VacA from *Helicobacter pylori*: a hexameric chloride channel

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Abstract VacA is a unique protein toxin secreted by the human pathogen *Helicobacter pylori*. At a neutral pH, the cytotoxin self-associates into predominantly dodecameric complexes. In this report, we show that at an acidic pH, VacA forms anion selective channels in planar phospholipid bilayers. Similar to several other chloride channels, the VacA channel exhibits a moderate selectivity for anions over cations ($P_{Cl}:P_{Na} = 4.2:1$), inhibition by the blocker 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid and a permeability sequence, $SCN^- \gg I^- > Br^- > Cl^- > F$, consistent with a 'weak field strength' binding site for the permeant anion. Single channel recordings reveal rapid transitions (486 s^{-1}) between the closed state and a single open state of 24 pS (+60 mV, 1.5 M NaCl). Evaluation of the rate of increase in macroscopic current as well as atomic force microscopy suggest that this VacA channel is a hexamer, formed by the assembly of membrane-bound monomers. Not only are these VacA channels likely to play an important role in the pathological activity of this toxin, but they may also serve as a model system to further investigate the mechanism of anion selectivity in general.

Key words: Ulcer; Vacuolating toxin; Anion selective; Atomic force microscopy

1. Introduction

The vacuolating toxin, VacA, is one of the primary virulence factors secreted by *Helicobacter pylori*, the bacteria whose prolonged colonization of the human gastric mucosa is a significant risk factor for the development of peptic ulcers and gastric carcinoma [1,2]. Although VacA has been observed to cause several alterations in the cellular structure and function [3,4], the best studied of these is a massive osmotic swelling, in the presence of weak bases, of intracellular acidic compartments that contain markers for late endosomes and lysosomes [4,5]. The mechanism by which VacA causes this extensive vacuolation is not understood, but the toxin is known to first bind to the plasma membrane [6] and there is some evidence to suggest an intracellular site of action [7]. In addition, it has recently been shown that VacA, a dodecameric complex at neutral pH, can cause a significant membrane conductance at a low pH [8].

In this report, we show that the low pH-induced membrane conductance is caused by well-defined hexameric anion selective VacA channels across the bilayer. The VacA channel is blocked by the typical chloride channel blocker, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and its permeability sequence for anions resembles that of many eukaryotic Cl^- channels, suggesting that this hexameric channel might be a useful model for elucidating the molecular mechanisms of anion selectivity of Cl^- channels in general. Moreover, we believe that the formation of these anion selective channels is likely to be an essential intermediate in the process of intoxication effected by VacA.

2. Materials and methods

2.1. Materials

VacA was purified from the broth supernatant of *H. pylori* 60190 as previously described [9]. Egg phosphatidylcholine (eggPC), dioleoylphosphatidylserine (DOPS) and the total lipid extract from bovine heart (HL) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol and all other reagents were purchased from Sigma Chemicals (St. Louis, MO, USA).

2.2. Electrophysiology

Planar lipid bilayers were formed by drawing a lipid solution over a small aperture (0.18 or 0.5 mm in diameter) separating two aqueous compartments in a teflon chamber. The lipid solution was 12 mg/ml eggPC/DOPS/cholesterol (55/15/30 mol%) in *n*-decane and the buffer compositions used in each experiment are described in the figure captions. All experiments were performed at room temperature. The chamber to which VacA was added is referred to as the *cis*-side. Membrane currents were recorded using a custom designed current amplifier optimized for high frequency response and the Ag/AgCl electrodes were connected to the chambers with a 1 M KCl agar bridge. The output was amplified and filtered between 2 kHz and 6 kHz (depending on the magnitude of channel conductance). Single channel analysis was carried out using the program Transit (Baylor College of Medicine). The ion selectivity was determined by measuring the reversal potential (membrane voltage for zero current) in asymmetric ionic solutions and the permeability ratios were calculated using the Goldman-Hodgkin-Katz equation [10]. The macroscopic conductance traces were fitted to Eq. 1 (see below) using standard least squares techniques with the program, SigmaPlot (SPSS).

