The *Helicobacter pylori* fatty acid *cis*-9,10-methyleneoctadecanoic acid stimulates protein kinase C and increases DNA synthesis of gastric HM02 cells

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Summary
Protein kinase C (PKC) has been implicated in the control of epithelial proliferative activity and in the process of malignant transformation. *Helicobacter pylori* (H.p.) infection is associated with increased gastric epithelial cell proliferation and has been linked with gastric carcinoma. In the present study, we report that the H.p. fatty acid *cis*-9,10-methyleneoctadecanoic acid (MOA) directly activates PKC ($K_c$ 3.3 $\mu$M). The effect of MOA upon PKC activation was Ca²⁺ dependent but did not require phosphatidyserine as phospholipid cofactor. MOA increased the stimulatory effect of phosphatidyserine at low Ca²⁺ (1 $\mu$M) concentrations. These findings indicate that MOA interacts at the phospholipid- and the diacylglycerol-binding domain to elicit PKC activation. Treatment of gastric mucous cells HM02 caused translocation of PKC from the cytosol to the nuclear, mitochondrial and membrane fraction. Furthermore, MOA stimulated [³H]thymidine incorporation into the DNA of HM02 cells. Our results show that the H.p. fatty acid MOA activates PKC and increases DNA synthesis in gastric epithelial cells.

Keywords: *Helicobacter pylori*; fatty acid; protein kinase C; cell proliferation

Protein kinase C (PKC) is a family of enzymes that plays a pivotal role in transmembrane signalling, cell growth and cell division (Nishizuka, 1986; Clemens et al., 1992). Diacylglycerol (DAG), a breakdown product of polyphosphoinositides, is a physiological effector of this(ese) enzyme(s). Tumour-promoting phorbol esters, such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA), which interact with the DAG site, are activators of the enzyme and several lines of evidence indicate that tumour promotion is related to PKC activation (Martelly and Castagna, 1989). Other tumour promoters structurally unrelated to TPA have also been described as activators of PKC, such as teleocidin B (Fujiki et al., 1984), chloroform (Roghani et al., 1987) and bile acids (Huang et al., 1992).

*Helicobacter pylori* (H.p.) is a Gram-negative bacterium that causes chronic gastritis and peptic ulcer diseases (NIH Consensus Conference, 1994). Moreover, an association between the microorganism and gastric cancer has been demonstrated by epidemiological studies (Forman, 1993), but a causal link between gastric cancer and H.p. infection has not been proven. H.p. produces unusual fatty acids such as *cis*-9,10-methyleneoctadecanoic acid (Goodwin et al., 1985). *Cis*-unsaturated fatty acids are known as tumour promoters (Bull et al., 1981) and PKC activators (McPhail et al., 1984).

There is some evidence that PKC plays a crucial role in gastric epithelial proliferation. Epidermal growth factor (EGF) and transforming growth factor α (TGF-α) have been reported to be mitogenic for primary cultured gastric parietal, chief and mucous cells (Chen et al., 1991; Rutten et al., 1993). In addition to the ability of these growth factors to stimulate tyrosine kinase activity, to increase inositol trisphosphate and to interact with G proteins, EGF has been shown to activate PKC (Reynolds et al., 1993; Wang et al., 1996). Overexpression of EGF, TGF-α and their receptor genes are thought to participate in the rapid cell proliferation seen in gastric cancer (Pfeiffer et al., 1990).

Based on these observations, we have examined the characteristics of *cis*-9,10-methyleneoctadecanoic acid (MOA)-mediated PKC activation and the effect of MOA on cellular DNA synthesis.

**MATERIALS AND METHODS**

**Materials**

Rat brain PKC (a mixture of alpha-, beta- and gamma-isoforms) purified to greater than 97% by the method of Allen and Katz (1991) was purchased from Biomol (Hamburg, Germany). MOA (purity 99%) was synthesized by J Holzkampf (Department of Organic Chemistry, University of Hanover, Hanover, Germany). Glycogen synthase peptide was obtained from Bachem (Heidelberg, Germany). Fetal calf serum was obtained from Life Technologies (Eggenstein, Germany). [γ-³²P]ATP and [³H]thymidine was obtained from Hartmann Analytic (Braunschweig, Germany). All other chemicals and media were purchased from Sigma (Munich, Germany).

