>31–82</td>
<td>M</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>65</td>
<td>34–96</td>
<td>M</td>
<td>29</td>
<td>36</td>
</tr>
</tbody>
</table>

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

---

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). PKCa, PKCd and PKCq control H. pylori-induced MMP-1 expression through c-Fos

Given our results, which demonstrate that H. pylori induces phosphorylation of PKCa, PKCd and PKCq, 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 PKCq and PKCq, but not PKCa, partially inhibited induction of MMP-1 by PMA (figure 5A, B).

A prominent decrease of c-Fos expression in PKC targeting 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 PKCa, PKCd and PKCq contribute to c-Fos up-regulation during infection with H. pylori. In PMA-exposed cells, PKCq 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 PKCa, PKCd and PKCq regulate AP-1 in AGS cells, gene reporter assays were performed (figure 5E). Overexpression of constitutively active PKCa, δ and θ led to the transactivation of the AP-1 reporter gene 48 h post-transfection (1.8-, 1.5- 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 PKCq-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
shows that co-culturing of AGS cells with *H. pylori* led to enhanced cellular invasion, which was less prominent on infection with the *virB7* mutant strain in comparison to the wt and *cagA* strains. To assess the role of MMP-1 in *H. pylori*-induced invasiveness, AGS cells were transfected with siRNA targeting MMP-1. Depletion of MMP-1 inhibited both basal and *H. pylori*-induced MMP-1 expression in AGS cells (figure 6B) and suppressed invasion in response to *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 PKCa, PKCb and PKCq, which is crucial for MMP-1 production in response to *H. pylori*, abolished transmigration of infected cells, indicating a functional role for these
isozymes in invasion (figure 6D). Depletion of PKCa, PKCd 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, PKCd 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, PKCd 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.36 Autophosphorylation of the hydrophobic motif of PKCa has been reported to stabilise the enzyme37 and to be triggered by the mammalian target of rapamycin complex 2 and HSP90.5 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...
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, we propose that T4SS is implicated in the regulation of Ca\(^{2+}\)-activated PKC isozymes. Consistently, phosphorylation of Ca\(^{2+}\)-regulated PKC\(\alpha\) is T4SS-dependent.

It has previously been shown that PI3K signalling is activated by *H. pylori*, which phosphorylates PI(3,4,5)P\(_3\) and leads to PI3 generation, has been implicated in PDK-1 activation. Here, PI3K inhibition diminished the phosphorylation of PKC\(\alpha\), 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\(\alpha\), PKC\(\beta\) and PKC\(\delta\), we focused on their role in infected gastric epithelial cells. All of these PKC isoforms are involved in regulation of the cytoskeleton, adhesion junctions and barrier permeability in the gastrointestinal epithelium. PKC may play a role in the pathogenesis of *H. pylori*-caused diseases by affecting the integrity of the gastric epithelium.
Gastric mucosa disturbances in response to H pylori imply a range of MMPs, including MMP-1.\textsuperscript{10} MMP-1 not only degrades collagens I-III, VII, VIII and X, gelatin, and entactin,\textsuperscript{10} 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.\textsuperscript{32} Cytokines, growth factors and LPS induce MMP-1 synthesis via MAPK cascades in different cell types.\textsuperscript{43,44} MMP-1 is often up-regulated in gastric ulcers and cancer.\textsuperscript{19,45} Our data indicate that H pylori stimulates MMP-1 synthesis in gastric epithelial cells, which is in accordance with published data.\textsuperscript{11,18,46} Although both P1 and P12 belong to the type I cagA 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 membrane-bound 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.\textsuperscript{34} Using the PKC inhibitor BIS I or PKC-specific siRNAs, we discovered that PKC\textalpha, PKC\delta and PKC\varnothing 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.\textsuperscript{39} In this study, PMA stimulated MMP-1 synthesis, invasion and migration of AGS cells. Depletion of one particular PKC isoform (eg, PKC\delta) 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,\textsuperscript{47} leading to AP-1 assembly on the MMP-1 promoter.\textsuperscript{30,43} 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.\textsuperscript{33,48} 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.\textsuperscript{19,46} 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\textsuperscript{2+}-dependent kinases, including PKC.\textsuperscript{49} Further, depletion of PKC\alpha, PKC\delta and PKC\varnothing 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 PKC\alpha, PKC\delta and PKC\varnothing 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.\textsuperscript{48} 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.

