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Formation of anion-selective channels in the cell plasma membrane by the toxin VacA of *Helicobacter pylori* is required for its biological activity

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The vacuolating toxin VacA, a major determinant of *Helicobacter pylori*-associated gastric diseases, forms anion-selective channels in artificial planar lipid bilayers. Here we show that VacA increases the anion permeability of the HeLa cell plasma membrane and determines membrane depolarization. Electrophysiological and pharmacological approaches indicated that this effect is due to the formation of low-conductance VacA pores in the cell plasma membrane and not to the opening of Ca^{2+}- or volume-activated chloride channels. VacA-dependent increase of current conduction both in artificial planar lipid bilayers and in the cellular system was effectively inhibited by the chloride channel blocker 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), while2-[(2-cyclopentenyl-6,7-dichloro-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)oxy] acetic acid (IAA-94) was less effective. NPPB inhibited and partially reversed the vacuolation of HeLa cells and the increase of ion conductivity of polarized Madine Darby canine kidney cell monolayers induced by VacA, while IAA-94 had a weaker effect. We conclude that pore formation by VacA accounts for plasma membrane permeabilization and is required for both cell vacuolation and increase of trans-epithelial conductivity.

Keywords: anion channel/epithelial permeability/*Helicobacter pylori*/VacA/vacuolation

Introduction

The protein toxin VacA produced by pathogenic *Helicobacter pylori* strains plays a major role in the pathogenesis of gastroduodenal diseases associated with infection by this bacterium (Blaser, 1993; Cover *et al.*, 1993a; Marchetti *et al.*, 1995; Xiang *et al.*, 1995). Consistently, gastric epithelial erosion observed in these pathologies is mimicked in animal models by the oral administration of VacA (Telford *et al.*, 1994). In non-polarized cells, at low cell density in the presence of ammonium ions, VacA induces cytoplasmic vacuoles (Leunk *et al.*, 1988; Cover and Blaser, 1992) derived from late endosomes and lysosomes (Papini *et al.*, 1994; Molinari *et al.*, 1997). VacA also causes a size-selective, vacuolation-independent permeability increase of polarized epithelial cell monolayers *in vitro*, a phenomenon proposed to favour *H.pylori* survival *in vivo* by increasing nutrient efflux from the gastric sub-mucosa (Papini *et al.*, 1998; Pelicic *et al.*, 1999).

The polymorphic *vacA* gene (Cover *et al.*, 1994) encodes a toxin of 95 kDa, which is partly released into the extracellular medium as oligomers (Telford *et al.*, 1994; Lupetti *et al.*, 1996; Cover *et al.*, 1997) and partly retained on the bacterial surface (Telford *et al.*, 1994; Pelicic *et al.*, 1999). Pre-treatment at acidic pH of soluble toxin oligomers strongly promotes the cell vacuolation and epithelium permeabilization activities of the toxin (de Bernard *et al.*, 1995; Papini *et al.*, 1998), while bacterium-bound VacA appears to be constitutively active at neutral pH (Pelicic *et al.*, 1999).

Like many A-B type toxins with an intracellular target (Montecucco *et al.*, 1994), VacA monomers are formed by two distinct domains (p37 and p58) linked by a protease-sensitive loop (Telford *et al.*, 1994). The 37 kDa N-terminal domain (p37) has been proposed to correspond to the enzymatically active portion of VacA (de Bernard *et al.*, 1997, 1998a). In fact, this domain, plus a substantial contiguous portion of the C-terminal domain (p58), induced vacuolation when expressed in the cell cytosol (de Bernard *et al.*, 1998a; Ye *et al.*, 1999). According to this model, the p58 domain mediates cell binding and the interaction with the membrane hydrophobic core in the process of p37 translocation (Moll *et al.*, 1995). Although direct evidence of enzymatic activity is lacking, it is assumed that VacA modifies membrane traffic along the endocytic pathway (Papini *et al.*, 1997; Montecucco, 1998).

On the other hand, VacA also shows features of a pore-forming toxin, since, in artificial planar lipid bilayers, toxin monomers form anion-selective channels (Tombola *et al.*, 1999) by assembling into hexameric, ring-like structures (Czajkowsky *et al.*, 1999; Iwamoto *et al.*, 1999). Hence we suggested (Tombola *et al.*, 1999) that VacA-dependent vacuolation results from an osmotic imbalance of late endosomes, in agreement with the inhibitory action of V-ATPase blockers (Cover *et al.*, 1993b; Papini *et al.*, 1993) and with the synergistic action of acidotropic weak bases (Cover *et al.*, 1992; Papini *et al.*, 1996, Ricci *et al.*, 1997; Sommi *et al.*, 1998) on vacuole formation and maintenance.

