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TPA and butyrate increase cell sensitivity to the vacuolating toxin of *Helicobacter pylori*

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Abstract The *Helicobacter pylori* toxin VacA induces large membrane-bound vacuolar compartments of late endosomal/lysosomal origin. Pre-treatment of cells with TPA and butyrate enhances the toxin induced vacuolisation up to 20 times, depending on the cell line, whereas other differentiating factors such as DMSO, EGF, valeric and retinoic acid have no effect. The higher toxin sensitivity induced by TPA does not result from an increased surface binding or endocytosis. The effect of TPA is apparent after several hours from addition and is inhibited by a PKC specific inhibitor. These data suggest that expression of cellular proteins, other than the toxin receptor(s), influences the vacuolating activity of VacA and may contribute to the sensitivity of different cell lines. The present findings define the most sensitive in vitro assay of the activity of VacA.

Key words: Vacuolating cytotoxin A; TPA; Butyrate; Cell sensitivity

1. Introduction

Infection of a large proportion of the human population with pathogenic strains of the bacterium *Helicobacter pylori* is associated with severe gastro-duodenal diseases [1–4]. Most of these parasites produce the protein toxin VacA, known to induce in cultured cells enlarged acidic compartments, or vacuoles, which originate from late endocytic compartments [5–10]. Secreted extracellularly as a 95-kDa polypeptide, VacA assembles into inactive oligomers formed by 6–12 subunits, which are dissociated and activated by low pH pretreatments [11–14]. Since VacA is (i) cleaved by protease into two fragments of 37 kDa (NH_2 terminal) and 58 kDa (COOH terminal) still bound by non-covalent interactions [11], (ii) endocytosed by cells [15] and (iii) able to vacuolate cells when expressed in the cytosol [16], it was proposed to be an A-B type protein toxin [17] with a yet unknown catalytic activity and cytosolic target.

In the course of studies aimed at the identification of the cellular substrate(s) of VacA we assayed the effect of a series of compounds of defined cellular activity. In the present work, we report the ability of two cell differentiating agents, TPA and butyrate, to enhance, in some cases dramatically, the sensitivity of different cell types to VacA, without increasing toxin cell binding. Time course, inhibition and binding experiments suggest that the TPA-induced increase of cell sensitivity to VacA is due to newly expressed proteins, not involved in toxin reception. This suggests that VacA cell vacuolation is the result of multiple factors that may vary not only according to the cell type but also to its differentiation state.

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Abbreviations: VacA, vacuolating cytotoxin A; TPA, 12-*O*-tetradecanoylphorbol-13-*O*-acetate; DMSO, dimethyl sulfoxide; NaBu, sodium butyrate; EGF, human epidermal growth factor

2. Materials and methods

2.1. Proteins and reagents

VacA toxin was isolated from *Helicobacter pylori* CCUG strain as described [18] and stored as liquid solution in PBS at 4°C. Activation by low pH treatment was achieved as reported [25]. Cell culture media, neutral red and rabbit polyclonal antibody against the α isoform of PKC were from Sigma; foetal calf serum (FCS) was from PAA Laboratories. Iodination of VacA was performed according to [19]. Bolton and Hunter reagent for iodination was from Amersham (2000 μCi/mmol). Bis-indolyl-maleimide, fumonisine, TPA, DMSO, human recombinant EGF, valeric acid, retinoic acid, butyric acid were from Sigma.

2.2. Cells

HeLa, MDCK, NRK, BHK, Vero, HL60 were cultured in plastic flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS under 5% CO_2 at 37°C. Jurkat cells were grown in RPMI, plus 10% FCS. Twenty-four hours before experiments cells were released with trypsin/EDTA and seeded in 24-well titration plates in their culture medium at a density of 4×10^4 per cm^2. When indicated, cells were pre-incubated for 1 or for 15 h with TPA (5–100 nM), or for 15 h with sodium butyrate (2 mM), DMSO (1% v/v), EGF (10 nM), valeric acid (5 mM), retinoic acid (5 μM) and further challenged with 20 nM VacA for 4 h. The extent of vacuolation was then determined by measuring the uptake of neutral red, as detailed [7]. In some experiments cells were incubated with bis-indolyl-maleimide (20–100 nM) for 30 min before TPA treatment as above.

2.3. VacA cell binding and uptake

VacA was labelled with Bolton-Hunter reagent in a ratio of 15 μg VacA/100 μCi ^{125}I. The iodinated protein was added to cold VacA to give a stock solution of 5 μM VacA.