**Acknowledgements** We thank G Baier and S Shaw for the PKC expression constructs, U Landeckel and S Krueger for the MMP-1 primers, S Kahlert for the antibody to H+, K+-ATPase, and B Peters for help in ANDVA.

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

**Funding** The work was funded in part by the Deutsche Forschungsgemeinschaft (SFB 854) and the Bundesministerium für Bildung und Forschung (FORSYS, BMBF-0313922) by grants to MN.

**Competing interests** None.

**Provenance and peer review** Not commissioned; externally peer reviewed.

**Open Access** This is an Open Access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 3.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/3.0/

**REFERENCES**

34. Martelli AM, Ding SZ, 33.
Chakraborti S, 25.
### A

**H. pylori (P12)**

<table>
<thead>
<tr>
<th></th>
<th>cytosol</th>
<th>membranes</th>
<th>nuclei</th>
<th>wl</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td><img src="image1" alt="Western blot" /></td>
<td><img src="image2" alt="Western blot" /></td>
<td><img src="image3" alt="Western blot" /></td>
<td><img src="image4" alt="Western blot" /></td>
</tr>
<tr>
<td>0.3 h</td>
<td><img src="image5" alt="Western blot" /></td>
<td><img src="image6" alt="Western blot" /></td>
<td><img src="image7" alt="Western blot" /></td>
<td><img src="image8" alt="Western blot" /></td>
</tr>
<tr>
<td>0.5 h</td>
<td><img src="image9" alt="Western blot" /></td>
<td><img src="image10" alt="Western blot" /></td>
<td><img src="image11" alt="Western blot" /></td>
<td><img src="image12" alt="Western blot" /></td>
</tr>
<tr>
<td>1 h</td>
<td><img src="image13" alt="Western blot" /></td>
<td><img src="image14" alt="Western blot" /></td>
<td><img src="image15" alt="Western blot" /></td>
<td><img src="image16" alt="Western blot" /></td>
</tr>
<tr>
<td>3 h</td>
<td><img src="image17" alt="Western blot" /></td>
<td><img src="image18" alt="Western blot" /></td>
<td><img src="image19" alt="Western blot" /></td>
<td><img src="image20" alt="Western blot" /></td>
</tr>
</tbody>
</table>

- **p-PKCα**
- **PKCα**
- **PKCδ**
- **PKCθ**
- **GAPDH**
- **Occludin**
- **Histone H3**

### B

**PMA**

<table>
<thead>
<tr>
<th></th>
<th>cytosol</th>
<th>membranes</th>
<th>nuclei</th>
<th>wl</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td><img src="image21" alt="Western blot" /></td>
<td><img src="image22" alt="Western blot" /></td>
<td><img src="image23" alt="Western blot" /></td>
<td><img src="image24" alt="Western blot" /></td>
</tr>
<tr>
<td>0.3 h</td>
<td><img src="image25" alt="Western blot" /></td>
<td><img src="image26" alt="Western blot" /></td>
<td><img src="image27" alt="Western blot" /></td>
<td><img src="image28" alt="Western blot" /></td>
</tr>
<tr>
<td>0.5 h</td>
<td><img src="image29" alt="Western blot" /></td>
<td><img src="image30" alt="Western blot" /></td>
<td><img src="image31" alt="Western blot" /></td>
<td><img src="image32" alt="Western blot" /></td>
</tr>
<tr>
<td>1 h</td>
<td><img src="image33" alt="Western blot" /></td>
<td><img src="image34" alt="Western blot" /></td>
<td><img src="image35" alt="Western blot" /></td>
<td><img src="image36" alt="Western blot" /></td>
</tr>
<tr>
<td>3 h</td>
<td><img src="image37" alt="Western blot" /></td>
<td><img src="image38" alt="Western blot" /></td>
<td><img src="image39" alt="Western blot" /></td>
<td><img src="image40" alt="Western blot" /></td>
</tr>
</tbody>
</table>