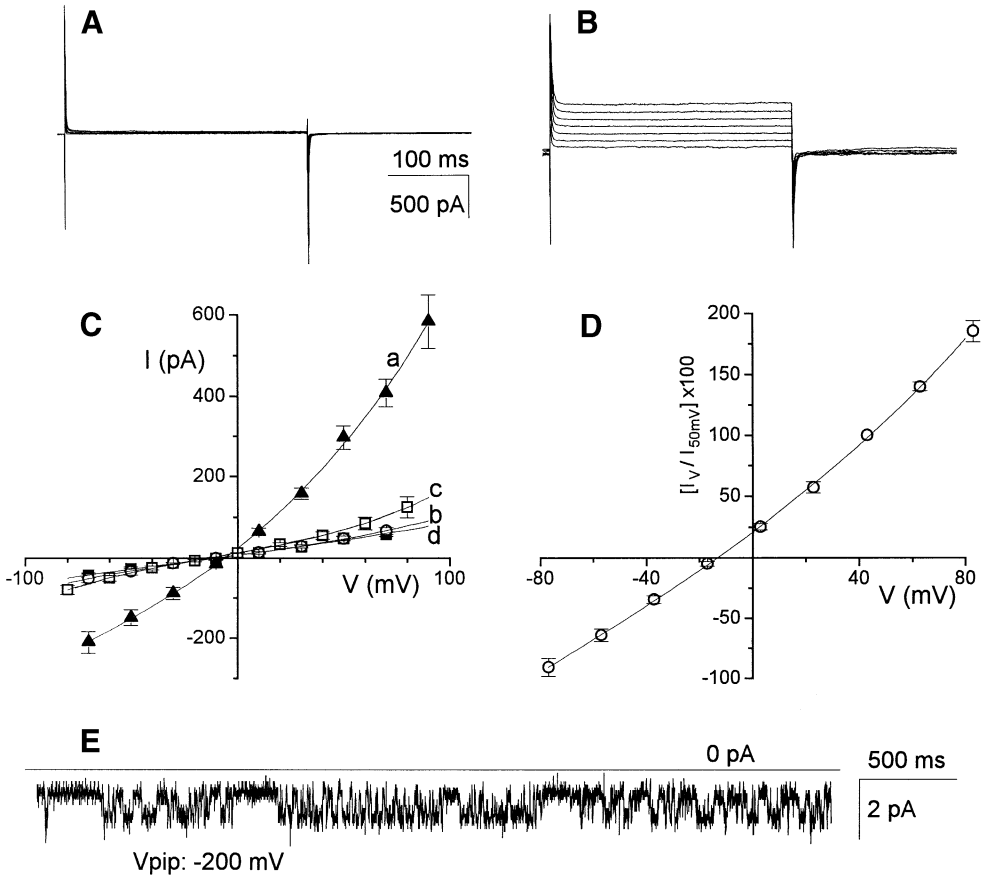

Fig. 1. VacA-treated cells exhibit an increased, anion-selective membrane permeability. (**A**) An example control experiment. A set of whole-cell current traces from an untreated HeLa cell is shown. Voltage protocol and media: see Materials and methods. Filtering: 1 kHz. Sampling: 4 kHz. (**B**) Current traces obtained from a HeLa cell exposed for 30 min to 0.5 μg/ml pre-activated VacA. Conditions as in (A). (**C**) Averaged I/V plots for cells treated with pre-activated (curve a, ▲, $n = 22$ independent experiments) or non-pre-activated (curve b, ○, $n = 6$) VacA (0.5 μg/ml, 30 min), or with pre-activated VacA incubated for 30 min with anti-VacA IgG before addition to the cells (curve c, □, $n = 8$) and for untreated cells (curve d, ■, $n = 31$). (**D**) Selectivity. Averaged I/V curve obtained from cells exposed to pre-activated VacA. Media: pipette: 150 mM TEACl; bath: 300 mM TEACl. The data are expressed as the percentage of the current value measured at V = 50 mV. $E_{rev} = –14.8 \pm 3.2$ mV ($n = 8$). (**E**) Single-channel activity in a cell-attached patch of a HeLa cell exposed to pre-activated VacA. Pipette voltage, –200 mV; filtering, 0.5 kHz; sampling, 2 kHz. Error bars represent ± SEM.

In this study, we provide direct evidence that anion-selective VacA channels are formed efficiently in the plasma membrane of cells, and that this results in depolarization of the membrane. Using known chloride channel inhibitors, we then demonstrate that blocking VacA channels inhibits and partially reverses both cellular vacuolation and the increase of the permeability of polarized epithelia caused by the toxin.

Results

Activated VacA increases the permeability of HeLa cell plasma membrane

Whole-cell patch–clamp experiments were performed on HeLa cells because this cell line is very sensitive to VacA (de Bernard *et al.*, 1998b) and because so far most of the research on the cellular effects of the toxin has been conducted using this model (Cover, 1997; Montecucco, 1998). Furthermore, HeLa cells lack the cystic fibrosis transmembrane conductance regulator (CFTR) channel (Anderson *et al.*, 1991), whose activity might have interfered with the quantification of VacA-induced currents. Incubation of HeLa cells for 30 min at 37°C with 0.5 μg/ml acid-pre-activated VacA resulted in a marked increase of the whole-cell current, as shown in Figure 1A, B and C (curve a). The VacA-induced conductance decayed with a $t_{1/2}$ of >1 h, independently of the presence of ATP in the pipette. No current was elicited, applying the same voltage pulse protocol, in untreated control cells (Figure 1A and C, curve d) or in cells exposed to non-pre-activated toxin (Figure 1C, curve b). When the toxin was pre-incubated with IgG isolated from VacA-immunized rabbits, which blocks cell vacuolation (Manetti *et al.*, 1995), only a very low current developed (Figure 1C, curve c). IgG from pre-immune serum showed no such inhibitory effect. No inhibition was observed, however, if the same antibodies were added to cells already exhibiting VacA-elicited current (not shown; $n = 5$). Analogous experiments at the planar bilayer gave the same results (not shown). No intracellular vacuoles developed under the conditions and over the time required (0.5–1 h) for patch–clamp experiments, as determined by both microscopic inspection and Neutral red uptake (NRU) assay (not shown).

The VacA-induced current is slightly outwardly rectifying, in agreement with the results obtained with VacA

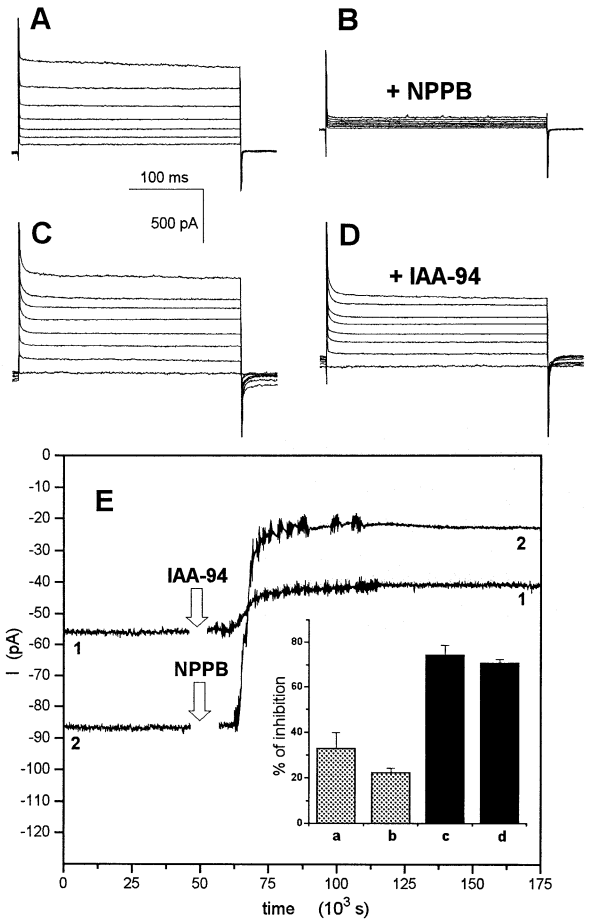

Fig. 2. Effect of chloride channel inhibitors on VacA-elicited currents. Inhibition of VacA-induced whole-cell currents by 100 μM NPPB (**A** and **B**) or 100 μM IAA-94 (**C** and **D**) added to the bath. Traces (B) and (D) were recorded from the same cells as (A) and (C) respectively, after addition of the inhibitor. Conditions are as described in Figure 1A and B. (**E**) Trans-planar bilayer current traces illustrating inhibition of VacA-mediated current conduction by IAA-94 (trace 1) and NPPB (trace 2). Inhibition is indicated by the rapid decrease of the absolute value of the current upon stirring after the addition of the inhibitor (indicated by the arrows). The amplitude of stirring-induced noise has been reduced for clarity. Medium: symmetrical 500 mM KCl. V_{cis}: –40 mV. Filtering and sampling: 100 Hz. Inset: Comparison of current inhibition in patch–clamp (a and c) and bilayer (b and d) experiments by 100 μM IAA-94 (a and b) and 100 μM NPPB (c and d). V: –40 mV (bilayer, *cis*); –50 mV (patch–clamp, bath). $n = 5$ (a and c), $n = 10$ (b), $n = 11$(d). Error bars represent ± SEM.

inserted into bilayers (Tombola *et al.*, 1999). The variability reflects cell-to-cell variation in the current conducted at any given potential, due to differences in cell membrane area, in the activity of toxin preparations, and possibly also in toxin binding. Accurate dose- and time-course analysis was hampered by this intrinsic variability. Higher toxin doses or longer incubation times could not be used since tight seals became difficult to establish.