HeLa cells were incubated for 1 h at 4°C or at 37°C with ^{125}I-VacA pre-activated at pH 2.0 and diluted in DMEM medium (90 000 cpm/ml). Cells were washed with ice-cold PBS, plus 0.5% BSA and suspended by incubating in PBS plus 5 mM Na-EDTA at 4°C for 5 min and the radioactivity determined by γ-counting using a 1600 TR Camberra Packard counter.

2.4. Immunofluorescence microscopy

Cells were seeded in 24-well titration plates on round glass coverslips at a density of 4×10^4 per cm^2 24 h before the experiment; they were fixed with 3% (w/v) paraformaldehyde for 20 min, treated with 0.27% (w/v) NH_4Cl, 0.38% (w/v) glycine for 10 min and permeabilized with 0.2% (w/v) saponin, 0.5% (w/v) BSA in PBS for 30 min. The first antibody was diluted in the permeabilization medium and incubated with cells for 1 h. After several washes, rhodaminated secondary antibody was added for 30 min in the same medium and washed. Samples

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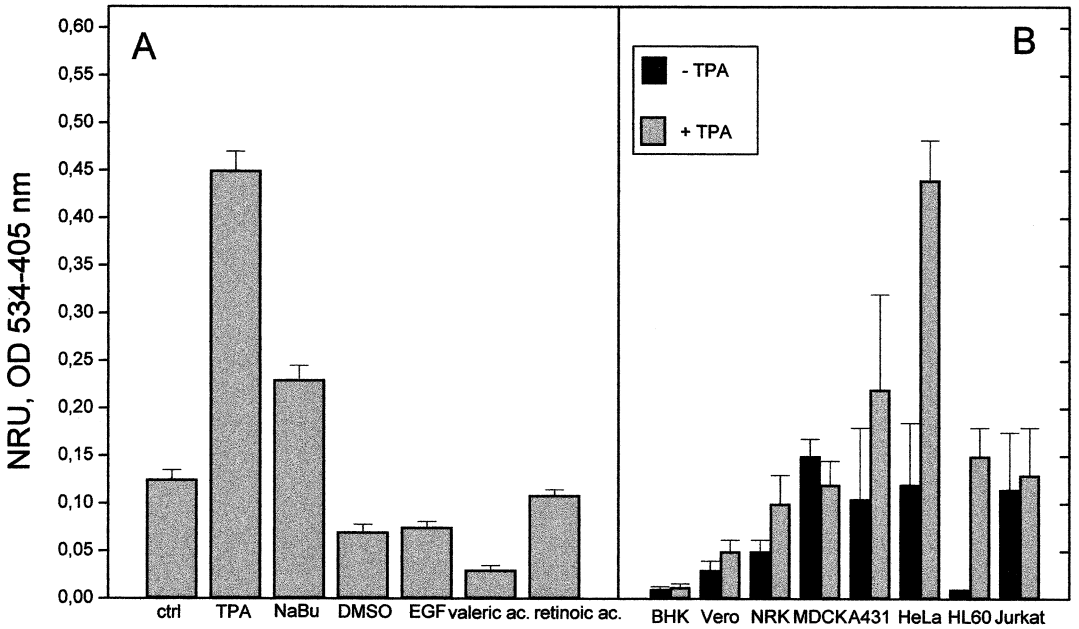

Fig. 1. A: Effect of various differentiating agents on the sensitivity of HeLa cells to VacA. Cells were incubated for 15 h with the agents indicated in the abscissa at the concentrations given in the experimental section and then challenged with the toxin (20 nM) for 4 h. The extent of vacuolation was estimated by measuring the amount of neutral red taken up by the cells. B: Comparison of the effect of TPA treatment on the sensitivity of various cell lines to VacA. The cell lines indicated in the abscissa were incubated with TPA and then challenged with VacA under the same conditions as in A and the amount of neutral red taken up by the cells without (filled bars) and with (grey bars) TPA pre-treatment is reported. Bars indicate the S.D. values obtained in three or more determinations run in duplicate.

were mounted on 90% (v/v) glycerol, 0.2% (w/v) *N*-propylgallate in PBS and observed with a fluorescence microscope (Zeiss Axioplan).

3. Results

Fig. 1A shows the effect of pre-treating HeLa cells for 15 h with various differentiating agents on intracellular vacuolation induced by subsequent treatment with VacA for 4 h. VacA activity, measured by neutral red uptake (NRU), increased sensibly after TPA and butyric acid treatment, while retinoic acid had a negligible effect. DMSO, EGF and valeric acid were, on the contrary, slightly inhibitory. TPA and butyrate actions are not cumulative since co-incubation of these two agents resulted in a VacA activity increase similar to that of TPA alone (not shown).