2.3. Atomic force microscopy (AFM)

A supported lipid membrane, composed of HL in the leaflet facing the solution and eggPC in the leaflet facing the mica substrate, was prepared as previously described [8,11]. The final protein concentration during the 1–2 h incubation period with the bilayer was ~0.3 μM. The sample was extensively washed with 5 mM citric acid, pH 5.0 after incubation, and maintained in this buffer during transport to the AFM. Imaging was performed in 10 mM sodium phosphate, pH 7.0, with a Nanoscope II AFM (Digital Instruments, Santa Barbara,

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Abbreviations: VacA, vacuolating toxin; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; eggPC, egg phosphatidylcholine; DOPS, dioleoylphosphatidylserine; HL, total lipid extract from bovine heart; AFM, atomic force microscopy

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CA, USA), in the contact mode, using oxide-sharpened 'twin tip' Si_3N_4 cantilevers with a spring constant of 0.06 N/m. The scan rate was ~9 Hz and the force applied during scanning was minimized to ~0.1 nN. The AFM piezoscanner was calibrated using mica and the pentameric cholera toxin B subunit.

3. Results and discussion

To investigate the properties of the VacA channel, we have used a standard planar lipid bilayer setup, where the membrane is formed on an aperture in a teflon partition separating two aqueous compartments [12]. In agreement with our previous observations [8], addition of VacA to the *cis*-chamber produced a large increase in the membrane conductance after a delay of several minutes. This macroscopic conductance was observed only at a low pH and with bilayers containing anionic lipids. At a higher salt concentration (1.5 M NaBr, 2 mM EDTA, 5 mM citric acid, pH 4.0), the initial increase of the membrane current could be resolved as a series of distinct steps, each an integral multiple of 7.7 pA (at −50 mV), indicative of conductance through individually well-defined VacA channels (Fig. 1). Indeed, at lower protein concentrations (3 nM monomers) in the same high salt/low pH buffer, single channels were routinely detected and reproducibly observed (Fig. 1, inset).

These single VacA channels are quite small. As shown in the current-voltage relationship (Fig. 2A), the single channel conductance in 1.5 M NaCl at a *cis*-positive potential of 60 mV was only 24 pS. Since most channels show a roughly linear relationship between the conductance and salt concentration, the single channel conductance at physiologically relevant NaCl concentrations would be expected to be only 2–3 pS, which is too small for direct single channel measurements. These VacA channels are also among the fastest yet identified. Analysis of the single channel events indicate a mean open time of 1.8 ± 0.4 ms ($n = 14$) and a mean closed time of only 0.4 ± 0.1 ms ($n = 14$).

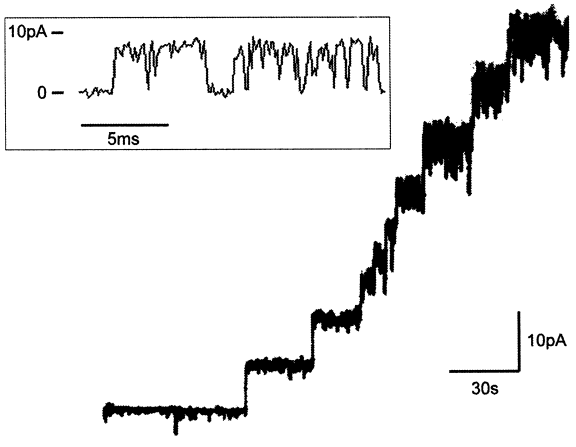

Fig. 1. Conductance of VacA channels in planar lipid bilayers. After the bilayer, composed of eggPC/DOPC/cholesterol (55/15/30 mol%), was formed across the aperture separating two compartments containing 1.5 M NaBr, 2 mM EDTA, 5 mM citric acid, pH 4.0, the addition of VacA (to a final monomer concentration of 15 nM) in the *cis*-chamber produced a series of distinct steps, each of roughly 7.7 pA (at −50 mV), that evolved into a macroscopic current described already [8]. At lower protein concentrations (3 nM) and using electronics providing a better time resolution, individual channels could be observed routinely (inset).

