Helicobacter pylori cytotoxin VacA increases alkaline secretion in gastric epithelial cells

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Debells, Lucantonio, Emanuele Papini, Rosa Caroppo, Cesare Montecucco, and Silvana Curci. Helicobacter pylori cytotoxin VacA increases alkaline secretion in gastric epithelial cells. Am J Physiol Gastrointest Liver Physiol 281: G1440–G1448, 2001.—Human infection by the bacteriumHelicobacter pylori(Hp) may lead to severe gastric diseases by an ill-understood process involving several virulence factors. Among these, the cytotoxin VacA is associated with higher tissue damage. In this study, the isolated frog stomach model was used to characterize the acute effects of VacA on the gastric epithelium. Our results show that VacA partially inhibits gastric acid output by increasing HCO3- efflux. Experiments conducted with double-barreled pH or Cl−-selective microelectrodes on surface epithelial gastric cells (SECs) and single gastric glands show that VacA does not impair the activity of the oxyntic cells but renders the apical membrane of SECs more permeable to HCO3− and Cl−. Inhibition of this permeation by 5-nitro-2-(3-phenylpro-pyridinylamino) benzoic acid indicates that this may be due to the formation of anion-selective pores by the toxin. We suggest that VacA-dependent HCO3− efflux from SECs improves the environmental conditions (pH, CO2 concentration) of the niche parasitized by Hp, that is the gastric surface. This may favor Hp persistence in the tissue and the secondary development of a chronic inflammation.

gastric secretion; anion channel

INFECTION BY THE GRAM-NEGATIVE bacterium Helicobacter pylori (Hp) is very common in humans (2). Hp colonizes the stomach and establishes a life-long chronic inflammation, which can evolve into severe gastritis, ulcer, and gastric adenocarcinoma (9).

Studies conducted in recent years clearly show that a plethora of different adaptations and virulence factors are needed to guarantee Hp early colonization and persistence within and below the mucus layer covering surface epithelial gastric cells (SECs) (39). A cytosolic bacterial urease, which generates NH3 and HCO3−, is essential for neutralization of gastric pH during early infection steps and presumably also during the whole parasitic cycle (19, 33, 57). This is confirmed by the recent discovery that Hp expresses a membrane urea transporter, which is activated by acidic conditions, allowing an increased supply of urease substrate (58).

A set of ~30 genes grouped in the so-called pathogenicity island cag (PAI cag) encodes for different factors essential in gastric colonization (9). Among these are the protein cag A and a type IV secretory apparatus that is responsible for its injection into host epithelial cells (49, 50).

Two other factors encoded outside PAI cag have been identified and intensively studied: the Hp-neutrophil-activating protein, which stimulates polymorphonucle-}

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bound to the surface of gastric cells (43, 44, 52, 55). Therefore, experimental approaches exploiting an intact gastric tissue are needed not only to test hypotheses generated in simpler models but also to identify the consequences of a given molecular action on the complex functional performance of a whole tissue. Indeed, very little is known about the acute functional alterations induced by the toxin on the actual target of the bacterium: the gastric epithelium.

To gather novel insight into the role played by VacA in the Hp parasitic cycle, we have challenged the intact gastric mucosa from Rana esculenta and analyzed one of its major physiological functions: the acid and alkaline secretion balance. Our data show that, despite the phylogenetic distance, this amphibian gastric model can be very useful in the study of Hp virulence factors. Moreover, we discovered that an early effect of VacA consists of a modification of the permeability of the apical plasma membrane of SECs to HCO$_3^-$, an event that results in alkali efflux and a significant decrease of apparent acid secretion by the tissue. The correlation of this action with the known pore-forming activity of VacA and its possible role for Hp adaptation strategy are discussed.

**MATERIALS AND METHODS**

**Tissue preparation.** Experiments on gastric mucosa of Rana esculenta were performed in accordance with the Italian guidelines for animal experimentation. The frogs were kept in an aquarium at room temperature and killed by decapitation and destruction of the spinal cord and brain. The stomach was removed, and the fundic mucosa was separated from the muscle layer by blunt dissection. The isolated stomach preparation was mounted in one of two different types of Lucite chambers for measuring either acid/alkaline secretion or transepithelial and cell membrane electrical parameters.