- **p-PKCα**
- **PKCα**
- **PKCδ**
- **PKCθ**
- **GAPDH**
- **Occludin**
- **Histone H3**

### C

**H. pylori (P12)**

<table>
<thead>
<tr>
<th></th>
<th>cytosol</th>
<th>membranes</th>
<th>nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td><img src="image41" alt="Bar graph" /></td>
<td><img src="image42" alt="Bar graph" /></td>
<td><img src="image43" alt="Bar graph" /></td>
</tr>
<tr>
<td>0.3 h</td>
<td><img src="image44" alt="Bar graph" /></td>
<td><img src="image45" alt="Bar graph" /></td>
<td><img src="image46" alt="Bar graph" /></td>
</tr>
<tr>
<td>0.5 h</td>
<td><img src="image47" alt="Bar graph" /></td>
<td><img src="image48" alt="Bar graph" /></td>
<td><img src="image49" alt="Bar graph" /></td>
</tr>
<tr>
<td>1 h</td>
<td><img src="image50" alt="Bar graph" /></td>
<td><img src="image51" alt="Bar graph" /></td>
<td><img src="image52" alt="Bar graph" /></td>
</tr>
<tr>
<td>3 h</td>
<td><img src="image53" alt="Bar graph" /></td>
<td><img src="image54" alt="Bar graph" /></td>
<td><img src="image55" alt="Bar graph" /></td>
</tr>
</tbody>
</table>

- **p-PKCα**, fold change

### D

**Transwell plates**

- PMA
- P1 wt
- P12 wt
- P12 vacA
- P12 wt hi
- P12 vacA hi

**p-Substrates of PKC**

- ![Western blot](image56)
- ![Western blot](image57)
- ![Western blot](image58)
- ![Western blot](image59)

- **p-MARCKS**
- **GAPDH**
Sokolova et al. Supplementary Figure 2

Non-infected

Hp-Gastritis

Adenoma

Adenocarcinoma
DMSO          BIS I + *H. pylori* (P12), 1 h          Rottlerin + *H. pylori* (P12), 1 h
Supplementary Figure 4

A

HCA-7

MMP-1 mRNA, fold change

Non-stimulated
BIS I

H. pylori (P12)

**

PMA

**

HSC

MMP-1 mRNA, fold change

Non-stimulated
BIS I

H. pylori (P12)

**

PMA

**

B

<table>
<thead>
<tr>
<th></th>
<th>HSC</th>
<th>HCA-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIS I</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H. pylori (P12)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

p-Substrates of PKC

GAPDH

C

AGS HSC

RevTr - + - +

Muc 5AC

HSC

H+, K+-ATPase

Muc 6

Cytokeratins

GAPDH
Sokolova et al. Supplementary Figure 7

A

-  \( H. pylori \) (P12)  

BIS I

PKC\( \alpha \) siRNA

PKC\( \delta \) siRNA

PKC\( \theta \) siRNA

B

-  PMA
DATA SUPPLEMENT

SUPPLEMENTARY MATERIAL AND METHODS

Preparation of cell lysates and immunoblotting

Whole-cell extracts were prepared with a modified RIPA buffer as described.[1] Aliquots of the lysates were boiled with sample buffer comprising 50 mM Tris-HCl, pH 6.8, 2% SDS, 10% Glycerol, 100 mM DTT, and 0.1% bromphenol blue for 5 min. Sub-cellular fractions of AGS cells were prepared with a ProteoExtract kit (Calbiochem/Merk KGaA) according to the manufacturer’s instructions. The proteins were separated by SDS-PAGE, electrotransferred onto Immobilon-P transfer polyvinylidene fluoride membranes (Millipore, Schwalbach, Germany) and stained with antibodies overnight at +4°C. Immunoreactivity was detected using the enhanced chemiluminescence detection kit Amersham™ ECL™ (GE Healthcare, Buckinghamshire, UK).