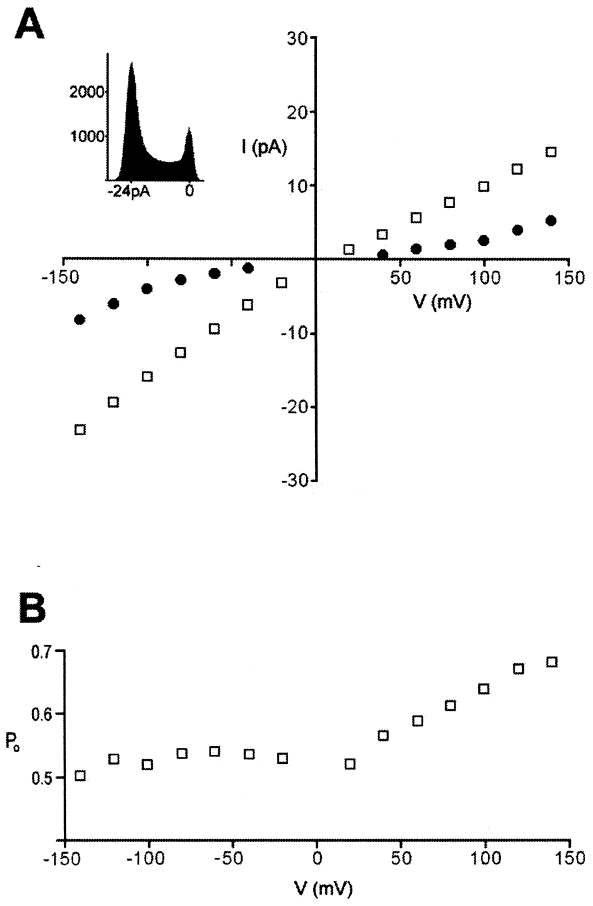

Fig. 2. Influence of the voltage polarity and salt composition on VacA channel properties. A: Current-voltage relationship of single VacA channels in 2 mM EDTA, 5 mM citric acid, pH 4.0 and either 1.5 M NaBr (open squares) or 1.5 M NaCl (filled circles). The current observed at negative potentials was found to be ~1.6 times greater than that observed at positive potentials and the current measured in NaBr was greater (from 3 to 5 times) than that measured in NaCl. The histogram in the inset was determined from single channel recordings and was used to calculate the current magnitude and the open probability. B: The single channel open probability is slightly greater at positive potentials than at negative potentials in 1.5 M NaBr, 2 mM EDTA, 5 mM citric acid, pH 4.0. The asymmetric dependency of the applied voltage polarity on the channel conductance and open probability suggests that the channel is asymmetrically oriented across the bilayer.

The current-voltage curve (Fig. 2A) also shows that the magnitude of single channel conductance was dependent both on the polarity of the applied membrane potential (~1.6 times greater for *cis*-negative potentials) and the type of salt present (3–5 times greater in 1.5 M NaBr than in 1.5 M NaCl). The asymmetric dependence on the membrane potential polarity implies that the channel is oriented asymmetrically across the bilayer, in agreement with the observed slight polarity-dependence on the open probability (Fig. 2B).

The ion selectivity of this channel was assessed by determining the reversal potential for macroscopic currents in asymmetric salt solutions (ranging in concentration from 100–150 mM), from which the permeability ratios were calculated. To our surprise, the VacA channel was found to be anion selective, with a Cl:Na permeability ratio of 4.2:1 (Table 1). These channels, once formed, could not be dissociated from the bilayer and, furthermore, remained anion selective from pH