**Acid and alkaline secretion measurements.** Tissues were mounted vertically between two halves of a Lucite chamber having an exposed area of 0.64 cm$^2$. Each half-chamber consisted of a circular fluid canal of 2.5 ml total volume filled with modified Ringer solution that was constantly recirculated by means of a bubble lift. The control Ringer solution on the serosal side contained (in mM): 102.4 Na$^+$, 4.0 K$^+$, 1.8 Ca$^{2+}$, 0.8 Mg$^{2+}$, 91.4 Cl$^-$, 17.8 HCO$_3^-$, 0.8 SO$_4^{2-}$, 0.8 H$_2$PO$_4^-$, and 11 D-glucose. To prevent accumulation of CO$_2$ this solution was gassed with 100% O$_2$ that was passed through a bottle containing Ba(OH)$_2$ solution (50 mM). Acid secretion and the alkaline secretion rate (ASR) was passed through a bottle containing Ba(OH)$_2$ solution (50 mM). Acid secretion and the alkaline secretion rate (ASR) was measured with an isolation voltage of 9.12 V and a 1-s duration. The voltage divider ratio (VDR) was determined as $V_a/V_b$ where $V_a$ and $V_b$ are voltage displacements recorded in response to transepithelial current pulses across the apical and basolateral cell membrane, respectively. All $R_t$ and VDR values given are corrected for the in-series resistances of the mucosal and serosal bath solutions.

Cells were impaled with a double-barreled microelectrode mounted on a Leitz micromanipulator connected to a model FD-223 dual-channel electrometer (WPI, New Haven, CT) and to a strip-chart recorder (Kipp & Zonen, Delft, Holland).

**Microelectrodes.** Double-barreled pH microelectrodes were constructed as previously described (16). Briefly, two molten pieces of filament-containing aluminum silicate glass tubing of different diameters (Hilgenberg, Malsfeld, Germany) were twisted together. The capillaries were then pulled (tip length $\approx$ 20 mm) in a PE2 vertical puller (Narishige, Tokyo, Japan). The thick channel was silanized in dimethylchlorosilane vapor (Serva, Heidelberg, Germany). The tip was back-filled with H$^+$ ligand (Hydrogen Ionophore II, Cocktail A/Cocktail B; Fluka, Buchs, Switzerland), and the shaft was then filled with a buffer solution containing (in mM): 500 KCl, 64.7 NaH$_2$PO$_4$, and 85.3 Na$_2$HPO$_4$, pH 7.0. The reference channel contained 500 mM KCl. Average slope resistance of the electrodes were 55.6 $\pm$ 0.4 MΩ (selective channel), and 187 $\pm$ 19 MΩ (reference channel). All microelectrodes were calibrated in the upper half of the chamber before and after each puncture by flushing the chamber with NaCl solutions containing a mixture of KH$_2$PO$_4$ and Na$_2$HPO$_4$ to yield pH values between 6.8 and 7.8 (osmolarity: 230 mosM). For the Cl$^-$ microelectrodes, the selective channel tip was filled with a Cl$^-$ ligand (Cl$^-$-selective liquid ion-exchanger, Cocktail A, Fluka), and the shaft contained 91.4 mM NaCl solution. Cl$^-$ microelectrodes were calibrated in Ringer solutions containing 10, 50, or 91.4 mM Cl$^-$ and the sensitivity was 57.3 $\pm$ 0.1 mV unit ($n = 6$) for a 10-fold change in Cl$^-$ concentration.
In some experiments, the luminal perfusate contained: 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB; Research Biochemical International, Natick, MA), papain, and L-cysteine.

All chemicals were of reagent grade and, when not specified, were purchased from either Farmitalia Carlo Erba (Milano, Italy), Fluka Chemie, Merck (Darmstadt, Germany), or Sigma Chemical (St. Louis, MO).

**Toxin.** VacA was extracted from the extracellular medium from cultures of the Hp strain CCUG 17874 as previously described (34) and purified by fractionized precipitation, ion exchange, and HPLC chromatography and stored at 4°C in PBS at concentrations of 0.1–0.2 mg/ml. Activation was achieved by pretreatment at pH 2.0 for 5 min at 37°C (15). The toxin concentration was chosen according to the results of pilot experiments, in which the range of 10–60 nM was tested, with the aim of achieving maximal effects with the lowest toxin concentration.