RNA isolation and RT-PCR

Total RNA was extracted with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and further cleaned using RNeasy® Plus Micro kit (Qiagen GmbH, Hilden, Germany). cDNA was synthesized from 1 µg of RNA using a random hexamer primer and RevertAid™ First Strand cDNA Synthesis kit (Fermentas, EU). cDNA was amplified as described¹ using following primers: 5’-CTGAAGGTGATGAAGCAGCC-3’ (forward) and 5’-AGTCCAAGAGAATGGCCGAG-3’ (reverse) for MMP-1, 5’-TCCAAAATCAAGTGGGGCGATGCT-3’ (forward) and 5’-CCACCTGGTGCTGACGTGACC-3’ (reverse) for GAPDH, 5’-CTAACCTAGCAGTCAGGTGAGATCCAC-3’ (forward) and 5’-GCACGGGATGTTGGAGTAGG-3’ (reverse) for mucin 5AC, 5’-
CCAATGACAGTGACCACCAG-3’ (forward) and 5’-CTCAAGTGGGGGAGTTGTGT-3’ (reverse) for mucin 6 (BioTeZ Berlin-Buch GmbH, Berlin, Germany). Serial dilutions of the dipeptidyl peptidase IV gene cloned into a pCR².1-TOPO vector and primers 5’-GATGCTACAGCTGACAGTCGC-3’ (forward), 5’-TGGTGACCATGTGACCCACTG-TGGTGACCATGTGACC’(reverse) served for the generation of a calibration curve.

Transfection

AGS cells (1 x 10⁵ cells/35 mm dish) were transfected with 20 nM siRNA (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) using siLentFecTM Lipid Reagent (BioRad) in Opti-MEM™ I culture medium (Invitrogen) supplemented with 5% FCS. A scrambled sequence that does not lead to the specific degradation of any known cellular mRNA was used as a control. For overexpression experiments, the cells were transfected with 0.2 µg of DNA using Effectene® transfection reagent (Qiagen) (DNA/Effectene ratio was 1:10). At 24 h of transfection, cells were starved in RPMI 1640 medium for 16-18 h and then infected with *H. pylori* or stimulated with PMA. Constitutively active forms of bovine PKCα (A25E) and human PKCθ (A148E) cloned into pEF vector were donated by G. Baier (Innsbruck Medical University, Austria). Alanine to glutamic acid substitution at the pseudosubstrate sequence prevents its binding to the kinase domain and, thus, supports an “open” active conformation of PKC.[2] Constitutively active (T507E) human PKCδ in the pCEFL vector was a gift of S. Shaw (National Cancer Institute, Bethesda, USA). Replacement of the threonine residue with a glutamic acid residue mimics phosphorylation and activates PKCδ.[3]

Reporter gene assay

AGS cells were seeded onto a 24-well plate at a density of 3.5 x 10⁴ cells per well in Opti-MEM™ I culture medium supplemented with 5% FCS. Reverse transfection was performed using 0.04:0.001 µg of Firefly AP-1:Renilla Luciferase plasmids (Cignal™ AP1 Reporter Assay Kit, SABiosciences,
Frederick, MD, USA) and 0.2 µg of a vector of interest using SureFECT™ transfection reagent (SABiosciences). A construct that encodes firefly luciferase under the control of a TATA box without any additional tandem repeats of the AP-1 transcription response element was used as a negative control. At 48 or 72 h post-transfection, cells were either left untreated or were stimulated with *H. pylori* or PMA and harvested further with Passive Lysis Buffer (Promega, Madison, WI, USA). Luciferase activity was estimated using the Dual-Luciferase Reporter Assay System (Promega) with a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). The firefly luciferase activity was normalised relative to *Renilla*'s activity. In some experiments, cell lysates were boiled with the sample buffer and used for immunoblotting.

**Invasion assay**

AGS cells (1 x 10⁵) were loaded into the upper chamber of a 24-well Transwell® plate (Costar, Corning, NY, USA) and cultured on polycarbonate filters (8-µM pore size) coated with collagen I (0.01 mg/insert) in RPMI1640 medium containing 0.5% FCS. After 2 h, cells were left untreated or were stimulated with *H. pylori* or PMA in the presence/absence of BIS I. The cells were allowed to invade toward 5% FCS for 18 h. Invaded cells on the bottom side of the membrane were washed, exposed briefly to trypsin, collected with PBS and counted. Percent invasion was determined by dividing the number of cells that translocated through the collagen I-coated filters by the number of total cells per well. Experimental replicates were performed in triplicate.

