Inhibition of the vacuolating and anion channel activities of the VacA toxin of *Helicobacter pylori*

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Abstract VacA, the vacuolating cytotoxin secreted by *Helicobacter pylori*, is believed to be a major causative factor in the development of gastroduodenal ulcers. This toxin causes vacuolation of cultured cells and it has recently been found to form anion-selective channels upon insertion into planar bilayers as well as in the plasmamembrane of HeLa cells. Here, we identify a series of inhibitors of VacA channels and we compare their effectiveness as channel blockers and as inhibitors of VacA-induced vacuolation, confirming that the two phenomena are linked. This characterization opens the way to studies in other experimental systems and to the search for a specific inhibitor of VacA action.

Key words: Gastritis; Ulcer; VacA; Ion channel inhibition; *Helicobacter pylori*

1. Introduction

Infection by *Helicobacter pylori* is at the origin of most cases of gastritis and gastric ulcers in humans [1,2]. Among the virulence factors produced by the pathogenic strains of the bacterium, CagA and VacA appear to be the most important [3]. The latter is a secreted and partially bacterium surface-bound [4] protein with a mass of about 95 kDa in its mature form, comprising N-terminal (p37) and C-terminal (p58) domains [5,6]. The secreted monomers assemble to form hexamers and heptamers, which at neutral pH may further associate to form dodecamers and tetradecamers [7–9]. Exposure of the isolated toxin to acidic conditions (pH < 5) leads to structural alterations, with dissociation of the complex into monomers [8–11]. Low pH exposure strongly increases the insertion of VacA into membranes [11] as well as its ability to induce the formation of vacuoles in cells incubated in media containing membrane-permeant amines [10]. The vacuoles originate from late endosomal/lysosomal compartments [12–14].

The mechanism of VacA-induced vacuolization has not yet been established, but the process presumably involves internalized toxin, since vacuoles develop following expression of the toxin in the cell cytosol [15,16]. VacA is known to be slowly endocytosed after binding to the plasmamembrane and to be localized intracellularly in the endosome-derived acidic compartments [17]. Recently, VacA has been reported to form anion-selective, low-conductance, voltage-dependent channels in planar membranes [18,19] and in the plasmamembrane of HeLa cells exposed to the toxin [20]. The pore-forming activity of the toxin is greatly enhanced in acidic media or if the toxin is pre-activated by exposure to low pH. Channel formation is probably due to hexameric complexes, which reform from the monomers prevailing at acidic pH [9,19].

It is thus important to identify and characterize inhibitors of the channel-forming and vacuolating activities of the toxin, aiming at (i) a better understanding of the properties of this novel channel, (ii) providing further evidence of the correlation between channel activity and vacuolization, (iii) identifying molecules which may be of potential value as toxin-specific inhibitors.

2. Materials and methods

2.1. Toxin and reagents

VacA was purified from *H. pylori* CCUG 17874 as described [21]. All planar bilayer experiments reported were performed using synthetic diphytanoyl-phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA; purity $> 99\%$) as the membrane lipid. *R*(+)-2-((2-cyclopentenyl-6,7-dichloro-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)oxy)-acetic acid (IAA-94) and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) were purchased from Research Biochemicals International (Natick, MA, USA). *N*-phenylanthranilic acid (NPA), 2-(3-(trifluoromethyl)anilino)benzoic acid (flufenamic acid), 2-(3-(trifluoromethyl)anilino)nicotinic acid (niflumic acid), acetylsalicylic acid, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (disodium salt) (DIDS), 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (disodium salt) (SITS) and *N*-*p*-(2-(5-chloro-2-methoxybenzamido)ethyl)benzene-sulfonyl-*N*′-cyclohexylurea (glibenclamide) were from Sigma (Milan, Italy). All the compounds listed, with the exception of acetylsalicylic acid, are known inhibitors of chloride channels. They were used as 25, 50 or 100 mM stock solutions in DMSO, except for DIDS and SITS, which were dissolved in water, and acetylsalicylic acid in ethanol.