**Data analysis and statistics.** All measurements are expressed as mean values ± SE of n individual transmural experiments or n individual micropuncture recordings on m tissues from which data were analyzed. The significance of the observation was evaluated by Student’s t-test for paired or unpaired data as appropriate and a P value <0.05 denoted a statistical difference.

## RESULTS

**Studies in the whole intact epithelium.** Hp resides within the mucus layer or is anchored to the luminal surface by means of the adhesines (3, 18, 27, 28). Hence, VacA toxin is presumably released by the bacterium directly onto the apical cell membrane. To mimic these in vivo conditions, before VacA addition, the mucus layer of stomach preparations was removed either by gentle suction or by pretreatment with a mucolytic solution (see *Measurements in the gastric gland lumen*). The gastric fundus mucosa was bathed in HCO₃⁻-containing Ringer solution and constantly bubbled with 5% CO₂ in O₂. When acid or alkaline secretion was measured by titration (pH-stat method), the mucosal surface was bathed in unbuffered HCO₃⁻-free Ringer solution constantly bubbled with 5% CO₂ in O₂ or pure O₂, respectively. Purified VacA was suspended in PBS and used either in the oligomeric non-activated form (na-VacA) or in the activated form (a-VacA). The toxin was added to the mucosal bath.

**Effect of VacA on acid secretion.** Stimulation of control gastric tissue with histamine (500 μM) elicited an average rate of acid secretion of 3.86 ± 0.22 μeq·cm⁻²·h⁻¹, whereas the Vᵢ in these conditions was −23.0 ± 1.5 mV (lumen negative; m = 25). Tissue exposition to a placebo control solution (consisting of 60 μl PBS and 6 μl 0.1 N HCl, a cocktail that represents the VacA solvent) did not modify acid secretion (from 3.91 ± 0.43 to 3.66 ± 0.35 μeq·cm⁻²·h⁻¹; m = 9). Whereas similar results were obtained when the epithelia were exposed to na-VacA, exposure to 40 nM α-VacA significantly reduced HCl secretion by ~25% within 3 h (from 3.82 ± 0.37 to 2.66 ± 0.38 μeq·cm⁻²·h⁻¹; m = 9; P < 0.001; Fig. 1A). During this period, Vᵢ depolarized (∆Vᵢ = 4.5 ± 1.5 mV; P < 0.01) in control conditions. Vᵢ changes either in a-VacA or na-VacA were not significant.

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**Effect of VacA on alkaline secretion.** ASR of the whole tissue was measured after inhibition of spontaneous acid secretion by the H2 receptor antagonist cimetidine (100 μM in the serosal bath). Under control conditions, i.e., in the presence of the placebo cocktail (see *Effect of VacA on acid secretion*), ASR decreased significantly over a period of 3 h, from 1.15 ± 0.1 to 0.65 ± 0.09 μeq·cm⁻²·h⁻¹ (n = 13; P < 0.01). After correction for the control data, ASR values recorded following addition of toxin indicated that na-VacA does not induce significant changes in ASR. On the contrary, a-VacA elicited a significant increase of ~50% in the alkaline output (Fig. 1B).

Whereas in all experiments Vᵢ was not significantly displaced from the mean value of ~30.1 ± 1.2 mV (m = 42), in the presence of a-VacA there was a slight hyperpolarization (∆Vᵢ = 1.6 ± 0.6 mV; m = 23).