Based on these data, we chose TPA as the best vacuolation-promoting agent, and challenged several commonly used cell lines. Results, summarised in Fig. 1B, revealed a varied response pattern. In fact, while the sensitivity to VacA of some cell types (HL60, HeLa, A431, NRK, Vero, Jurkat) increased upon TPA pre-treatment, that of others (MDCK, BHK) was minimally inhibited or not affected. No correlation was found between the gain in sensitivity to the toxin by the studied cell lines and their basal vacuolation value. The most remarkable effect of TPA is displayed on HL60 cells, which are virtually insensitive to VacA in resting conditions. On the contrary, MDCK cells, which are toxin sensitive, were hardly affected by TPA treatment.

HeLa cells, a line extensively used either for standard quantification of vacuolation or for studying the mechanism of cellular intoxication, were used in subsequent experiments. The dose-response curve (Fig. 2, panel A) shows that the increased VacA intoxication induced by TPA occurs in the 5–15-nM range, and saturates at 20–30 nM, in agreement with the TPA activating doses of PKC [20]. In fact, TPA rapidly activates the intracellular regulatory protein protein kinase C (PKC) [21,22], which is then down-regulated [23]. In parallel, downstream signals activated by PKC change the pattern of gene expression generally leading to differentiation and inhibition of cell proliferation [24,25]. Time-course experiments (Fig. 2, panel B) showed that the increase of VacA intoxication is a late effect of TPA, occurring only after 8–10 h of treatment. Interestingly this feature was emphasised by pulsing cells with TPA for 1 h, followed by washing, instead of incubating cells continuously with the drug (Fig. 2, panel B). In fact, VacA sensitivity increased with a similar slow kinetics, but the intensity of such change was much higher. This suggests that maintaining TPA in the medium has a detrimental action on VacA which superimposes and counterbalances its activatory effect, consistent with the fact that continuous TPA treatment inhibited at early times (<5 h) VacA action. Immunofluorescence analysis (Fig. 3) confirmed that at incubation times >5 h PKC intracellular level was strongly down-regulated. Hence, in order to test if a decreased PKC activity, following down-regulation, was responsible for the higher vacuolating activity of VacA, the effect of the PKC inhibitor bis-indolyl-maleimide [26] was assessed. Fig. 4 shows that the basal activity of VacA on HeLa cells is scarcely modified by treatment with this PKC inhibitor, while TPA dependent increase of VacA action was completely abolished. This result proves that the initial activation of PKC is necessary to subsequent VacA sensitisation and suggests that regulation of gene expression, with possible neo-synthesis of protein factors, rather than the decrease of PKC activity due to down-regulation, might account for this action.