**Migration assay**

Migration of AGS cells was studied using the Oris™ Cell Migration Assay (AMS Biotechnology Ltd., Abingdon, UK) according to the manufacturer instructions. Briefly, 5 x 10⁴ cells/well were seeded into a 96-well plate containing cell seeding stoppers. After overnight incubation, a 2-mm diameter cell-free zone at the centre of each well was created by removing of the cell seeding stoppers. Cell growth medium was replaced with fresh RPMI 1640 containing 5% FCS, and cells
were incubated with *H. pylori* or PMA. After 24 h of treatment, cells were rinsed with PBS and stained with Calcein AM reagent. Fluorescent cells in the detection zone (applying the detection mask) were imaged by fluorescence microscope BZ-8100 (Objective CFI Plan Apo 4x; Keyence Corporation, Osaka, Japan). The cell-free area was measured using BZ-Analyzer software (Keyence Corporation).

**Immunostaining**

HSC (8.4 x 10^4) were seeded onto glass slides coated with 0.04 mg Matrigel™ in a 24-well plate. After 4 days in culture, the cells were fixed with 4% paraformaldehyde (Sigma, Saint Louis, USA) and permeabilized with 0.1% Triton® X-100 (Sigma) in PBS. Unspecific binding was blocked with 10% normal goat serum (Sigma). Mouse anti-pan cytokeratin antibody (recognizes human cytokeratins 1, 4, 5, 6, 8, 10, 13, 18, 19; Sigma) and anti-H⁺, K⁺-ATPase (recognizes α subunit; Antibodies-online GmbH, Aachen, Germany) were used in dilutions 1:200 and 1:100, respectively. Cy3-conjugated anti-mouse antibody was from Dianova (Jackson ImmunoResearch Laboratories, West Grove, USA). Cells were also stained in the absence of primary antibodies to evaluate non-specific secondary antibody reaction. Hoechst 33342 (Invitrogen, Carlsbad, USA) in dilution 1:1000 was used to stain nuclei. Images were taken using a fluorescence microscope BZ-8100 (Keyence Corporation, Osaka, Japan).

**Immunohistochemistry**

For retrieval of antigens, deparaffinised sections were heated in citrate buffer (pH 6.0) using a microwave oven for 20 min. Endogenous peroxidase was blocked by 20 min-incubation in 0.3% hydrogen peroxide within absolute methanol. Sections were washed, and non-specific binding was blocked with a pre-immune serum (Merck, Germany). Overnight incubation at +4°C was carried for binding of the primary rabbit polyclonal antibodies (dilution 1:50), including anti-phospho-PKC (pan) (αThr497, δThr505, θThr538) (Abcam, Cambrige, UK) and PKC0 (Abnova, Taipei City,
Taiwan). Then, 30 min-incubation with biotinylated secondary antibody was performed, followed by substrate binding by using streptavdin-biotin-peroxidase method. Counterstaining with haemalaun was carried out additionally in all cases. For all stains, negative and positive controls were performed, and staining was repeated until internal controls showed appropriate results.

PKCθ immunostaining was evaluated semiquantitatively according to the Remmele immunoreactive score (IRS).[4, 5] Briefly, the percentage of positively stained epithelial cells was divided into five grades of 0-4 (0%, <10%, 10-50%, 51-80%, >80%) and multiplied by the intensity the immunohistochemical reaction scaled from 0 to 3. To quantify phospho-PKC (pan) immunostaining, an evaluation of positively stained cells per high power field (hpf) was carried out.