**Measurements in the gastric gland lumen.** VacA-induced reduction of acid secretion and relative increase of ASR may depend on the targeting of either the SECs, most directly exposed to the toxin, and/or of acid-secreting oxyntopeptic cells (OCs) located deeply in the glands. To discriminate between the two possibilities, the action of VacA on the OC secretory activity was followed directly by monitoring HCl secretion in...
the lumen of single gastric glands with a method recently developed in our laboratory (16). Unique observations of the glandular pH (pH$_{g}$) were made in situ using double-barreled proton-sensitive microelectrodes. The insertion of the microelectrode in the gland lumen was achieved by first impaling an oxytropical cell and then gradually advancing the electrode until the tip broke into the gland lumen (Fig. 2A). The correct positioning of the microelectrode tip was established by the following criteria: 1) the near identity of the glandular potential (V$_{g}$), the voltage recorded via the nonselective channel of the microelectrode, with the V$_{t}$; 2) the near identity of the electrical resistance recorded between the microelectrode reference channel and serosal bath macroelectrode with R$_{t}$ (the resistance recorded between serosal and mucosal bath macroelectrodes); 3) the strong acidification of the gland lumen in response to stimulation with histamine; and 4) the eventual, but not immediate, response to pH changes in the luminal bath. The latter was also employed to evaluate whether mucosally applied toxin might penetrate the gastric pits and contact the OCs directly. The time course of the change in pH$_{g}$ shows that the gland content may be effectively replaced by the bathing solution in ~5 min. Because protons are much more diffusible in aqueous solution than other molecules, we repeated the same type of experiment with Ca$^{2+}$-sensitive microelectrodes. By exposing the luminal side of the epithelium to solutions with different Ca$^{2+}$ concentrations, it was found that a change in Ca$^{2+}$ concentration was also recorded within 5 min after the change of solutions (Caroppo R, Debellis L, and Curci S, unpublished results). It is therefore likely that the apical membrane of the OC can be slowly reached by the cytotoxin.

Nevertheless, to further improve VacA diffusion toward the OC apical surface, the luminal mucus layer was removed by incubation in a mucolytic solution containing papain (5 U/100 ml) plus L-cysteine (5 mM) (23) for 20–30 min before the addition of the acid-activated toxin. The enzyme concentration and the exposure time were kept low to avoid damage to the mucosa. As shown in Fig. 3, A and B, ~5 min after stimulation with histamine (500 μM), pH$_{g}$ began to acidify rapidly, reaching values below pH 5. On reaching a stable value, the tissue was exposed to papain plus L-cysteine. Subsequent exposure to 40 nM a-VacA for 90 min did not further reduce acid secretion; in fact, sometimes a continuous slight lowering of the pH$_{g}$ was observed (Fig. 3A).

We considered the possibility that, in the stimulated tissue, the flux of secreted fluid from the gastric pits might impair the VacA diffusion down into the glands. The toxin was therefore also applied to the luminal side of tissues maintained in the resting state. As reported in Fig. 3C, under these conditions, pH$_{g}$ also did not increase within 90 min of exposure to a-VacA. These experiments demonstrate that, in our model and over the duration of the experiments, the toxin does not alter the secretory properties of the OCs.

Measurements in surface epithelial cells. Given that the OCs appeared to be functionally intact following treatment with the toxin, we next investigated SECs as the targets of VacA. Double-barrelled proton-sensitive microelectrodes were used to measure SEC apical membrane potential (V$_{a}$) and intracellular pH (pH$_{i}$) in resting tissues. In these experiments, the dissected mucosa was mounted horizontally, with the mucosal side facing up. Micropunctures were preceded by removal of the surface mucus layer by gentle suction to improve the VacA efficacy, as explained above. The intracellular pH measured in six cells averaged 7.35 ± 0.06 pH units and remained unchanged ~90 min after exposure to 40 nM a-VacA.

The effect of the toxin was hence evaluated by measuring the pH$_{i}$ response to sudden changes in luminal HCO$_{3}^{-}$ concentration ([HCO$_{3}^{-}$]). Records presented in Fig. 4 show that under control conditions, pH$_{i}$ remained unchanged when luminal [HCO$_{3}^{-}$] was either reduced from 17.8 to 2 mM or elevated from 17.8 to 36 mM (Fig. 4A). However, on exposure to 40 nM a-VacA, a significant intracellular acidification (by 0.12 ± 0.01 pH units; n = 8; P < 0.001) or alkalization (by 0.11 ± 0.03 pH units; n = 5; P < 0.001) was observed when luminal [HCO$_{3}^{-}$] was reduced or elevated, respectively (Fig. 4B). Na-VacA did not elicit any response (Fig. 4C). These data indicate that after VacA addition, the api-