2.2. Planar lipid bilayer experiments

Experiments were carried out as already described [18]. The *cis* compartment is defined as the one into which the toxin was added, which was also the one containing the active electrode, whose voltage relative to ground (in *trans*) is reported. Current (cations) flowing from the *cis* to the *trans* side was considered positive and plotted upward. The standard medium used was 0.5 M KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES/K^+, pH 7.2, in both chambers. In all cases, VacA was pre-activated by incubation in phosphate-buffered

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saline (PBS)-HCl, pH 2, at 37°C for 8 min, immediately before use. We have observed that the process of pore formation is accelerated at alkaline medium pH values. In part of the experiments therefore, after VacA addition, the pH of the bilayer chambers was shifted to 7.5 with KOH and re-adjusted to 7.2 with HCl after a sizable current had developed. Inhibitors were added to the same compartment into which the toxin was introduced, as a few μl of stock solution, after the current had reached a nearly stable level. In some cases, the measured current levels were corrected for a residual incorporation of VacA channels during the experiment as described in [18].

2.3. Patch-clamp experiments

HeLa cells were cultured at 37°C in plastic flasks in DMEM, supplemented with 10% (v/v) FCS and gentamicin (50 μg/ml) in a 5% CO_2 humidified atmosphere. They 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. Immediately before the experiment, the cells were treated at 37°C for 30 min with 0.5 μg/ml 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). The pipette 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+}). 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 partially cancelled manually and series resistance was compensated when necessary. Anionic inward fluxes are plotted as outward currents. Membrane potentials are reported as intracellular with respect to ground, i.e. as pipette potentials, equivalent to the potential in the *trans* compartment of bilayer experiments. In most experiments, the membrane potential was clamped at −80 mV and 300 ms pulses were applied in 20 mV steps from −80 to +80 mV, with 40 or 10 s intervals between pulses. Addition of the inhibitors was performed manually with a micropipette, followed by thorough mixing of the chamber contents (0.5 ml) by withdrawal and re-addition of 300 μl aliquots.

2.4. Quantification of vacuolation

Vacuole formation in cultured HeLa cells was determined by the neutral red uptake assay [22], as described [18]. To induce vacuolization, after overnight culture, the cells were incubated for 3 h at 37°C with 5 μg/ml acid-activated VacA plus 5 mM NH_4Cl, with or without inhibitors. The cells were then washed with PBS plus 0.3% (w/v) bovine serum albumin (BSA) and further incubated for 8 min at room temperature with 8 mM neutral red in PBS plus 5 mM NH_4Cl and 0.3% BSA. After three washes with the same medium, the dye was extracted with 70% (v/v) ethanol/0.37% (v/v) hydrochloric acid and quantified by determining the absorbance at 405 nm.

3. Results

3.1. Chloride channel inhibitors reduce current conduction and cell vacuolization by VacA with the same order of potency

Since VacA pores are anion-selective, we tested known inhibitors of chloride channels. They included four arylaminobenzoates related to diphenylamine-2-carboxylate, the indanyl alkanoic acid IAA-94 and the two stilbene sulfonate derivatives SITS and DIDS. Their effect on VacA-mediated current conduction was determined in planar bilayer experiments.