SUPPLEMENTARY TABLES

Supplementary Table 1 Antibodies used for immunoblotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-PKC (pan)</td>
<td>Rabbit</td>
<td>Abcam</td>
</tr>
<tr>
<td>Phospho-PKCα (Thr497)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occludin</td>
<td>Mouse</td>
<td>BD Biosciences Pharmingen, San Jose, CA, USA</td>
</tr>
<tr>
<td>PKCδ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKCθ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLCγ1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-1</td>
<td>Rabbit</td>
<td>Calbiochem/Merk KGaA, Darmstadt, Germany</td>
</tr>
<tr>
<td>c-Fos</td>
<td>Rabbit</td>
<td>Cell Signalling Technology Inc., Danvers, MA</td>
</tr>
<tr>
<td>Histone H3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-ATF-2 (Thr71)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-ERK1/2 (Thr202/Tyr204)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-MARCKS (Ser152/156)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-JNK (Thr183/Tyr185)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-c-Jun (Ser63)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-PKC (pan) (βII Ser660)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-PKCδ (Thr505)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-PKCδ (Ser643)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-PKCμ (Ser744/748)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-PKC0 (Thr538)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-PKCζ/λ (Thr410/403)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-SEK1/MKK4 (Thr261)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-Ser-substrates of PKC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKCμ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Mouse</td>
<td>Chemicon International, Temecula, CA, USA</td>
</tr>
</tbody>
</table>
### Supplementary Table 2 Inhibitors and activators used in this work

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Manufacturer</th>
<th>Cellular targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>D609</td>
<td>50 µg/mL</td>
<td>Biomol GmbH, Hamburg, Germany</td>
<td>PC-PLC</td>
</tr>
<tr>
<td>BIS I, LY294002, Rottlerin, U73122</td>
<td>5 µM, 20 µM, 10 µM, 5 µM</td>
<td>Calbiochem/Merk KGaA, Darmstadt, Germany</td>
<td>cPKC, nPKC, PI3K</td>
</tr>
<tr>
<td></td>
<td>5 µM</td>
<td>Cell Signalling Technology Inc., Danvers, MA, USA</td>
<td>PI-PLC</td>
</tr>
<tr>
<td>PMA</td>
<td>66 nM</td>
<td>Cell Signalling Technology Inc., Danvers, MA, USA</td>
<td>DAG-binding proteins</td>
</tr>
<tr>
<td>BAPTA-AM, Genistein</td>
<td>10 µM, 150 µM</td>
<td>SIGMA-ALDRICH CHEMIE GmbH, Steinheim, Germany</td>
<td>Ca²⁺Tyrosin kinases</td>
</tr>
</tbody>
</table>

NOTE. Inhibitors were added to the cells 30 min prior to *H. pylori* or PMA treatment.

### Supplementary Table 3 PKCθ expression 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 IRS +SEM</th>
<th>IRS interpretation, (number of specimens/%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td></td>
<td>n</td>
</tr>
<tr>
<td>Non-infected</td>
<td>32</td>
<td>19-72</td>
<td>20</td>
<td>12</td>
<td>4.72 ± 0.37</td>
</tr>
<tr>
<td>Hp-Gastritis</td>
<td>31</td>
<td>32-82</td>
<td>20</td>
<td>11</td>
<td>4.74 ± 0.40</td>
</tr>
<tr>
<td>Adenoma</td>
<td>21</td>
<td>31-82</td>
<td>11</td>
<td>10</td>
<td>5.81 ± 0.53</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>59</td>
<td>40-96</td>
<td>26</td>
<td>33</td>
<td>5.36 ± 0.30</td>
</tr>
</tbody>
</table>

NOTE. No significant variance between the groups was determined by the ANOVA global test. M, male; F, female; n, negative; w, weak; m, moderate; s, strong expression.
**SUPPLEMENTARY FIGURE LEGENDS**