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Fig. 2. A: schematic drawing illustrating the technique employed for the micropuncture of the gastric gland lumen. 1) microelectrode approaching a microdissected gland, 2) microelectrode in the cytoplasm of an oxytropical cell (OC), 3) microelectrode placed in the gastric gland lumen. SEC, surface epithelial gastric cells. B: micropuncture of gastric gland lumen: test for the accessibility of gland space by luminal perfusate. Changes of glandular pH (pH$_{g}$) in response to luminal exposure to HCO$_{3}^{-}$-free buffer solutions at different pH. Note that 90% of the maximum value was reached within 5 min and that the response was proportional to the luminal perfusate pH. Subsequent stimulation with histamine (500 μM) acidified gland lumen.
The cal membrane of SECs became permeable to \( \text{HCO}_3^- \).

Although transepithelial electrical parameters such as \( V_t \) and \( R_t \) were not significantly changed on exposure to the toxin, the \( V_a \) hyperpolarized by \( 0.8 \pm 0.3 \) mV \((n = 8; \ P < 0.05)\) in response to reduction of luminal \([\text{HCO}_3^-]\) but changed slightly in response to \([\text{HCO}_3^-]\) rise. The VDR, which represents the ratio between the relative resistance of apical/basolateral membranes, did increase significantly from \( 12.64 \pm 3.99 \) to \( 14.78 \pm 3.90 \) \((n = 8; \ P < 0.05)\) and from \( 12.61 \pm 5.37 \) to \( 13.03 \pm 5.64 \) \((n = 5; \ P < 0.01)\) in response to reduction or increase, respectively, of luminal \([\text{HCO}_3^-]\) in a-VacA-treated tissues.

In four tissues, the effect of VacA was also tested on intracellular \( \text{Cl}^- \) activity \((a_{\text{Cl}}^i)\) in SECs and on the cell membrane potential response to sudden luminal \( \text{Cl}^- \) reduction \((2 \text{ mM Cl}^-; \text{ion replaced with gluconate})\), using double-barrelled \( \text{Cl}^- \) sensitive microelectrodes. Interestingly, the basal intracellular \( \text{Cl}^- \) activity was significantly increased by exposure to the toxin \((13.24 \pm 1.16 \) to \(21.16 \pm 2.36; \ n = 8; \ P < 0.01)\).

Figure 5 shows that in control conditions, \( a_{\text{Cl}}^i \) decreased in response to a reduction of luminal \( \text{Cl}^- \) (by

![Fig. 3. Effect of 40 nM a-VacA on pH of gastric gland lumen. A: micropuncture of gastric gland lumen; top trace (left scale): transepithelial potential difference \((V_t)\) in mV (mucosal surface negative); middle trace (right scale): gland lumen potential \((V_{gl})\) in mV; bottom trace (left scale): \( \text{pH}_{gl} \) in pH units. The superimposed voltage pulses indicate response to transepithelial constant current pulses \((50 \mu\text{A/cm}^2, 1\text{-s duration})\) used for transepithelial resistance measurements. Note that \( V_{gl} \) equals \( V_t \). After serosal perfusion was changed from cimetidine \((100 \mu\text{M})\) to histamine \((500 \mu\text{M})\)-containing solution, \( \text{pH}_{gl} \) decreased. Exposure to a-VacA did not impair acid secretion. Toxin application was preceded by mucolytic treatment \((5 \text{ U/100 ml papain}) \pm 5 \text{ mM L-cysteine})\). B: time course of the change in pH of the gland fluid in 5 stimulated mucosae. Details as in A. Data expressed as means \pm SE. C: time course of the change in pH of the gland fluid in 3 mucosae in the “resting” state \((\text{cimetidine})\). Details as in A. Data expressed as means \pm SE.

![Fig. 4. Effect of changes in luminal \( \text{HCO}_3^- \) concentration from 17.8 to 2 or 36 mM on intracellular pH \((\text{pH}_i)\) of surface epithelial cells in control conditions \((A)\), after exposure to 40 nM a-VacA \((B)\), and after exposure to 40 nM na-VacA \((C)\).](image)

![Fig. 5. Effect of changes in luminal \( \text{Cl}^- \) concentration from 91.4 to 2 mM on intracellular \( \text{Cl}^- \) activity \((a_{\text{Cl}}^i)\) of surface epithelial cells in control conditions and after exposure to 40 nM a-VacA.](image)
shown). VacA-induced increase of anion permeability in the SEC apical membrane was also blocked by NPPB. As shown in the experiment of Fig. 6 and in analogy with the previous observations on other cell types, exposure to 50 μM NPPB decreased the pH response to luminal [HCO₃⁻] reduction from 0.11 ± 0.03 to −0.04 ± 0.05 pH units (n = 3; P < 0.01). At variance, IAA-94 did not modify the VacA effect (not shown).