The VacA-induced ion permeation pathway is anion-selective (Figure 1D). The whole-cell current exhibited a reversal potential of –14.8 ± 3.2 mV ($n = 8$) in 150/300 mM tetraethylammonium chloride (TEACl), close to the predicted reversal potential for Cl^- under these conditions (–17.9 mV), consistent with chloride selectivity (calculated $P_{Cl}/P_{TEA} = 12$). The facts that we observed the VacA-induced current also in the presence of TEA^+ as the cationic species, and that the reversal potential of the current was 0 mV in 150 mM NaCl/150 mM KCl (not shown) are also consistent with an anion selectivity of the channel. In planar lipid bilayers VacA forms an anion-selective, voltage-dependent channel of 10–30 pS (2 M KCl) (Iwamoto *et al.*, 1999; Tombola *et al.*, 1999). The properties of the whole-cell conductance and of toxin single channels observed in the cell-attached configuration (Figure 1E) of VacA-treated cells are essentially identical to those exhibited by VacA-treated planar bilayers. These observations indicate that the conductance increase is due to the insertion of VacA channels into the lipid bilayer portion of the plasma membrane.

To characterize further VacA channels, we studied the effect of two well-known chloride channel inhibitors, namely 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and 2-[(2-cyclopentenyl-6,7-dichloro-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)oxy]acetic acid (IAA-94). Figure 2 shows representative current traces recorded from VacA-intoxicated patched HeLa cells (Figure 2A–D) or from VacA-doped planar lipid bilayers (Figure 2E) before and after addition of these drugs (100 μM). In both systems NPPB was much more effective than IAA-94 (70–75% versus 20–30% inhibition; see inset of Figure 2E). Although this correlation further supports the idea that the toxin itself forms channels in the plasma membrane, other experiments were performed to test the possibility that VacA activates endogenous chloride channels. Since CFTR is not expressed in HeLa cells (Anderson *et al.*, 1991), the two known channels that might contribute to current conduction are the swelling-activated chloride channel (SWAC) and the Ca^{2+}-activated chloride channel. These channels have an intrinsic I/V relationship similar to that of VacA currents (Figure 3D). However, in contrast to its effect on VacA currents, IAA-94 (100 μM) strongly inhibited (70.2 ± 3.3%; $n = 5$) HeLa SWACs, as shown in Figure 3A and B. Moreover, SWAC currents showed a rapid, almost complete rundown within 3–5 min when ATP was omitted from the pipette solution, while VacA current, as mentioned above, was much more stable. Importantly, VacA current was observed also when using a pipette solution strongly hypo-osmotic with respect to the bath ($n = 8$), conditions under which SWAC is not active (Lepple-Wienhues *et al.*, 1998). These experiments and observations exclude the possibility that the effect of VacA on plasma membrane ion conductance is mediated by the opening/activation of SWAC.

In order to investigate the involvement of calcium-activated channels, we studied for the first time this channel activity [calcium-induced chloride current (ICaCl)] in HeLa cells. As shown in Figure 3C, when the pipette contained 500 nM free Ca^{2+}, HeLa cells exhibited a rapidly inactivating current at negative potentials and a slowly activating current at positive potentials ($n = 6$), in agreement with studies performed in other cell lines (Nilius *et al.*, 1997a,b). This behaviour was clearly different from that of VacA-induced currents (see Figures 1B, 2A and 2C). Most importantly, we always observed VacA-induced current conduction ($n = 22$), even though the Ca^{2+} concentration (10 nM) in the standard pipette medium was well below the activation threshold of ICaCl (Nilius, 1997a). An involvement of Ca^{2+}-activated chloride channels can thus be excluded. Nilius and co-workers (1997a)

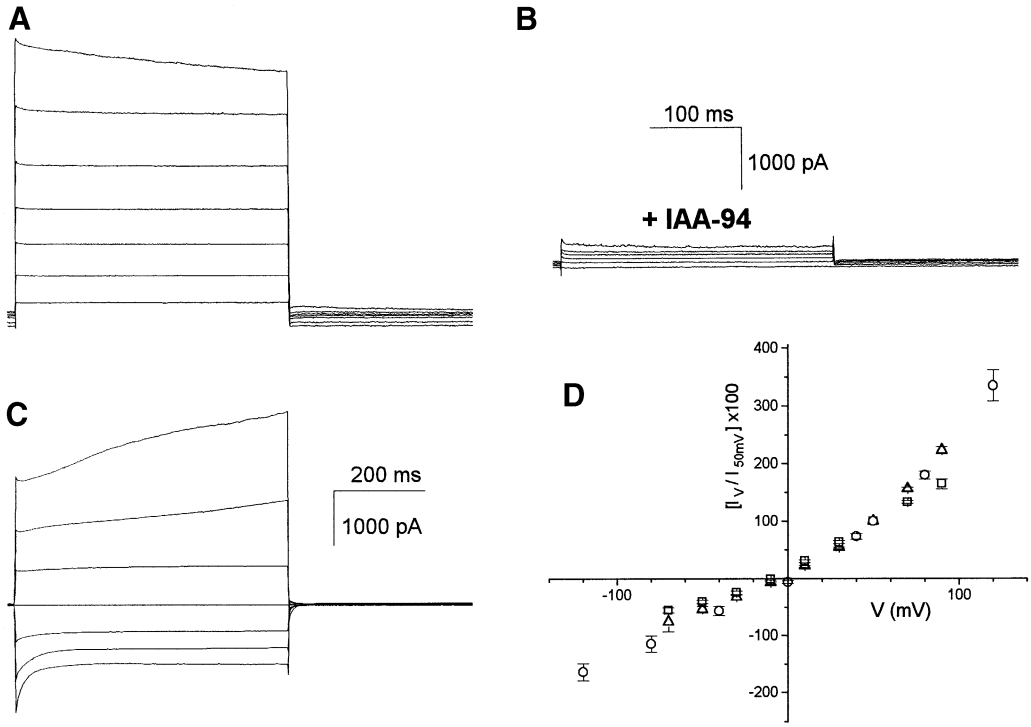

Fig. 3. VacA-elicited currents are not due to endogenous swelling- or Ca^{2+}-activated chloride channels. (**A**) Current traces recorded in the whole-cell configuration from a HeLa cell after activation of SWACs. Pipette, 164 mM TEACl medium; bath, 150 mM NaCl (see Materials and methods). Voltage protocol as in Figures 1 and 2. (**B**) A set of traces recorded from the same cell as (A), after addition of 100 μM IAA-94. (**C**) Whole-cell current traces recorded with 500 nM Ca^{2+} in the pipette medium. Holding potential: 0 mV. Voltage pulses of 600 ms duration were applied from –120 to +120 in 40-mV steps. (**D**) Intrinsic voltage dependences of Ca^{2+}- (○; $n = 6$), swelling- (□; $n = 7$) and VacA- (△; $n = 22$) induced currents. The data are plotted as the percentage of the whole-cell currents flowing at +50 mV. Error bars represent ± SEM.

have reported that ICaCl is potently blocked by 100 μM NPPB. Experiments aimed at testing the effect of IAA-94 on these channels were hampered by the reproducible appearance of high leak-like currents upon addition of the compound to patched cells in the presence of 500 nM intracellular Ca^{2+} ($n = 4$).