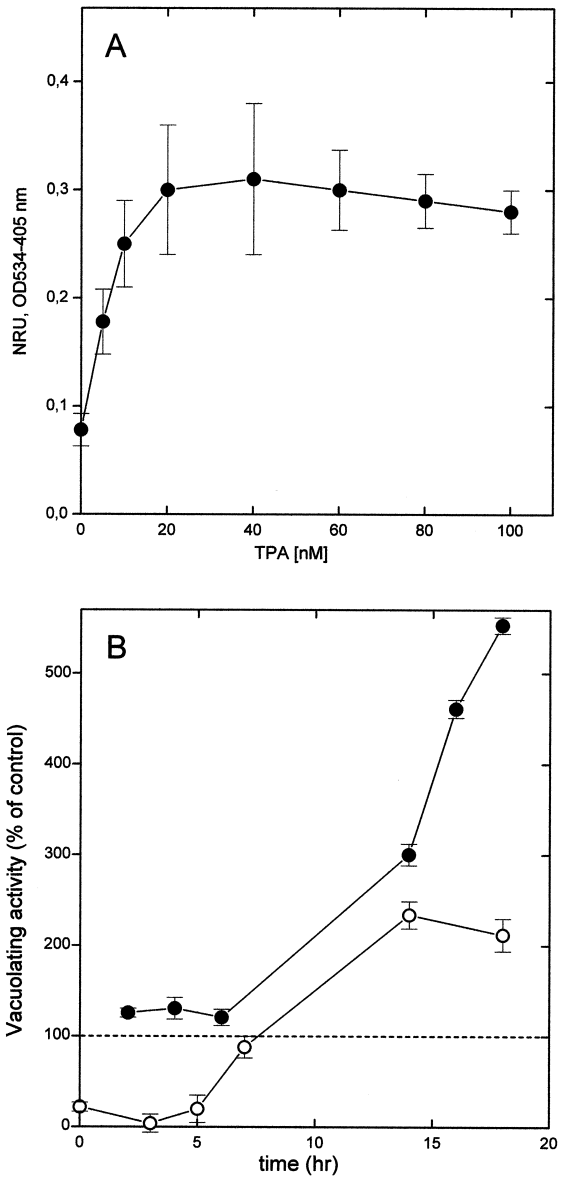

Fig. 2. Dose-response and kinetics of TPA-induced increase of VacA sensitivity. A: Effect of different doses of TPA on the sensitivity of HeLa cells to VacA determined as in Fig. 1A. B: Kinetics of the TPA effect on HeLa cells continuously exposed to TPA until the end of the incubation with the toxin (open circles) or on HeLa cells exposed to TPA for 1 h, washed and then challenged with the toxin at the indicated times for an additional 4 h (filled circles). Time 0 refers to the time of addition of TPA (20 nM). The upper trace (filled circles) begins with the time of addition of the toxin, which was performed after a 1-h incubation with TPA, followed by washings. Bars represent S.D. values obtained in three different experiments run as duplicates.
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Binding and internalisation experiments with ^{125}I-labelled VacA showed no increase in toxin association to cells and in the rate of endocytosis after TPA incubation, but, rather, a partial inhibition, ruling out the possibility that an increase expression of a surface receptor for the toxin is responsible for its increased vacuolation (Fig. 5). These results were confirmed by indirect immunofluorescence experiments with VacA specific antibodies, which failed to reveal differences in binding and internalisation extents after TPA incubation (not shown).

4. Discussion

Vacuolation of cultured cells is a well-established quantitative method of assay of the toxic activity of VacA, a major virulence factor of pathogenic strains of *Helicobacter pylori* [5,6]. However, although available data indicate that both osmotic and membrane traffic regulation can influence the extent of intracellular vacuolation [7,27], the knowledge of the events leading to vacuole formation, is still very incomplete.

Here, we show that in some cell types VacA sensitivity is highly increased by TPA and butyrate, two compounds belonging to the heterogeneous class of differentiating agents, which stimulate intracellular transduction events and modulate gene expression. A systematic analysis revealed that TPA affects differently the most widely used stabilised cell lines, in agreement with their genotypic and phenotypic differences. This observation suggests that TPA switches on or off some cellular function(s), which is initially absent, partially or maximally activated, according to the cell type.

In the case of TPA stimulation, the onset of increased sensitivity to VacA requires PKC activation, as shown by the effect of a specific inhibitor, with a rather long time (10–15 h), indicating that a change in gene expression is likely

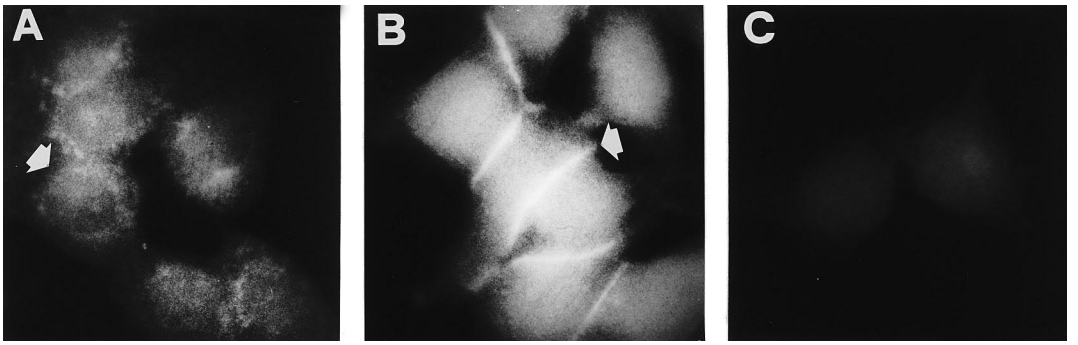

Fig. 3. Down-regulation of PCK by TPA in HeLa cells. HeLa cells were seeded on glass coverslips and treated with normal DMEM medium for 5 h (A), or with TPA (20 nM) for either 5 min (B) or 5 h (C). Indirect immunofluorescence with antibodies to PKC (α-isophorm) was then performed after cell fixation as described in Section 2. Samples were viewed with an Axioplan fluorescence microscope (Zeiss) and photographed. Arrowheads point to cytoplasmic PKC in panel A and to plasma membrane associated PKC in panel B. Magnification 200×.

to be involved. Biochemical and morphological evidence shows that this effect is not due to an increased cell association or endocytosis of VacA.