**DISCUSSION**

Detailed studies of the action of Hp virulence factors have been limited by the lack of suitable experimental systems for measurements at the cellular level. The amphibian model used here affords the possibility to study directly, with electrophysiological techniques applied to OCs and SECs, the effects of acute topical addition of the cytotoxin VacA to the luminal surface of the isolated gastric mucosa perfused in vitro. In the same preparation, we also developed an approach that allowed us to monitor pH changes in the lumen of single gastric glands in situ. The present study tried to mimic as closely as possible the in vivo situation to ensure a rapid contact of the toxin with the cell surface. In fact, our data demonstrate that VacA is capable of interacting efficiently with amphibian gastric cells.

The most important implication of our observations is that one of the early events in the Hp infection appears to be the membrane insertion of VacA with the formation of anion-selective channels that can increase efflux of HCO₃⁻ across the apical membrane of the surface cells.

A similar result was also obtained in a recent study performed on the rat duodenum perfused in vivo, where an increase in alkaline secretion was described in response to treatment with Hp water extracts (23).

Our data clearly indicate that, at least in the early stage of intoxication, VacA gives rise to an increased alkali efflux in the amphibian gastric mucosa. This conclusion is based on the following lines of evidence: 1) the significant reduction in HCl secretion during stimulation with histamine (Fig. 1A); 2) the functional integrity of the OCs as tested in the experiments with pH microelectrodes in the gland lumen (Fig. 3); 3) the increase in ASR monitored in response to the cytotoxin (Fig. 1B); and 4) the observation that the transepithelial response to luminal Cl⁻ reduction (Fig. 5) or to HCO₃⁻ steps (not shown) remained unchanged after treatment with the toxin.

The fact that the decrease in acid secretion is not quantitatively comparable with the increase in alkali output is not against this conclusion, because direct quantitative comparison between these two phenomena cannot be accomplished. In fact, as discussed in a previous work (11), ASR is most probably underestimated due to CO₂ diffusion from the HCO₃⁻/CO₂-containing serosal perfusate.

Furthermore, according to previous studies, alkaline secretion seems to originate not only from the SECs (22, 54) by mechanisms that are not fully understood (6) but also from the OCs (11, 17). The fact that pHgl, either at rest or after stimulation with histamine, did not change in response to the toxin clearly precludes any short-term involvement of these cells in the change in ASR. Therefore, because amphibian gastric mucosa is composed of only SECs and OCs, with the exception of a few neck cells (see Fig. 2A), the SECs are the most likely cell type to be involved in the change in ASR.

The experiments using micropuncture of the SECs with pH microelectrodes confirmed that these cells are responsible for the change in alkaline output and that this effect has to be attributed to a single virulence factor: VacA. As shown here and also in previous studies from our laboratories, the anionic permeability of the SEC apical membrane is negligible under control conditions. Reducing luminal [Cl⁻] does not significantly alter SEC intracellular Cl⁻ activity (12, 13) (see also Fig. 5), and reduction of luminal [HCO₃⁻] is ineffective in changing pHl (see also Fig. 4) (6). After incubation with VacA, however, SEC pH increased or decreased, respectively, in response to an increase or reduction of luminal [HCO₃⁻], and SEC RCl⁻ decreased in response to luminal Cl⁻ reduction. This implies that exposure to the cytotoxin resulted in a modification of the SEC apical membrane anion permeability.

An additional point of evidence in support of this conclusion is provided by the changes observed in the Vₐ and in the ratio of the SEC apical/basolateral membrane resistance, expressed as VDR. After mucosal perfusion with low or high HCO₃⁻ during exposure to

![Figure 6](http://ajpgi.physiology.org) Effect of reduction of luminal HCO₃⁻ concentration from 17.8 to 2 mM on the pH, of surface epithelial cells in the presence of 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB; 50 μM) and during exposure to 40 nM a-VacA.
a-VacA, VDR increased significantly, whereas it remained unchanged in control experiments (na-VacA).