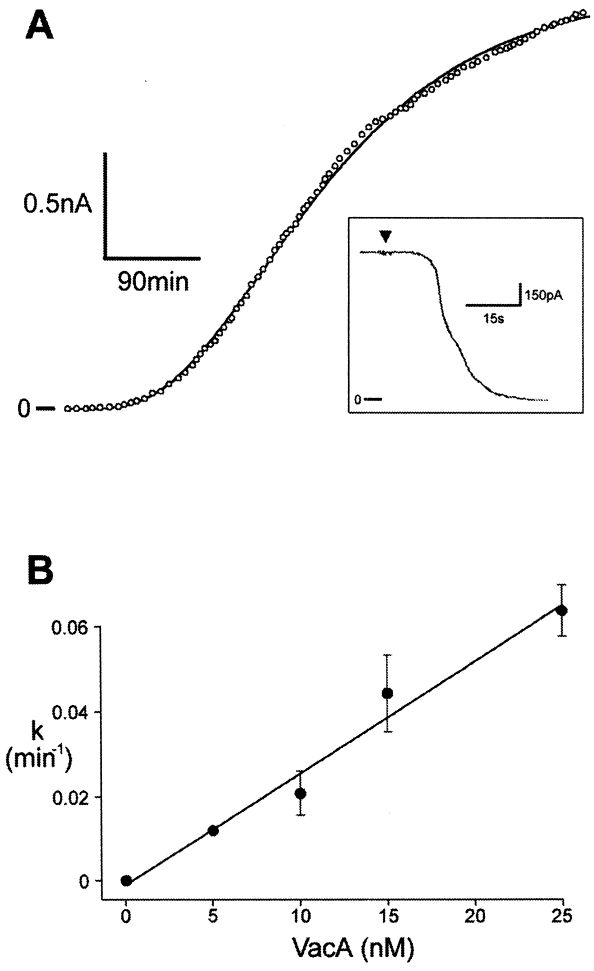

Fig. 3. Analysis of the rise of macroscopic VacA current and the effect of the specific chloride channel blocker, DIDS. A: The macroscopic current produced by 25 nM VacA in 1.5 M NaCl, 2 mM EDTA, 5 mM citric acid, pH 4.0 (at −50 mV) is well described by Eq. 1, with $n = 5.3$ (dark line). Similar measurements conducted at protein concentrations ranging from 5 nM to 25 nM generated currents that could all be remarkably well-described by Eq. 1, with $n = 6 \pm 2$, suggesting that the VacA channel is hexameric. Inset, The macroscopic current was rapidly blocked by the addition of 20 μM DIDS (triangle) to the *cis*-chamber containing 1.5 M NaCl, 2 mM EDTA, 5 mM citric acid, pH 4.0. Addition of up to 200 μM DIDS to the *trans*-chamber did not reduce the VacA current. B: The rate constant, k, of the rise of the macroscopic current is directly proportional to the protein concentration, consistent with the hypothesis that the rate-limiting step in the formation of VacA channels is the binding of the monomers to the membrane.

4 through pH 7 ($P_{Cl}:P_{Na} \sim 3:1$ at pH 7). The selectivity of the VacA channel is clearly different from that observed for diphtheria toxin, which is weakly anion selective at pH 3.5, but becomes weakly cation selective when the pH is raised to 5.5 [13].

Table 1
Ion permeability ratios

x	P_x/P_{Cl}
SCN^-	6.80 ± 2.64 ($n = 4$)
I^-	1.87 ± 0.57 ($n = 4$)
Br^-	1.29 ± 0.25 ($n = 4$)
F^-	0.35 ± 0.06 ($n = 3$)
Na^+	0.24 ± 0.03 ($n = 5$)

Since previous observations [9,14,15] indicated that VacA dodecamers disassemble into monomers in solution at a low pH, it is reasonable to assume that the VacA channel is assembled directly from monomers. If so, the macroscopic current should have a well-defined relationship with the subunit stoichiometry, as described by the following equation:

$$I = a(1-\exp(-kt))^n \quad (1)$$

where a is the maximum current, k is the concentration-dependent rate of current increase and n is the number of monomers required to form a conductive channel. The only explicit assumptions here are that the rate-limiting step is the binding of the monomers to the membrane and that this binding is followed by rapid intermolecular interactions that assemble the functional channel. As shown in Fig. 3A, the evolution of the macroscopic VacA current was found to be remarkably well-described by this equation, with $n = 6 \pm 2$ (number of experiments = 13), regardless of the protein concentration, suggesting that the VacA channel is most probably formed by six subunits. Furthermore, the rate constant, k, is found to be a linear function of the toxin concentration (Fig. 3B), in agreement with the assumption that the rate-limiting step is the binding of the monomers to the membrane.

To directly determine the subunit stoichiometry, we have applied AFM to image membrane-bound VacA channels. As shown in the unprocessed image of Fig. 4, oligomers, both those isolated and those within the close-packed patch, are found to consist of a central hexagonal ring, surrounded by six peripheral domains. Taken together, the electrophysiological and AFM data demonstrate that the anion selective VacA channel is hexameric.