**Supplementary Figure 1**  *H. pylori* activates PKC. AGS cells were infected with *H. pylori* P12 wt (A, C) or were stimulated with PMA (B, C) for the various times indicated, and sub-cellular fractions were prepared. Immunoblotting was performed using antibodies against phosphorylated and unphosphorylated forms of PKC isozymes. GAPDH, occludin and histone H3 were immunodetected to show the appropriate fractionation and equal protein amount in the cell samples. (A, C) The graphs summarize the densitometric analysis of 3 independent immunoblots (experiments). *p<0.05, **p<0.01 vs non-stimulated cells (0 hours) within each subcellular fraction. (D) AGS cells were infected with P1wt, P12 wt, its isogenic VacA-deficient mutant or with heat-inactivated bacteria (hi) for 1 or 3 h. Heat-inactivated *H. pylori* were prepared by incubating the bacteria suspension at +70°C for 15 min. In a set of experiments, Transwell® permeable supports (pore size 0.4 µM) were used to separate *H. pylori* P1wt from AGS cells grown in the bottom chamber. Pore-permeable PMA was used as a positive control. The whole cell lysates were prepared and immunoblotting was performed using antibodies as indicated. GAPDH was immunodetected to show equal protein amounts in the cell samples.

**Supplementary Figure 2**  Histologic examination of phospho-PKC (pan) expression in human antral gastric mucosa. High-power field confocal images are shown. Magnification, 200x. Scale bares = 200 µM.

**Supplementary Figure 3**  In contrast to rottlerin, BIS I does not affect viability of *H. pylori*. *H. pylori* P12 wt was incubated in RPMI 1640 medium supplemented with 5 µM BIS I or 10 µM rottlerin for 1 h. The bacteria suspension was diluted in PBS and plated onto GC agar supplemented with horse serum, vancomycin, trimethoprim, nystatin and vitamins. The bacterial colonies grew in microaerophilic conditions for 3 days, and then the photos were taken using a VersaDoc™ Imaging System (BioRad, Hercules, CA, USA).
**Supplementary Figure 4** *H. pylori* up-regulates MMP-1 in a PKC-dependent manner. (A) BIS I-treated or non-treated HCA-7 cells or HSC were incubated with *H. pylori* P12 wt or PMA for 7 h. MMP-1 expression was analysed by qRT-PCR. *p<0.05, **p<0.01 vs non-stimulated cells; #p<0.05, ##p<0.01 vs BIS I-free stimulated cells. (B) BIS I-treated or non-treated HCA-7 cells or HSC were incubated with *H. pylori* P12 wt for 4 h. The whole cell lysates were prepared and immunoblotting was performed using antibodies as indicated. GAPDH was immunodetected to show equal protein amounts in the cell samples. (C) Characteristics of cultured human stomach cells (HSC) derived from prenatal stomach tissue. Expression of proteins typical for stomach epithelium (mucin 5AC, mucin 6, pan-cytokeratins and H⁺, K⁺-ATPase) was analysed in non-treated HSC by RT-PCR and immunostaining. GAPDH expression served as an internal control in RT-PCR. AGS cells served as a positive control. Muc 5AC, mucin 5AC; Muc 6, mucin 6. Cytokeratins and H⁺, K⁺-ATPase are pseudocolored in yellow; Hoechst 33342 nuclear stain is pseudocolored in margenta.

**Supplementary Figure 5** *H. pylori* activates MAPK. AGS cells were infected with *H. pylori* P1wt or the *cagA* and *virB7* mutants for various times indicated or were stimulated with PMA for 1 h, and the cell lysates were prepared. Immunoblotting was performed using antibodies as indicated.

**Supplementary Figure 6** In contrast to PMA, *H. pylori* does not accelerate wound healing. AGS cells grown on an uncoated 96-well plate were infected with different *H. pylori* strains as indicated or stimulated with 4 nM PMA in fresh RPMI 1640 containing 5% FCS in the presence or absence of BIS I for 24 h (A, B). The cells were labelled with Calcein AM and imaged (B). Relative area covered by the cells was calculated (A, C). (C) AGS cells were transfected with siRNAs against PKCα, PKCδ or PKCθ prior to stimulations. *p<0.05, **p<0.01, vs respective control; #p<0.05, vs PMA-stimulated cells.
**Supplementary Figure 7** *H. pylori* induces cell scattering in a PKC-independent manner. AGS cells were either pre-treated with BIS I or transfected with PKC-targeting siRNAs and then infected with *H. pylori* P12 wt (A) or PMA (B) for 3 h. Cell morphology was monitored with an inverted Nikon Eclipse TC100 microscope.

**SUPPLEMENTARY REFERENCES**