Effect of VacA on plasma membrane potential

Using the current-clamp method to measure the resting membrane potential of HeLa cells we obtained values in the –45 to –55 mV range, in agreement with the results obtained by Stein *et al.* (1996). The chloride concentration in HeLa cells has been reported to be in the 65–95 mM range (Ikehara *et al.*, 1991). Insertion of active chloride channels in the plasma membrane is expected to shift the value of the potential towards the equilibrium potential for Cl^-, i.e. to cause depolarization. To check whether exposure to VacA resulted in a change of this parameter we used the membrane potential-indicating probe bis-(3-propyl-5-oxoisoxazol-4-yl) pentamethine oxonol (bis-oxonol) (Apell and Bersch, 1987). As shown in Figure 4A, addition of pre-activated VacA reduced the cell plasma membrane potential with rapid kinetics, while non-activated VacA had no such effect. Depolarization exhibited a very short lag phase, and reached a plateau within 3–5 min. VacA-induced plasma membrane depolarization was also observed by using the voltage-sensitive probe [H^3]tetraphenylphosphonium and adherent HeLa cells (not shown). Uptake of DIS3-C(5), a voltage probe that accumulates almost exclusively inside mitochondria (Kimura *et al.*, 1999), was not modified by VacA on the same time scale (not shown). To substantiate a correlation between plasma membrane depolarization and formation of VacA channels, we investigated the inhibitory action of NPPB and IAA-94. Figure 4B shows that the presence of 100 μM NPPB significantly reduced the fluorescence change caused by VacA treatment. IAA-94 had a weaker effect, in agreement with its lower activity on VacA channels (see Figure 2).

Effect of NPPB and IAA-94 on cell vacuolation and epithelial ion permeability induced by VacA

The data presented above indicate that low doses of VacA, which do not lead to cell vacuolation, can rapidly alter the plasma membrane ion resistance, by forming anion-selective channels. In order to test whether cell vacuolation, obtained in the presence of weak bases, depends on the presence of VacA channels we exploited the ability of NPPB to inhibit them. HeLa cells were treated with 2.5 μg/ml of acid-activated VacA, in the presence of 5 mM NH_4Cl, and with or without 100 μM NPPB for 4 h. The extent of vacuolation was evaluated morphologically (Figure 5A–C) and quantitatively (Figure 5D) after NRU. NPPB strongly inhibited the development of typical Neutral red-labelled vacuoles induced by VacA. Since the electrogenic activity of the V-ATPase, necessary for vacuole formation (Papini *et al.*, 1996), is regulated by the intrinsic permeability of endosomal membranes to anions (Galloway *et al.*, 1983; van Dyke *et al.*, 1986), a property which may in principle be affected by Cl^- channel inhibitors, we checked the effect of NPPB and IAA-94 on basal cell NRU. The fraction of the cell volume occupied

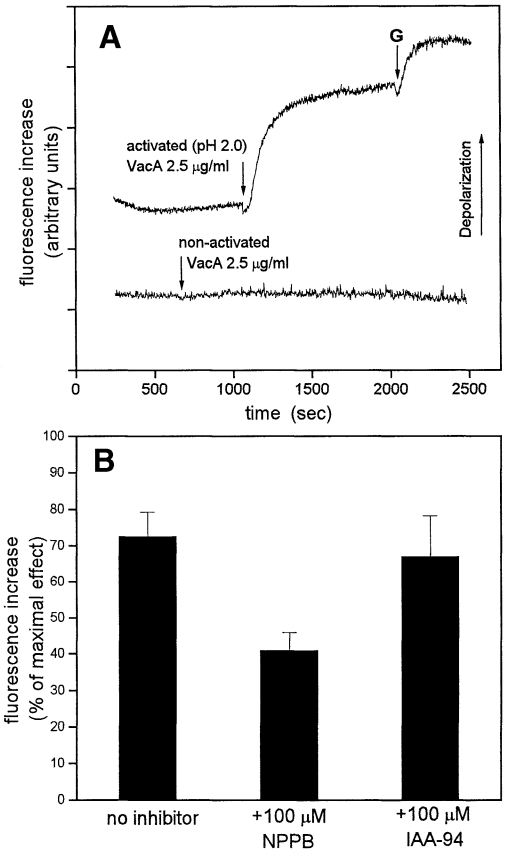

Fig. 4. Effect of VacA on the membrane potential of HeLa cells. (**A**) Traces from representative experiments. HeLa cells were detached from the plastic support by gentle trypsin–EDTA treatment and incubated with 100 nM bis-oxonol. After reaching a steady state, 2.5 μg/ml of acid-activated or non-activated VacA were added and the change in fluorescence was recorded. Gramicidin (4 μg/ml) was added when indicated for complete depolarization of the cells. (**B**) Effects of NPPB and IAA-94 on membrane depolarization. HeLa cells were treated as in (A) in the presence of the indicated inhibitors. Data are the mean of at least four experiments. Bars represent ± SE.

by these compartments is presumably constant in non-vacuolated cells and hence Neutral red indicates the endosomal and lysosomal pH values. Figure 6A shows that incubation of cells with 50 μM NPPB for 4 h does not affect endosomal and lysosomal acidification, while 50 μM IAA-94 or higher concentrations of NPPB (not shown) induce a decrease of NRU. As shown in Figure 6B, IAA-94 is a weaker inhibitor of VacA-dependent vacuolation than NPPB, in spite of its greater ability to increase endosomal and lysosomal pH in non-intoxicated cells. Figure 6B also shows that a given concentration of NPPB or IAA-94 inhibits vacuolation more effectively at lower toxin doses.

Since cell vacuolation depends on toxin binding to the cell surface and, possibly, on its subsequent endocytosis (Garner and Cover, 1996), we verified by indirect immunofluorescence analysis that endocytosis of VacA is not significantly affected by NPPB (Figure 6C and D). Cells fully vacuolated by treatment with VacA (0.5 μg/ml) in the presence of NH_4^+ for 18 h partially reversed the vacuolated phenotype upon addition of 50 μM NPPB to the incubation medium (30–40% decrease of NRU after 4 h; not shown).

The kinetics of vacuole development caused by cytosolic overexpression of VacA in HeLa cells (de Bernard *et al.*, 1998a) was also affected by NPPB. After 5 h of toxin expression, the increase of NRU was reduced to 34 ± 3% of control by 50 μM NPPB and almost abolished by 100 μM NPPB. After 7.5 h, the values were 82 ± 4% and 58 ± 7%, respectively ($n = 2$). The lower efficiency of the inhibitor at longer expression times can be explained by the higher amount of toxin accumulated intracellularly (de Bernard *et al.*, 1998a).

A recent study demonstrates that VacA permeabilizes epithelial cell monolayers to ions and neutral molecules with a molecular weight lower than ~340 Da, probably by modulating the resistance of the epithelial paracellular pathway (Papini *et al.*, 1998). Such an effect is independent of vacuolation and is not affected by V-ATPase inhibitors or by the presence in the medium of NH_4^+ (Papini *et al.*, 1998 and our unpublished observations). Co-incubation with 200 μM NPPB protected Madine Darby canine kidney (MDCK) cell epithelial monolayers from the VacA-induced increase of ion conductivity (Figure 7A). Addition of NPPB to the cell monolayer after VacA resulted in a significant reversion of the conductivity increase (Figure 7A). As in the case of HeLa cell vacuolation, epithelial conductivity increases were inhibited more efficiently by NPPB than by IAA-94 (Figure 7B).