**Determination of protein kinase C activity**

PKC was determined in a reaction mixture (50 μl) containing 20 mM Hepes (pH 7.4), 0.2 mM EGTA, 10 mM magnesium chloride, 40 μM

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[32P]ATP (500 c.p.m. pmol⁻¹), 100 μM glycogen synthase peptide, leupeptin 1 μg and 5 ng of brain enzyme. Calcium chloride, TPA (dissolved in dimethyl sulfoxide), sonicated suspensions of MOA, phosphatidylserine and 1,2-diocanoyl-sn-glycerol (DIC₈) were added separately, as described in the text. Incubation proceeded for 5 min at 30°C and was terminated with 20 μl of an acetic acid-trichloroacetic acid solution. An aliquot (35 μl) of the solution was spotted onto Whatman P-81 phosphocellulose paper, washed three times in acetic–phosphoric acid (30%/1%) and counted by liquid scintillation analysis.

The determination of PKC translocation in gastric HM02 cells, which are derived from a human well-differentiated mucous-producing gastric carcinoma (Wagner et al., 1994), were grown for 3 days in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) at 37°C in a 5% carbon dioxide atmosphere. The cell monolayers were then kept for 1 day in FCS-free medium and trypsinised. Cell suspensions (1 × 10⁶) were incubated with MOA at 37°C in 1 ml of buffer medium (pH 7.4) for the times indicated. The buffer composition was (in mm): sodium chloride 70, sodium bicarbonate 20, sodium dihydrogen phosphate 0.5, disodium hydrogen phosphate 1.0, Hepes 50, calcium chloride 1.0, magnesium chloride 1.5 and glucose 11 (buffer A). Afterwards, the cells were washed once with calcium chloride-free buffer A and resuspended in 0.5 ml of ice-cold 20 mM Tris (pH 7.4) and leupeptin at 20 μg ml⁻¹ (buffer B). All subsequent procedures were carried out at 4°C. The cells were lysed by three 20-s bursts of sonication (power 30 W) and centrifuged at 100 000 g for 30 min. The supernatant was retained as the cytosolic fraction, the pellet was rehomogenized in 0.5 ml of buffer B containing 1 mM 3-(3-cholamidopropyl)-dimethylammonio]propyl-sulphophate (CAPS) and stirred for 30 min.

To study translocation of PKC to different subcellular fractions, 10⁷ HM02 cells were treated with 50 μM MOA for 30 min and processed as described above. Subcellular fractions were obtained by differential centrifugation and defined by the distribution of markers as follows: 1500 g for 10 min, nuclear pellet (P₁); 15 000 g for 15 min, mitochondriai pellet (P₂); 100 000 g for 60 min, membrane pellet (P₃). The three pellets were rehomogenized in buffer B setting the protein concentration to 1 mg ml⁻¹ and PKC was solubilized with 1 mM CAPS. PKC activity was determined in the presence of 0.3 mM free calcium chloride and 50 μg ml⁻¹ phosphatidylserine. Non-PKC

activity, assayed in the absence of calcium chloride and of phospholipid was subtracted from the total values.

**Marker assays and marker distribution in subcellular fractions**

**DNA determination**

DNA content was analysed by the diphenylamine reaction (Richards, 1974) using calf thymus DNA as a standard.

**Cytochrome c oxidase**

Cytochrome c oxidase was used as a marker for mitochondria. The enzyme activity was determined according to the method described by Copperstein and Lazarow (1951).