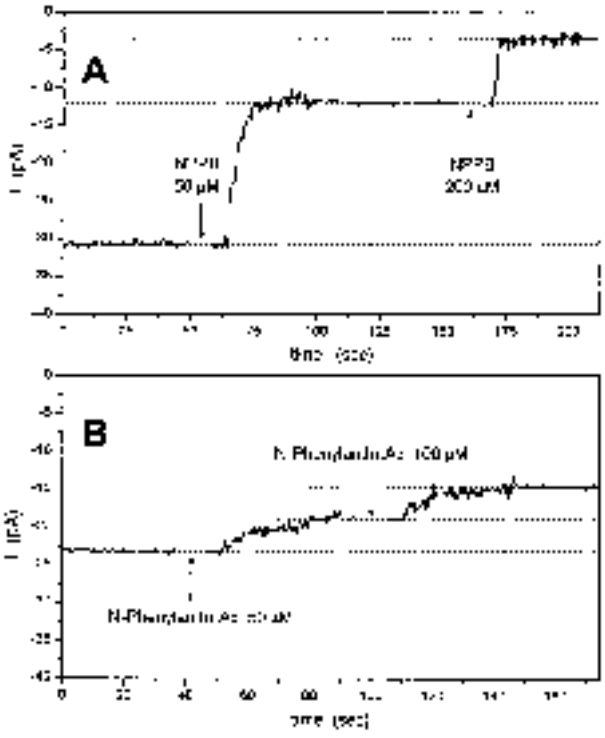

Fig. 1. Current records exemplifying measurements of inhibition of current conduction by VacA in planar bilayer experiments. NPPB (A) or NPA (B) were added at the indicated final concentration to the *cis* chamber. Inhibition took place upon stirring of the chamber contents. The disturbances caused by stirring have been partly eliminated for clarity. The origin of the time scale has been arbitrarily positioned at a moment following the attainment of a stable current level. Filtering: 10 Hz. Digital sampling: 50 Hz.

Fig. 1 illustrates data collection and the effects of NPPB and *N*-phenyl anthranilic acid. The current decreased rapidly upon stirring the contents of the chamber into which the inhibitor was added (*cis* side). The data obtained are summarized in Fig. 2, which presents titrations of current conduction in bilayer experiments. The data are fitted by curves described by the relationship $\%_{r.c.} = 100 - P_1/(1+K_D/[L])$, a form of the Langmuir isotherm, in which $\%_{r.c.}$ stands for the % residual current, [L] is the concentration of inhibitor and P_1 is a fitting parameter, whose physical meaning is the maximal percentage of inhibition theoretically observable at an infinite concentration of inhibitor. The values of the parameters K_D and P_1 are listed in Table 1. It is noteworthy that for three of the arylaminobenzoates and IAA-94, P_1 is significantly lower than 100, leading to the prediction that current conduction would not be completely inhibited even at infinite concentration of

Table 1
Tabulation of K_D, IC_{50} and P_1 values

Inhibitor	$K_D \pm$ S.D. (μM)	IC_{50} (μM)[a]	$P_1 \pm$ S.D.
NPPB	18 ± 2	24	88 ± 2
DIDS	36 ± 2	40	95 ± 2
Flufenamic acid	39 ± 5	57	84 ± 3
Niflumic acid	74 ± 16	99	87 ± 6
NPA	139 ± 27	134	102 ± 8
SITS	149 ± 19	156	98 ± 5
R(+)-IAA-94	190 ± 21	469	70 ± 4

[a]Calculated from the fitting equation.