Discussion

This study builds on our previous observation that VacA forms ion channels in artificial planar lipid bilayers (Tombola *et al.*, 1999) and analyses the toxin effect on cell plasma membranes, investigating its relationship with known cytotoxic actions. By patch–clamp, VacA was shown to form anion-selective channels in the plasma membrane of HeLa cells, which cause membrane depolarization. The alterations of HeLa cell plasma membrane and planar bilayer conductance induced by VacA were both prevented by anti-VacA antibodies and their biophysical properties were very similar. Furthermore, current conduction induced by VacA in both planar lipid bilayers and cell plasma membranes was efficiently inhibited by NPPB, while it was less sensitive to IAA-94. These inhibitors similarly affected cell membrane depolarization induced by VacA. A further pharmacological characterization of VacA activity is currently in progress (F.Tombola *et al.*, manuscript in preparation). VacA-induced activation of known endogenous Cl^- channels having comparable properties (swelling- and Ca^{2+}-activated chloride channels) can be excluded. Since toxin-neutralizing antibodies did not reduce current conduction by the pre-assembled VacA channels in the planar bilayer and the currents induced in cells, we cannot at present dismiss the remote possibility that an abundant, unknown endogenous channel, having biophysical and pharmacological properties very similar to VacA, is activated by the toxin.

The increase of plasma membrane ion permeability by VacA requires its pre-activation at acidic pH and is very efficient, taking place within minutes at concentrations and under conditions which do not give rise to vacuoles in HeLa cells even after several hours. Thus, provided that sufficiently low toxin doses are used and ammonium is omitted, channels can form in the cell plasma membrane without necessarily triggering cellular vacuolation.

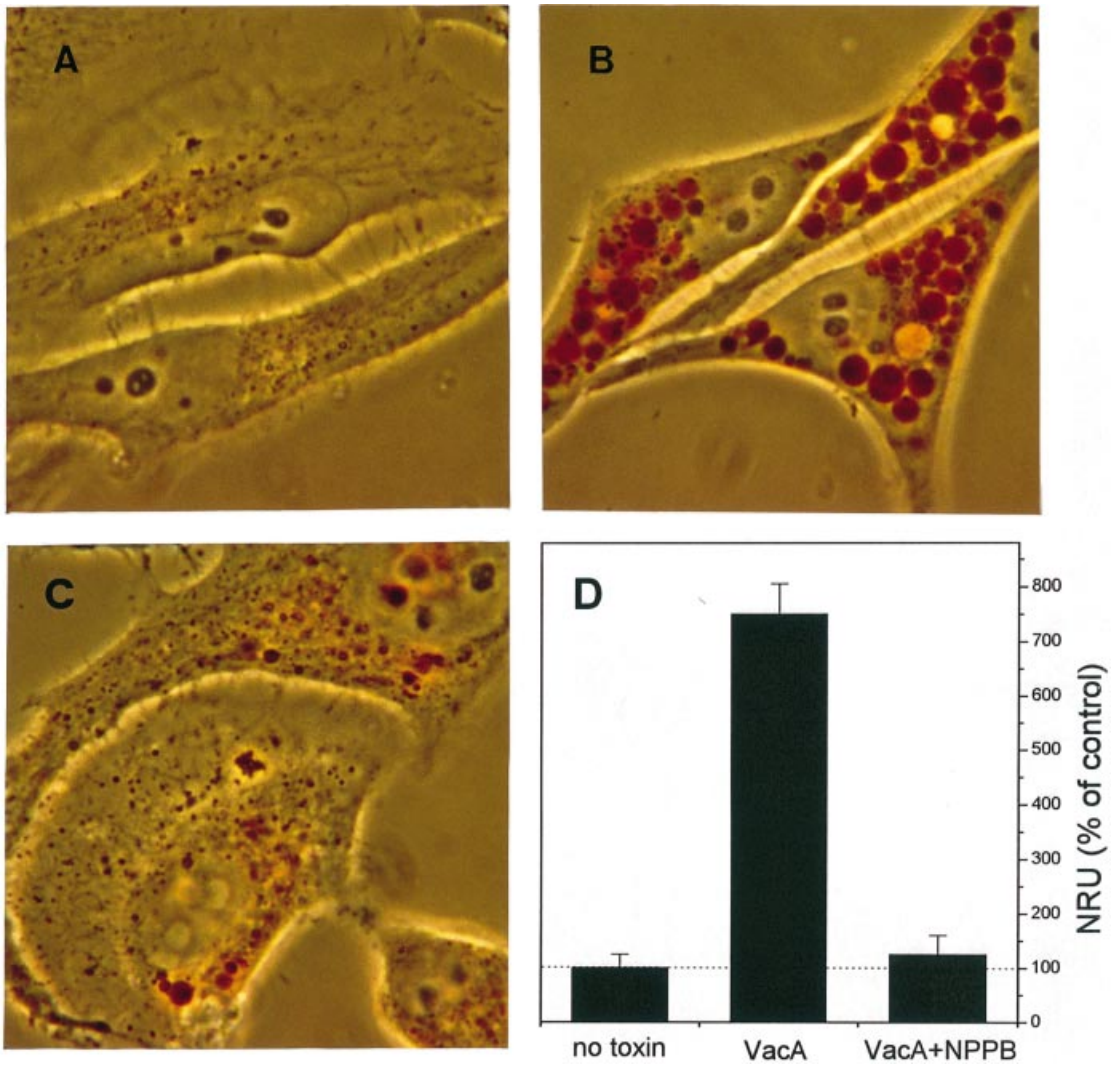

Fig. 5. Effect of NPPB on VacA-induced HeLa cell vacuolation. HeLa cells were grown for 2 days on glass coverslips and further incubated for 4 h in culture medium at 37°C, supplemented with 5 mM NH_4Cl, with no toxin (**A**), or in the presence of acid-activated VacA (5 μg/ml) (**B**) or with the acid-activated VacA and 100 μM NPPB (**C**) and photographed after staining of vacuoles with Neutral red. (**D**) Parallel cell samples, grown on plastic, were treated as above and NRU was measured as indicated in Materials and methods and expressed as a percentage of the basal value (no toxin). Data reported in the histogram are the mean of at least four experiments run in duplicate and bars are ± SE.

Nonetheless, vacuolation induced by higher toxin doses together with ammonium was inhibited by the same channel blockers, NPPB and IAA-94. Furthermore, NPPB was more effective than IAA-94 also in this case. NPPB, at the concentration used in the relevant experiments, did not affect endo/lysosomal pH in non-intoxicated cells. This suggests that inhibition of endosomal endogenous chloride channels is not responsible for the effects of these inhibitors, since IAA-94, which had a smaller effect on vacuolation, did affect endosomal pH.

The evidence summarized above suggests that formation of vacuoles occurs as a consequence of VacA channel activity and of the presence of weak bases, when the degree of the resulting change in ion permeability overcomes the homeostatic, compensatory cellular systems (Lang *et al.*, 1998). This is in keeping with the observation that blocking one such system, the Na^+/K^+ ATPase, dramatically increases cell sensitivity to VacA-induced vacuolation (Cover *et al.*, 1993b).