The data here reported add significantly to the understanding of VacA intoxication mechanisms and suggest that special caution must be paid in ranking cell types for their sensitivity to VacA. A striking example is provided by HL60 cells, which are toxin insensitive and become sensitive to VacA upon exposure to TPA. This finding may have major consequences because it provides a unique system for the identification of gene product(s) involved in cell vacuolisation. Another relevant implication of the present data is that the extent of toxin cell binding and endocytosis does not necessarily define the degree of cellular vacuolation, which is strongly influenced by still unknown protein factors. This may reflect the fact that the vacuolated phenotype is the result of a complex and articulated alteration of the organisation of the endocytic pathway. Alternatively, intracellular sorting, translocation and intracellular target modification might be modified by TPA treatment and favour cell intoxication by VacA.

Provided that cell lines such as HeLa or HL60 are employed, the present study has a major impact in the definition of a protocol to assay the toxic activity of VacA. In fact, here we have clearly shown that HeLa cells treated for 1 h with 20 mM TPA, before the addition of the toxin, in standard 96-well trays provide the most sensitive in vitro assay of the vacuolating activity of VacA presently available.

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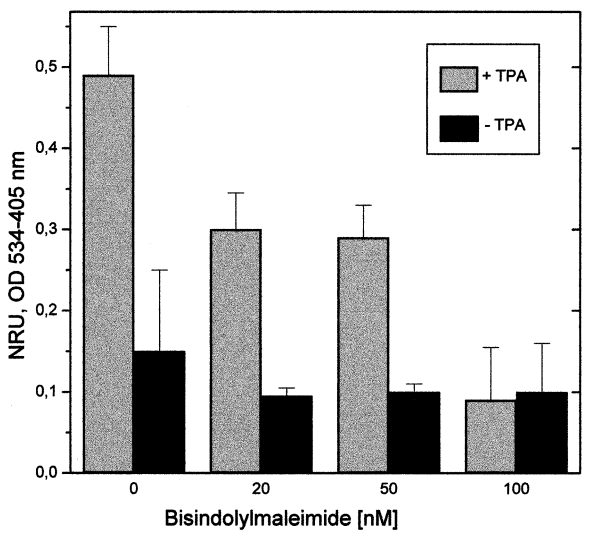

Fig. 4. Effect of PKC inhibitor bis-indolyl-maleimide on basal and TPA-enhanced VacA sensitivity. The inhibitor was added to HeLa cells at the indicated concentrations and after 30 min TPA (20 nM) was added. After 15 h cells were washed and incubated with VacA for 4 h. The extent of vacuolisation was measured as in Fig. 1 and bars represent S.D. values of two experiments run in duplicate.

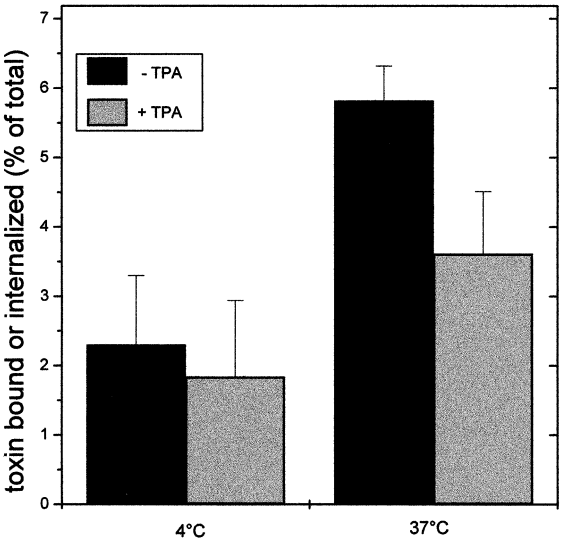

Fig. 5. Binding and uptake of ^{125}I-VacA by TPA-treated cells. HeLa cells were incubated at 4°C, treated with TPA (20 nM) and after 1 h ^{125}I-VacA was added and incubation was prolonged for 1 h. After four washings, the amount of radioactivity associated to the cells was determined. Other samples, after 1 h of incubation with the radioactive toxin at 37°C, were cooled, washed three times and counted. Cell associated radioactivity is expressed as percentage of the total radioactivity added in each well and bars represent S.D. values obtained from three different experiments run as duplicates.

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