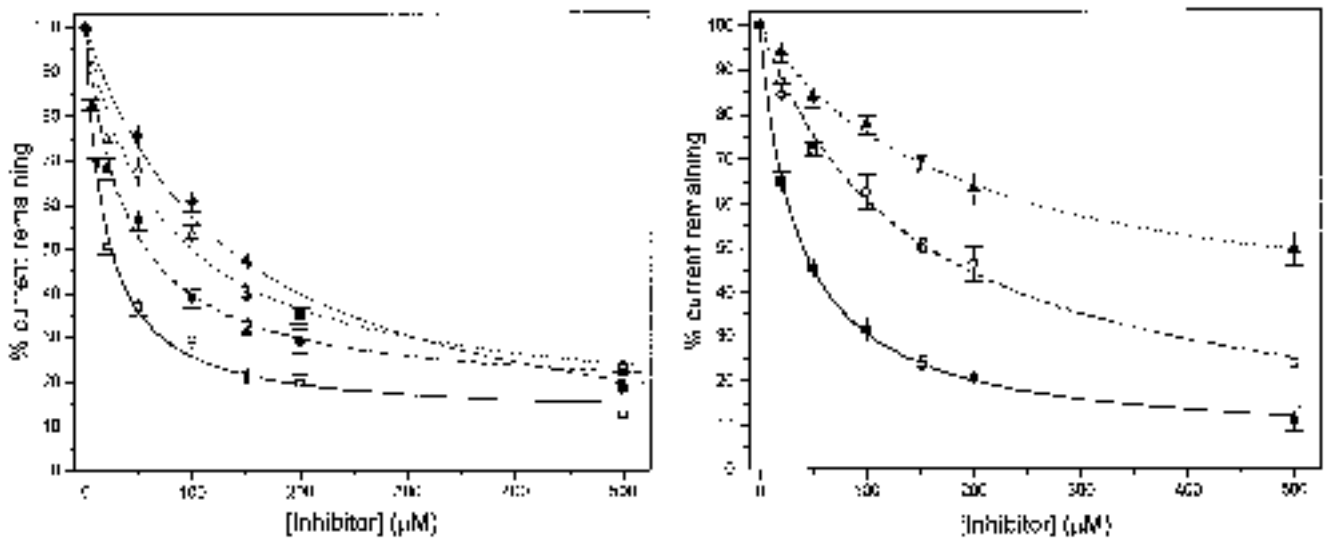

Fig. 2. Titration curves plotting the residual current vs. the concentration of the indicated inhibitors in the *cis* compartment. Each experimental point represents the average of 3–11 independent determinations, ± S.E.M. The voltage applied was −40 mV. Curves: 1 (□) NPPB; 2 (●) flufenamic acid; 3 (△) niflumic acid; 4 (♦) *N*-phenylanthranilic acid; 5 (■) DIDS; 6 (○) SITS; 7 (▲) IAA-94; (◇) glibenclamide.

the inhibitor. From the K_D values, the following ranking of inhibitor potency (at low concentrations) can be derived: NPPB > DIDS ≅ flufenamic acid > niflumic acid > NPA ≅ SITS > IAA-94.

This sequence can be used to distinguish VacA from other anion channels. For example, Ca^{2+}-activated chloride channels are inhibited with the order: niflumic acid > NPPB > NPA ≅ DIDS [23]. In the case of glibenclamide, only low concentrations were used because of the solubility characteristics of this compound and a K_D value could not be determined. Current conduction was reduced to 84.6 ± 0.3% (± S.E.M., $n = 4$) and 71.8 ± 1.9% ($n = 5$) of control values with 20 and 50 μM, respectively. Glibenclamide thus appears to be about as effective as NPA and SITS. Acetylsalicylic acid (aspirin), which has some structural resemblance to the fenamates used in this work, had no effect on either current conduction or vacuolation (not shown).

Fig. 3 presents a comparison of the inhibition of VacA-mediated current conduction exerted by five of the compounds in patch-clamp experiments on toxin-exposed HeLa cells and in planar lipid bilayer determinations.

It has previously been suggested that the VacA anion-selective channel plays a major role in vacuole formation and that the major sites of this action are late endosomal and lysosomal compartments [18]. If this is the case and if the various inhibitors have comparable membrane permeability properties, the VacA channel inhibitors identified here should (a) inhibit VacA-induced vacuolization measured quantitatively as neutral red uptake and (b) follow the same order of potency determined for their inhibition of current flow through the channel. Fig. 4 shows that this is indeed the case. With glibenclamide, neutral red uptake was reduced to 85.6 ± 3.2% and 73.8 ± 3.5% ($n = 5$) of control with 20 and 50 μM inhibitor, in agreement with the bilayer data (see above). DIDS and SITS were not tested as inhibitors of vacuolation, because