Vacuolation is not related to the observed decrease of TER in epithelial cell monolayers induced by the toxin, which probably results from a slight alteration of intercellular junctions (Papini *et al.*, 1998). Interestingly, however, NPPB efficiently prevented the increase of ion conductivity induced by VacA in MDCK cell monolayers. Hence the Cl^- channel blocker NPPB inhibits all known toxic actions of VacA. In the light of the present findings, recent data obtained with Caco-2 cell monolayers showing that VacA can induce a NPPB-sensitive change in the short-circuit current, attributed to an increase of anion apical secretion (Guarino *et al.*, 1998), can be explained by direct formation of VacA channels.

The VacA channel activity described here was shown to develop efficiently only after pre-exposure of the toxin to acidic pH, conditions that are necessary to activate both vacuolating activity in sparse cells and TER decrease in polarized epithelial monolayers (de Bernard *et al.*, 1995; Papini *et al.*, 1998). This common requirement for acid activation confirms the association between pore formation and toxic effects by VacA.

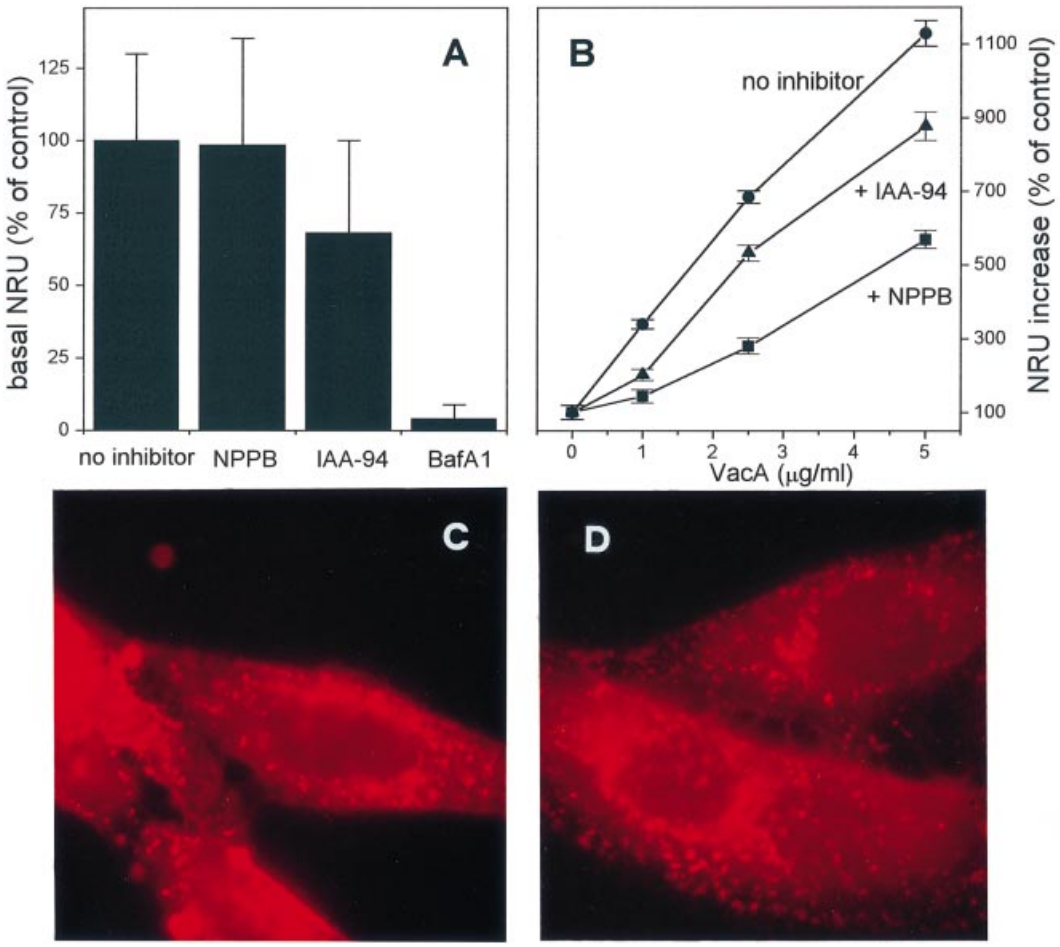

Fig. 6. Effects of NPPB and IAA-94 on basal NRU of HeLa cells and on VacA-induced vacuolation. Lack of an effect of NPPB on VacA internalization by cells. (**A**) HeLa cells were incubated for 4 h at 37°C with no inhibitor or with 50 μM NPPB, 50 μM IAA-94 and 100 nM bafilomycin A1, respectively. The uptake of Neutral red was then determined. Values are the mean of three experiments run in quadruplicate and bars represent ± SE. (**B**) HeLa cells with the indicated doses of VacA (pre-activated at pH 2.0) in the presence of 5 mM NH_4Cl for 4 h in the presence or not of NPPB and IAA-94 (50 μM), as indicated. Vacuolation was then quantified as the percentage increase of NRU with respect to control, non-VacA-treated cells. Values are the mean of three experiments run in triplicate and bars are ± SE. In the lower panels, HeLa cells were treated with acid-activated VacA (5 μg/ml) in the presence (**C**) or absence (**D**) of 100 μM NPPB for 4 h, fixed and permeabilized. The distribution of endocytosed VacA was determined by indirect immunofluorescence microscopy using a polyclonal antibody to VacA.

According to the hypothesis that VacA has an enzymatic activity, possibly associated with the N-terminus domain p37 (de Bernard *et al.*, 1998a), such a channel would be related to the translocation process. In fact, in the case of many cytosol-acting A-B type toxins the B domain engages the biological membrane in a low-pH-dependent manner, forming an ion channel (Menestrina *et al.*, 1994; Montecucco *et al.*, 1994). However, recent evidence suggests that the VacA channel is formed by toxin monomers assembled into hexameric rings, and that regions of both the putative A (p37) and B (p58) domains are required for the formation of these structures (Cover *et al.*, 1997; Lanzavecchia *et al.*, 1998; Czajkowsky *et al.*, 1999; Iwamoto *et al.*, 1999; Reyrat *et al.*, 1999). Indeed, p58 forms dimers rather than hexamers (Reyrat *et al.*, 1999) and does not form channels (Tombola *et al.*, 1999). Hence, we propose that VacA pores play a direct role in the typical vacuolation induced by the toxin. In fact, endocytosed VacA channels would be expected to stimulate the turnover of the endosomal V-ATPase by increasing the membrane permeability to anions (Cl^- and HCO_3^-), which in turn would lead to the accumulation of osmotically active species. This would account for the strong synergistic effect of extracellular weak bases on vacuole formation (Cover and Blaser, 1992; Papini *et al.*, 1996; Ricci *et al.*, 1997; Sommi *et al.*, 1998) and for the requirement of V-ATPase proton pumping activity (Cover *et al.*, 1993b; Papini *et al.*, 1993). VacA channels are sufficiently stable to reach late endosomes in an active state, and VacA persists undegraded for days in these compartments (Sommi *et al.*, 1998). The data presented here clearly show that VacA channels are required for vacuole formation. Consistently we have shown that NPPB also inhibits vacuolation induced by intracellular expression of VacA. This implies that VacA channels form and associate with endosomes also in this experimental model. Indeed, part of cytosolically produced VacA associates with V-ATPase-positive compartments and vacuoles (de Bernard *et al.*, 1998a). Furthermore, vacuolation by cytosolic VacA, like vacuolation by exogenous toxin, is inhibited by bafilo-mycin A1 and monensin, and depends on the presence of weak bases (de Bernard *et al.*, 1998a; Ye *et al.*, 1999). Cells expressing VacA-derived protein constructs vacuolate only if the construct comprises a