**Na⁺,K⁺-ATPase**

Na⁺,K⁺-ATPase was used as a marker for plasma membranes. Enzyme activity was assayed by the liberation of inorganic phosphate from ATP at 37°C in 1 ml of medium containing 20–50 μg of protein, 20 mM Tris buffer, pH 7.4, 2 mM magnesium chloride, 2 mM Tris-ATP, 100 mM sodium chloride and 20 mM potassium chloride in the absence and presence of 0.1 mM ouabain.
The assays were performed on P2-P3, the 100 000 g supernatant and the original cell homogenate. DNA was enriched in P2; 80 ± 4% of the DNA present in the original cell homogenate was found in this fraction, 15 ± 2.5% in P1. Cytochrome c oxidase was enriched in P2, 63 ± 12.6% of the total enzyme activity was present in P2, 30 ± 15% in P1. Na+,K+-ATPase was enriched in P1 (58 ± 1% of total enzyme activity), 34 ± 5% was detectable in P2 (values are means ± s.e.m. of three experiments). DNA, cytochrome c oxidase and Na+,K+-ATPase were not detectable in the 100 000 g supernatant.

Protein determination

Protein was determined using the method of Lowry et al (1951).

Determination of DNA synthesis

HM02 cells were plated at 2.5 × 10⁴ cells per well into 24-well plates and allowed to grow to confluence. Confluent cells were washed twice with phosphate-buffered saline and incubated for 24 h in 1 ml of serum-free RPMI-1640 medium. MOA (0.3–30 μM) was added to the cells. After 4 h, 0.5 μCi [³H]thymidine was added and incubation continued for an additional 4 h. Incorporated radioactivity was quantified by using an automated cell harvester, followed by scintillation spectrometry.

Statistics

Results are expressed as means of three independent experiments. For statistical analysis Student’s t-test was used. P-values less than 0.05 were considered to be significant.

RESULTS

Activation of protein kinase C by MOA

The direct activation of PKC by MOA in the presence of 200 μM free Ca²⁺ is shown in Figure 1. PKC was activated in the absence of a phospholipid and a diglyceride by the fatty acid in a concentration-dependent manner. The Kᵢ value was 3.3 μM. The maximal reaction velocity with MOA (1922 ± 128 nmol ³²P transferred min⁻¹ mg⁻¹ protein) was lower than that with saturating (50 μg ml⁻¹) concentrations of phosphatidylserine (4480 ± 336 nmol ³²P transferred min⁻¹ mg⁻¹ protein). In the absence of Ca²⁺ (0.2 mM EGTA) the fatty acid did not activate PKC.

The concentration dependence on Ca²⁺ of PKC activation in the presence of 5 μM MOA is shown in Figure 2A. Like the activation by phosphatidylserine, the fatty acid activates PKC in a Ca²⁺-dependent fashion. One characteristic of the phosphatidylserine stimulation of PKC is that this can be augmented at low Ca²⁺ concentrations by diacylglycerols or phorbol esters (TPA). Figure 2B shows that MOA, like 1,2-diocanoyl-sn-glycerol (DIO₂), greatly enhanced phosphatidylserine-induced activation of PKC at low (1 μM) Ca²⁺ concentrations. Although phosphatidylserine was not required for MOA-supported PKC activation, the phospholipid affected the reaction. In the presence of 200 μM free Ca²⁺ the effects of phosphatidylserine (1 and 5 μg ml⁻¹) were additive to those of MOA. However, no additive effects were found at saturating (10 and 50 μg ml⁻¹) concentrations of the phospholipid (Figure 3).