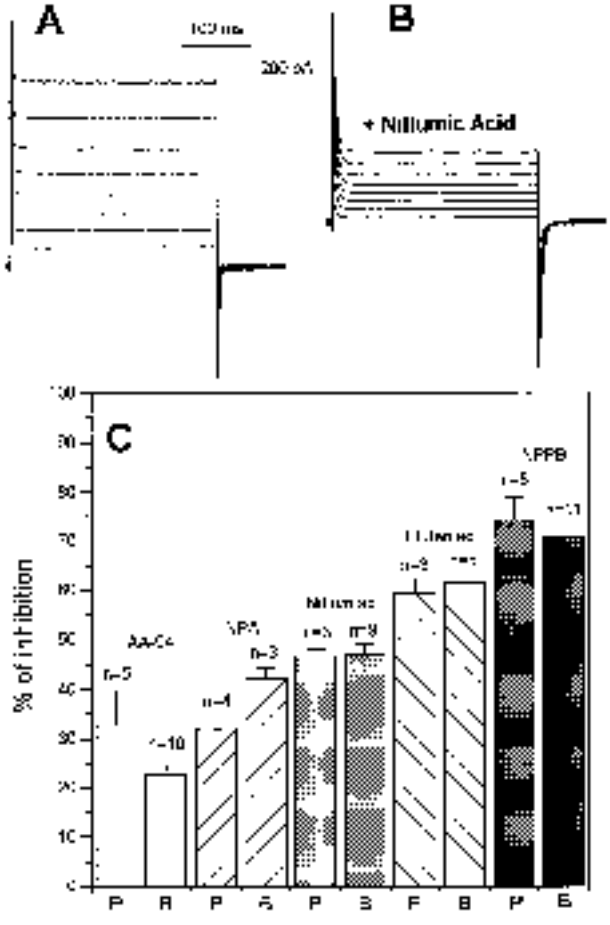

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Fig. 3. Inhibition of VacA-induced whole-cell currents and comparison with bilayer data. A and B: Exemplary current traces recorded from the same cell before (A) and after (B) the addition of 100 μM niflumic acid to the bath. C: A comparison of the inhibition by the specified compounds (100 μM) in patch-clamp (P) and bilayer (B) experiments. Applied voltage: −40 mV (bilayer, *cis* compartment), +40 mV (patch-clamp, pipette; +50 for NPPB and IAA-94). Averages ± S.E.M. The number of independent experiments averaged is indicated in the figure.

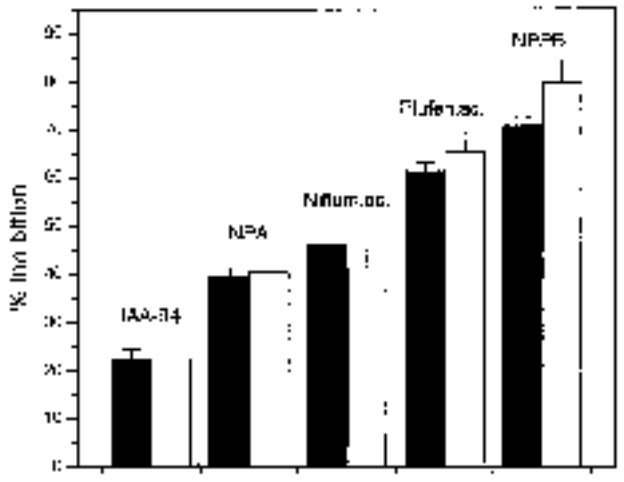

Fig. 4. Comparison of the inhibition of transbilayer current conduction (darker columns) and of NR uptake by VacA-exposed HeLa cells (lighter columns) by the indicated inhibitors, added (in the *cis* compartment only in bilayer experiments) at a 100 μM final concentration. In vacuolation experiments, 5 μg/ml of acid-activated toxin was used. Error bars: ± S.E.M. ($n = 5$ for vacuolation experiments).

these compounds are well known to covalently and unspecifically modify proteins over the long incubation times needed to assess effects on vacuolation.