To further characterize this unusual channel, we have de-

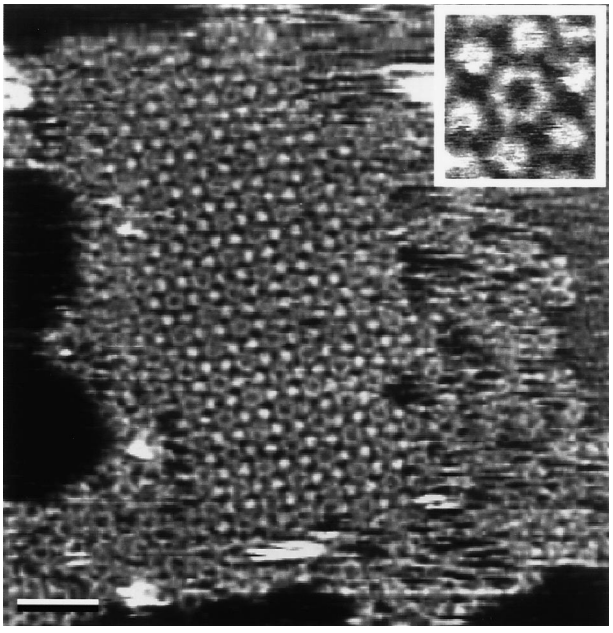

Fig. 4. This image shows a cluster of close-packed VacA oligomers. The hexameric complex consists of a central hexagonal ring surrounded by six peripheral domains (inset, 34×37 nm^2). Three peripheral domains form the contact region between neighboring oligomers [8]. These images were taken at a neutral pH after the hexamers were already assembled at a low pH. The channel remains anion selective under these conditions. Scale bar: 50 nm.

termined the permeability ratios for a series of anions, measuring the reversal potential under asymmetric salt concentrations. We found that, in general, the greater the ionic radius, the higher the permeability: $SCN^- \gg I^- > Br^- > Cl^- > F^-$. This particular permeability sequence is normally considered to reflect a 'weak field strength' binding site within the channel for the conducting anion [16], which is a common attribute among many chloride channels [17]. Because other chloride channels have been found to be specifically blocked by the disulfonic stilbene, DIDS, we have measured the macroscopic conductance of the VacA channels in the presence of the blocker. As shown in the inset to Fig. 3A, the addition of 20 μM DIDS to the *cis*-chamber of the lipid bilayer system indeed caused a rapid reduction in the current. However, when applied to the *trans*-side even up to 200 μM, DIDS failed to block the channel (data not shown). It should be noted that a blockage by DIDS on only one side of the channel is also a well-documented property of other Cl^- channels [17,18].

Thus, the VacA channel exhibits several fundamental characteristics common to most chloride channels: a moderate selectivity for anions over cations [16], a 'weak field strength' permeability sequence [17] and a blockage by asymmetric concentrations of DIDS [18]. In spite of this similarity though, there is no identifiable sequence homology between VacA and any known Cl^- channels [3]. Since the subunit stoichiometry of Cl^- channels has been suggested to range from monomeric to pentameric, the existence of a hexameric Cl^- channel (demonstrated in this work) and the lack of sequence homology with other known channels seem to suggest that anion selectivity may not require a highly conserved structure, as is suggested for cation channels based on the recent K^+ channel structure [19]. Rather, it may be that only a 'weak field strength' binding site, constructed from a variable arrangement of different amino acid residues, is required to achieve the defined yet only weakly anionic selectivity. To further substantiate this hypothesis, would require a detailed atomic structure for the ion selectivity filter. Since 2D crystals of the membrane-bound VacA hexamers have already been obtained [8], which should be amenable to high resolution structural determination by electron crystallography [20], this hexameric VacA Cl^- channel may be particularly useful to provide insight into the structural basis for anion selectivity, which may differ fundamentally from that of cation channels.

The formation of anion channels may also play an important role in the pathological activity mediated by this cytotoxin. For example, the apical surface of the gastric epithelium might experience, under certain conditions, a sufficiently low pH to trigger formation of these VacA channels directly within the plasma membrane of gastric epithelial cells or parietal cells. Acid secretion by parietal cells is thought to be regulated by an endogenous Cl^- channel [21] and, therefore, *H. pylori* VacA activity might alter the gastric acid secretion. Alternatively, the anion channel might form in the membranes of the late endosomes/lysosomes after internalization [6,22]. By allowing the influx of Cl^-, the inside positive potential might be lowered, leading to the stimulation of the electrogenic proton pump and, thereby, a lowering of the intralumenal pH. Because the extent of weak base accumulation within acidic compartments is determined by the lumenal pH [23], these VacA channels might thus directly cause the massive osmotic swelling that has been observed [4,5].

In summary, we have presented evidence that VacA monomers at a low pH assemble into hexameric anion selective channels on bilayers containing negatively-charged lipids. These anion channels exhibit several characteristics of identified eukaryotic chloride channels, including moderate selectivity and blockage by DIDS. Therefore, the VacA channel is the first hexameric chloride channel so far identified. It is expected that with targeted mutations, both the mechanism of anion selectivity and the pathological consequences of this VacA channel will become elucidated and the well-formed 2D crystals may afford the possibility of a high resolution structure, providing an architectural basis for understanding the general principles of anion selectivity.

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