Effect of MOA on intracellular distribution of protein kinase C in gastric HM02 cells

Fractionation of gastric HM02 cells yielded a soluble and a particulate fraction. Total PKC activity (soluble plus particulate) in
**Table 1** Effect of MOA (50 μM) on the distribution of protein kinase C in subcellular fractions of HMO2 cells

| Cell fraction       | PKC activity (pmol 32P mg⁻¹ protein min⁻¹) |
|---------------------|--------------------------------------------|
|                     | -MOA                                       | +MOA                                      |
| Nuclear (P₁)        | 58 ± 14 (5)                                | 170 ± 24* (15)                           |
| Mitochondrial (P₂)  | 213 ± 11 (19)                              | 304 ± 33* (27)                           |
| Membranes (P₃)      | 242 ± 14 (21)                              | 355 ± 32* (32)                           |
| Cytosol             | 609 ± 43 (54)                              | 294 ± 50* (26)                           |
| Total               | 1122 ± 23                                  | 1123 ± 123                                |

HM02 cells were treated for 30 min with 50 μM MOA and the cell homogenate was fractionated by differential centrifugation as described in Material and methods. Σ is the total PKC activity obtained by summation of the activity of each of the fractions. The percentage in each fraction of the total activity is given in parenthesis. Values are means ± s.e.m. of three experiments. *P < 0.05 vs control.

**Table 2** Effect of MOA on [³H]thymidine incorporation into HM02 cell DNA

| Treatment | [³H]thymidine uptake (c.p.m.) |
|-----------|------------------------------|
| None      | 4376 ± 390                   |
| MOA 0.3 μM| 4606 ± 318                   |
| MOA 1 μM  | 6572 ± 573                   |
| MOA 3 μM  | 7595 ± 167                   |
| MOA 10 μM | 8108 ± 400                   |
| MOA 30 μM | 6855 ± 253                   |
| TGF-α (10 ng ml⁻¹) | 8137 ± 204                   |

Values are means ± s.e.m. of four determinations. *P < 0.05 vs control.

untreated cells was 1002 ± 87 pmol ³P transferred min⁻¹ mg⁻¹ protein (n = three cell experiments). Approximately 50% of total PKC activity was in the soluble fraction. The intracellular distribution of PKC after 15 min incubation with increasing concentrations of MOA is shown in Figure 4. MOA at concentrations of 30, 50 and 100 μM caused a decrease in soluble PKC activity that was paralleled by an increase in enzyme activity in the particulate fraction. The effect of MOA on translocation of PKC was time dependent. Maximal decrease of soluble PKC activity was detectable 30 and 60 min after the addition of MOA (3 and 30 μM) to the cells. A return to control levels was noted after 120 min of incubation. The activity of PKC in the particulate cell fraction behaved reciprocally (Figure 5).

**Subcellular locations of translocated PKC**

Table 1 shows the distribution of PKC activity in the subcellular fractions of HM02 cells. PKC was found in P₁, P₂, and P₃; significant amounts were found in the mitochondrial (19%) and membrane (21%) fraction. Treatment of HM02 cells with MOA (50 μM) resulted in a significant increase in PKC activity in all particulate cell fractions. The enzyme activity increased threefold in P₁ and 1.5-fold in the mitochondrial P₂ and membrane pellet P₃.

**Effect of MOA on DNA synthesis**

Table 2 demonstrates that MOA stimulates DNA synthesis of HM02 cells. The threshold concentration was 1.0 μM, maximal response was seen at 10 μM. The effect of TGF-α (10 ng ml⁻¹) was similar to that of 10 μM MOA.

**DISCUSSION**

Our data demonstrate that the H.p. fatty acid MOA activates PKC, both at the level of the isolated enzyme and at the level of the intact cell and stimulates DNA synthesis.

Activation of PKC is a two-step process. The first step is the formation of an enzyme–Ca²⁺–phospholipid (phosphatidylycerine) complex; the second step in association is the binding of diacylglycerol (or phorbol esters), which through conformational changes increases the affinity of the enzyme for Ca²⁺ and thereby renders it fully active at submicromolar Ca²⁺ concentrations (Nishizuka, 1986). The characteristics of MOA-triggered PKC activation provide evidence that this fatty acid interacts with two binding sites of the enzyme: (a) MOA directly stimulates PKC in the absence of phosphatidylserine; (b) at subsaturating concentrations phosphatidylserine enhances the effect of MOA in a medium containing 200 μM Ca²⁺; and (c) MOA augments phosphatidylserine-stimulated PKC activation at low (1 μM) Ca²⁺ concentrations. The results (a) and (b) favour the hypothesis that MOA interacts with PKC in the phospholipid-binding domain, whereas (c) indicates that MOA in addition interacts with the diacylglycerol binding site of the enzyme. Therefore, MOA can be regarded as a novel and unique PKC activator. Other naturally occurring PKC activators, such as retinoic acid (Ohkubo et al., 1984), bile acids (Huang et al., 1992), lipid X (Wightman & Raetz, 1984) and unsaturated fatty acids such as linoleic acid (Lester, 1990) have been found to interact with PKC in the phospholipid-binding domain.