4. Discussion

The results reported here show that VacA channels are inhibited by a number of known chloride channel inhibitors. The agreement between the data obtained with patch-clamp and those derived from experiments with planar lipid bilayers (Fig. 3) provides additional evidence that the increased permeability of the plasma membrane of HeLa cells exposed to the activated toxin [20] is due to the insertion of VacA channels and not due to possible activating effects on endogenous chloride channels.

In the absence of detailed structural information on the VacA channel, it is difficult to rationalize the scale of potency of the inhibitors determined here in terms of the structures of the compounds. Most of the inhibitors we used are generally considered to be chloride channel blockers (e.g. [24–27]). Since most of the compounds tested here inhibit a variety of anion-selective channels besides VacA, which has no identifiable homology with known channels, it may be assumed that the block results from charge/dipole and hydrophobic interactions in the lumen of the channel, without the involvement of a binding site with a conserved structure. A different mechanism may apply in the case of glibenclamide, an uncharged (but dipole-rich) molecule mainly known as an inhibitor of K_{ATP} channels.

A major conclusion that can be drawn at the present stage is that a strict correlation exists between VacA channel activity and the major recognized effect of VacA at the cellular level, namely vacuole induction. All the six inhibitors of the channel tested also inhibited vacuolation. It is noteworthy in this context that acetylsalicylic acid was completely ineffective on either current conduction or vacuolation. This lack of activity, which might be related to its smaller size and/or lack of an apolar moiety, indicates that the inhibition of vacuolation by arylaminobenzoates, which are non-steroidal anti-inflammatory drugs and cyclooxygenase inhibitors (e.g. [28]), is not a consequence of the inhibition of cyclooxygenase, which is inhibited also by acetylsalicylate.

The fact that the compounds used exhibited the same effectiveness ranking as inhibitors of current conduction and as inhibitors of vacuolization is highly significant. We have already shown that another inhibitor of vacuolization, the histidine reagent DEPC, also inhibits current conduction and that the p58 domain of VacA is incapable of either forming channels or inducing vacuoles [18]. Furthermore, both pore formation and vacuolation are strongly increased upon exposure of the toxin to low pH. Taken together, these data lead to the conclusion that VacA channel activity and vacuole induction are inseparable. The facts that VacA ends up in the endosomal-vacuolar membrane [17] and that the pH of these compartments is acidic suggest that vacuolation may be a consequence of channel formation in the endosomal membrane. This hypothesis is consistent with the ability of VacA synthesized in the cytoplasm to cause vacuoles [15,16] if it is assumed that the toxin can enter this membrane directly from the cytoplasm and not only by endocytosis. This seems possible, given the demonstrated ability of VacA to form channels in artificial membranes, irrespective of their composition [18]. This suggestion is also in keeping with the evidence that the activating effect of low pH is due to the dissociation of oligomers into monomers [8,11], which would be the species capable of membrane insertion (alternatively, the hexamer might insert) [9]. Expression of VacA in the cytoplasm presumably results in the production of monomers, which might insert into the endosomal (and possibly other) membranes in a process competing with oligomerization to yield the 'unproductive' dodecamer. Once there, the toxin is expected to increase the membrane permeability to chloride and bicarbonate, causing depolarization, increased activity by the V-ATPase, accumulation of ammonium ions inside the vacuoles, increase of the osmotic pressure in these compartments, water influx and swelling [6,18,19]. If an adequate supply of membrane is assured by Rab7-dependent [14] fusion, the final outcome of the process would be the formation of large vacuoles.

In conclusion, the quantitative characterization of VacA inhibition by a spectrum of compounds presented above opens the way to the identification and the study of this channel and of the effects of its activity in systems more physiologically relevant than planar bilayers, such as gastric epithelia or animal models. This pharmacological characterization may serve as a starting point for a search for specific inhibitors, potentially useful in cases of antibiotic-resistant infection. Furthermore, the establishment of a correlation between channel formation and cytotoxicity by VacA offers a method to screen VacA-derived constructs in vitro to select the ones most promising for the formulation of an anti-*H. pylori* vaccine.

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