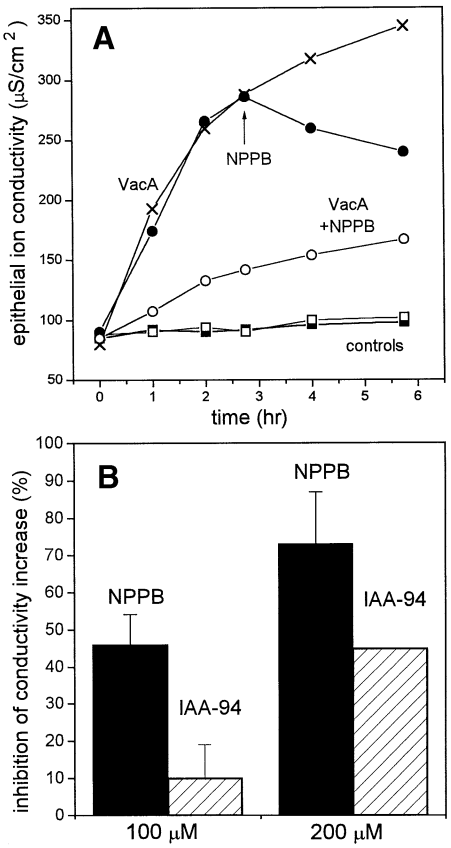

Fig. 7. Effect of NPPB and IAA-94 on epithelial ion conductivity. (**A**) MDCK cell monolayers were incubated in culture medium at 37°C with acid-activated VacA (2.5 μg/ml) and NPPB (200 μM) in the indicated combinations. Epithelial ion conductivity was determined at different times. In the experiment denoted by (●) NPPB was added at 3 h. (**B**) Cell monolayers were treated as in (A) with VacA and the indicated doses of NPPB or IAA-94. After 4 h, the inhibition of the VacA-induced conductivity increase was determined. Values are the mean of two independent experiments run in triplicate and bars are ± SE.

sizable portion of the p58 domain along with the whole p37 (de Bernard *et al.*, 1998a; Ye *et al.*, 1999). This also fits with channel involvement, since, as mentioned, structural determinants of pore assembly seem to be spread along the VacA molecule, in both p37 and p58.

Data obtained with epithelial monolayers also strengthen the notion that VacA channel formation is an essential aspect of its cellular action. In this case, however, hypotheses on the possible relationship between the channel-forming action and this intoxicated phenotype are less straightforward. Importantly, the partial reversion by NPPB of both intracellular vacuolation and epithelial ion conductivity increase suggests that the persistence of VacA channels is necessary to maintain these two altered states.

VacA resembles the CFTR not only in that it forms a low-conductance chloride channel (with, however, different properties), but also because it is localized in endosomal as well as in the plasma membranes (Bradbury, 1999 and references therein). The CFTR might therefore be expected to confer, upon activation by cyclic nucleotide-dependent phosphorylation, a phenotype somewhat similar to that induced by VacA. Some authors have reported that the intra-endosomal pH is slightly lower in CFTR-expressing cells in comparison with CFTR$^-$ controls (Barasch *et al.*, 1991; Biwersi and Verkman, 1994), while others found no such difference (Lukacs *et al.*, 1992; Dunn *et al.*, 1994; Biwersi *et al.*, 1996). In one study, CFTR activation was reported to increase modestly the permeability of the endosomal membrane to anions (Biwersi and Verkman, 1994). The whole-cell chloride currents induced by full activation of the CFTR are of the same order of magnitude as those observed by us using VacA concentrations not leading to vacuole formation (e.g. Anderson *et al.*, 1991; Vennekens *et al.*, 1999; Zhang *et al.*, 1999). This suggests that the contribution of the CFTR to the anion conductance of endosomal membranes might not be sufficient to trigger vacuolation. Interestingly, the activation of CFTR has been reported to stimulate endosome–endosome fusion slightly, by a mechanism proposed to depend on anion conduction (Biwersi *et al.*, 1996). It may be speculated, therefore, that VacA channels might display a similar but stronger effect that might play a role in vacuolation, in agreement with the observation that Rab7-dependent fusion events are involved in this process (Papini *et al.*, 1997).

The formation of anion-selective VacA pores in the apical plasma membrane of gastric epithelial cells might help *H.pylori* colonization of the stomach by allowing the efflux of potential metabolic substrates such as pyruvate and HCO_3^- (Mendz *et al.*, 1994; Burns *et al.*, 1995) from the host cell cytosol. This action would act in concert with the increase of paracellular diffusion of other low molecular weight molecules (Papini *et al.*, 1998) to provide a selective advantage to toxin-producing strains in the nutrient-poor environment of the gastric mucous layer. In addition, the predicted increased secretion of carboxylates and, more importantly, of HCO_3^- is expected to counteract, together with bacterial urease, the acidification of the mucous layer due to proton diffusion from the stomach lumen, or to alter acid secretion by oxyntic cells (Debellis *et al.*, 1998). A leak of the alkaline anion HCO_3^- through the apical membrane of gastric superficial epithelia would be a relevant physiological change mediated by VacA, since it has been shown that this membrane is non-permeable to this ion under physiological conditions (Caroppo *et al.*, 1997).

Finally, a particularly important aspect of the present study is that it opens the possibility of searching for NPPB analogues or other anion channel blockers potentially useful in the therapeutic treatment of diseases caused by *H.pylori*.

Materials and methods

Reagents

VacA was purified from the *H.pylori* strain CCUG 17874 as described (Manetti *et al.*, 1995), and stored at 4°C in PBS at concentrations of 0.1–0.2 mg/ml. The toxin was activated by pre-treatement at pH 2.0 for 5 min at 37°C. Rabbit pre-immune and anti-VacA sera were obtained as described elsewhere (Manetti *et al.*, 1995) and IgG fractions were purified by absorption on protein A–Sepharose (Sigma, Milan, Italy). PGEM plasmids with no insertion or containing the portion of the *vacA* gene encoding the mature toxin (1–913) (PGEM95) have been described elsewhere (de Bernard *et al.*, 1997). Recombinant vaccinia virus vT7 (Fuerst *et al.*, 1986) was a gift from Dr M.Zerial (EMBL). Bis-oxono was obtained from Molecular Probes Inc. (Eugene, OR). IAA-94 and NPPB were purchased from Research Biochemicals International (Natick, MA). 3,3′-Dipropylthiadicarbocyanine iodide [$DiSC_3$-(5)] was from Fluka (Milan, Italy).