In gastric mucous cells that are likely targets for H.p., MOA caused translocation of PKC from the cytosol to the particular cell fraction. In the nuclear cell fraction PKC activity was increased threefold. Nuclear PKC has been shown to play an essential role in the mitogenic effect of platelet-derived growth factor (Fields et al., 1990). In general, increased levels of nuclear PKC is associated with cell proliferation (Clements et al., 1992). However, PKC is a family of enzymes consisting of at least nine isotypes. Thus, certain signal transduction pathways may involve only single PKC species. In particular, translocation and activation of PKC-β at the nucleus has been suggested to play a role in growth regulation. In Swiss 3T3 cells an overexpression of PKC-β (but not PKC-α) enhanced growth rate (Eldar et al., 1990) and in K562 erythroleukaemia cells activation of PKC-β by the PKC activator bryostatin leads to a proliferative signal (Hocevar et al., 1992). The PKC isoenzymes expressed in HM 02 cells and the specific isoform(s) of PKC that mediates the action of MOA are not known. This question will require additional investigation.

Several properties of H.p. may facilitate cancer development without being specifically carcinogenic. H.p. gastritis is associated with a significant decrease in the concentration of ascorbic acid in gastric juice (Banerjee et al., 1994).

Ascorbic acid is an antioxidant that has important functions as a scavenger of reactive oxygen species and inhibits N-nitrosation (Licht et al., 1988). A further response to H.p. infection is a substantial increase in gastric epithelial cell turnover rates. Several studies have shown an approximate doubling of cell turnover rates associated with H.p. and a restitution to normal levels after successful eradication of the bacterium (Alam et al., 1994; Cahill et al., 1994, 1995; Lynch et al., 1995). An increase in epithelial cell proliferation is one of the earliest mucosal changes in the development of gastric cancer (Deschner et al., 1972) and an increased epithelial cell proliferation has been shown in macroscopically normal tissue remote from gastric carcinoma (Brito et al., 1992).
It has been suggested that ammonia, or ammonium-containing substances, abundantly produced as a result of H.p. urease activity, may act as cancer promoters enhancing rates of cell division (Tsujii et al., 1992). Recently, Fan and colleagues (1996) reported that H.p. can directly stimulate proliferation of the gastric epithelial cell line AGS. Our results demonstrate that the H.p. fatty acid MOA activates PKC and initiates DNA synthesis in the mucous cell line HM02. Therefore, we hypothesize that MOA may account, at least in part, for the cell proliferative effect of H.p. The concentration of MOA present in the H.p. infected mucosa is not known. We have found that approximately 30% (i.e. 630 ng of MOA from 10^4 H.p.) of intracellular MOA are released from the bacterium during 24-h log phase growth (C. Birkholz and W. Beil, unpublished observations). This fatty acid amount would correspond to approximately 2 μM MOA in a volume of 1 ml. At this concentration MOA clearly stimulates PKC activity and enhances DNA synthesis in mucous cells.

We conclude from this study that the H.p. fatty acid MOA activates PKC and increases DNA synthesis in the gastric epithelial cell line HM02. We suggest that MOA-induced enhancement of proliferative activity may render the gastric epithelial cells more susceptible to carcinogenic stimuli and thereby contribute to the enhanced risk of gastric cancer in H.p. infected populations.

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