Cells

MDCK I and HeLa cells were cultured at 37°C in plastic flasks in DMEM, supplemented with 10% (v/v) fetal calf serum (FCS) and gentamycin (50 μg/ml) in a 5% CO_2 humidified atmosphere. For patch–clamp experiments HeLa cells were detached by trypsin–EDTA treatment, re-seeded (20–30 000 cells/cm^2) and grown for 2 days on glass coverslips in six-well plates. In some experiments, cells were transiently transfected with PGEM or PGEM95 as described (de Bernard *et al.*, 1997). To form monolayers, MDCK I cells were seeded on Transwell porous filters (pore diameter 0.4 μm; Costar, Cambridge, MA), at a density of $0.6 \times 10^6/cm^2$. The medium of the upper and lower filter chambers was renewed every 24 h, and the cells were cultured until the formation of stable monolayers, as assessed by monitoring the trans-epithelial electrical resistance (TER) (10–12 000 $\Omega \times cm^2$).

Patch–clamp experiments

The whole-cell configuration was routinely used. Cells were treated at 37°C for 30 min with 0.5 μg/ml non-activated or acid-activated VacA diluted in DMEM, and subsequently washed with the bath solution (150 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES pH 7.3–7.4). When specified, activated VacA was brought to neutral pH by dilution in PBS and further incubated at 37°C for 30 min, before addition to cells, with 200 μg/ml (final concentration) of purified IgG from pre-immune or VacA-immunized rabbits. In some other cases, antibodies (200 μg/ml) were added to the extracellular medium after incubation with VacA and establishment of the whole-cell configuration. The pipette solution contained 134 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES pH 7.35 (10 nM free Ca^{2+}). Current–clamp measurements were performed with the same media. In some experiments 2 mM ATP was included in the pipette solution. For selectivity experiments the bath solution contained 300 mM TEACl instead of 150 mM NaCl, while in the pipette 150 mM TEACl substituted for 134 mM KCl. Swelling-activated chloride channel activity was induced by breaking into the cell, bathed with the 150 mM NaCl solution, with a hyperosmotic pipette solution containing 164 mM TEACl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 4 mM ATP (pH=7.3 with TEAOH). In the study of calcium-activated chloride channels the bath solution contained 225 mM TEACl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES, while the pipette solution was composed of 134 mM TEACl, 0.1 mM $MgCl_2$, 3.79 mM $CaCl_2$, 5 mM EGTA, 10 mM HEPES, 4 mM ATP pH 7.2 (free Ca^{2+} = 500 nM). Whole-cell currents were monitored with an EPC-7 amplifier (List) and pulse protocols were applied using the Pclamp6 program set (Axon). Capacitive currents were cancelled manually and series resistance was compensated when necessary. Anionic inward fluxes are shown as outward currents. Membrane potentials are reported as intracellular with respect to ground. In most experiments the membrane potential was clamped at –70 mV and 300 ms pulses were applied in 20 mV steps from –70 to +70 mV, with 40 or 10 s intervals between pulses.

Planar lipid bilayer experiments

Experiments were performed as described by Tombola *et al.* (1999) using diphytanoylphosphatidylcholine (DPhPC) as membrane lipid. The medium was 500 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES pH 7.2, in both chambers. VacA was pre-activated by exposure to pH 2.0. Inhibitors were added as stock solution in dimethylsulfoxide (DMSO) (final DMSO concentration 0.1% v/v) after the trans-bilayer current had stabilized (plateau), and the chamber contents were homogenized by repeatedly stirring with magnetic bars. In some cases, antibodies were preincubated with VacA or added to either the *cis* or the *trans* chamber in analogy to patch–clamp experiments.

Monitoring of the membrane potential

HeLa cells were detached by EDTA–trypsin treatment for 3 min at 37°C, washed with DMEM supplemented with 10% (v/v) FCS and gentamycin (50 μg/ml) and re-suspended in 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM D-glucose, 20 mM HEPES–Tris buffer pH 7.4. Cells were placed in a thermostatted cuvette (2×10^6 cells in 2 ml of medium) containing a magnetic stirring bar, in a Perkin Elmer Luminescence Spectrometer LS 5B at 37°C. Bis-oxonol from a 100 μM stock solution in DMSO was added at a final concentration of 100 nM. After stabilization of the fluorescence signal (excitation, 540 nm; emission, 580 nm) non-activated or acid-activated (pH 2.0) VacA was added at a final concentration of 0.5–5 μg/ml. After the fluorescence signal had reached a plateau gramicidin A (4 μg/ml) was added to cause complete depolarization. In some experiments the medium contained 100 μM NPPB or IAA-94. Mitochondrial membrane potential was monitored with the cationic dye DiS-C3-(5), added to cells at the final concentration of 100 nM. After stabilization of the fluorescence signal, acid-activated (pH 2.0) or non-acid-activated VacA (0.5–5 μg/ml) was added and the change of the fluorescence signal was recorded (excitation, 620 nm; emission, 660 nM). Complete dissipation of mitochondrial potential was achieved by addition of 1 μM carbonylcyanide *p*-fluoromethoxyphenylhydrazone (FCCP).

Neutral red uptake

Cells were incubated in culture medium plus 5 mM NH_4Cl for 3 h at 37°C, without or with VacA (1–10 μg/ml, pre-activated at pH 2.0) and without or with 50 or 100 μM NPPB or IAA-94. The cells transfected with PGEM or PGEM95 were incubated in culture medium supplemented with 5 mM NH_4Cl and 10 mM hydroxyurea at 37°C for 5 or 7.5 h in the absence or in the presence of NPPB (50 or 100 μM). Cells were subsequently incubated with 8 mM Neutral red in PBS plus 5 mM NH_4Cl and 0.3% BSA for 8 min at room temperature. Whenever NPPB or IAA-94 was present, the inhibitor was maintained during NRU. After three washes with PBS plus 0.3% BSA and 5 mM NH_4Cl, the dye was extracted out of the cells with 70% (v/v) ethanol–0.37% (v/v) hydrochloric acid and quantified by absorbance determination at 405 nm. Data were expressed as the percentage of NRU values of cells treated with no toxin (Figure 5) or transfected with PGEM plasmids. In some experiments, parallel samples of cells grown on glass coverslips were treated for NRU assay as above, mounted upside down on slides and photographed with an optical microscope (Zeiss-Axioplan).

Measurement of the epithelial cell monolayer ion permeability

Acid (pH 2.0)-activated VacA (2.5–10 μg/ml) with or without NPPB or IAA-94 (100 or 200 μM) was added to the upper filter chamber of MDCK I monolayers. For each filter the TER value ($\Omega \times cm^2$) was determined at different time points at 37°C with a Millipore apparatus (see Papini *et al.*, 1998). Epithelial ion conductivity ($\mu S/cm^2$) was obtained by calculating the reciprocal of TER.

Indirect immunofluorescence

HeLa cells on glass coverslips were incubated for 3 h in DMEM plus 10% FCS, with VacA (2.5 μg/ml), or with the toxin plus NPPB (100 μM). They were then washed with PBS and fixed by treatment for 20 min with 3% (w/v) paraformaldehyde in PBS at room temperature. After washes with PBS containing 0.38% glycine (w/v) and 0.27% NH_4Cl (w/v) and with PBS, cells were permeabilized with 0.2% (w/v) saponine in PBS plus 3% BSA for 30 min, and incubated for 1 h with rabbit polyclonal antibodies to VacA diluted in PBS plus 3% BSA. After extensive washing cells were incubated with Texas-red conjugated secondary antibodies, washed again and mounted on coverslips in 90% (v/v) glycerol containing 3% (w/v) propylgallate and viewed with a fluorescence microscope (Zeiss-Axioplan).

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