On the other hand, the observation that the responses of $V_t$ and of SEC $V_a$ to luminal low Cl$^{-}$ were not influenced by the toxin confirms that the OCs were not affected by VacA. Such responses originate from the OC apical membrane (6, 12, 31) rather than from the SECs, which scarcely contribute to the tissue conductance (31).

Furthermore, the observation that $V_t$ and $V_a$ responses to luminal Cl$^{-}$ reduction were larger than the responses to HCO$_3^-$ steps is possibly explained by the fact that the Cl$^{-}$ conductance of the OC apical membrane is larger than the conductance to other anions, such as the HCO$_3^-$ (10, 47).

In view of the finding that the specific target cell of the toxin is the SEC, it is not surprising that VacA does not elicit significant changes in transepithelial electrical parameters such as $V_t$ and $R_t$. In fact, SECs represent a low-conductance pathway across the gastric mucosa, which operates in parallel with the prevalent OC conductance, and therefore the amount of SEC contribution does not exceed 10% of total transepithelial conductance (31). In such a situation, any electrical signal that originates at the apical membrane of SECs will be strongly attenuated.

Moreover, in a study on confluent monolayers of different cell lines (MDCK, T84, epH4, Caco-2), the paracellular permeability increased and the $R_i$ decreased (43) only when the monolayer $R_t$ values were $>1$ kΩ·cm$^2$. Therefore, the electrical resistance of the amphibian stomach having, as observed, a value of $\sim 600$ Ω·cm$^2$ is expected not to be modified by VacA.

The most straightforward interpretation of our results is that anion-selective channels made by VacA directly modify the apical plasma membrane permeability of SECs. In fact, the VacA channel conducts Cl$^-$ and HCO$_3^-$ with equal efficiency (55). This possibility is backed by the observation that, in both gastric cells in situ and in HeLa cells in culture, the anionic conductance elicited by VacA is sensitive to NPPB and insensitive to IAA-94.

An important aspect of these findings is their possible significance for clinical observations. The reduction in HCl secretion during stimulation with histamine observed in our experiments appears to fit with previous clinical studies on humans (20, 24, 26, 30) and in animals in vivo (4, 29). Nevertheless, it is also evident that the amount of alkali that may leave the SECs through VacA channels in our model (1 μeq·cm$^{-2}$·h$^{-1}$) is too modest to explain the significant pH changes observed in the gastric lumens of Hp-infected patients. In these studies, such a decrease was attributed to either a dysfunction of secreting cells or to a marked reduction of their number after Hp infection. In our model, on the contrary, the decrease in acid secretion observed on the whole tissue was not the result of an altered secretory activity of the OCs. However, we document that HCO$_3^-$ secretion is increased by VacA and that the permeability of SEC membranes to HCO$_3^-$, normally very low, becomes evident in these conditions. This permeabilization of the gastric epithelium to HCO$_3^-$ cannot be ascribed to cell damage and loss of tissue integrity, because parameters such as $V_t$ and resistance remain quasi-stable.

Therefore, it seems unlikely that, at least in the short term, VacA is responsible for the impairment of the secretory function of OCs observed in chronic infections (8, 21, 25, 32, 51).

A relevant implication of the alkali exit from SECs, to which bacterial cells are intimately bound, can be envisaged anyway inasmuch as an increased HCO$_3^-$ efflux from the gastric superficial cells may represent a strategy employed by Hp to better colonize the stomach. In fact, under normal conditions, there is a pH gradient across the mucus layer covering the gastric epithelium, ranging from slightly acidic to very acid pH values moving from the epithelial cells to the gastric lumen (7, 45, 53). Such a gradient depends on the balance between acid and alkali diffusion from the gastric lumen and from surface cells, respectively. The increased net HCO$_3^-$ flux may modify this gradient, thus creating conditions more compatible with life for Hp, which depend strictly on the activation of bacterial urease and on maintenance of the proton motive force (58). Moreover, because it is known that HCO$_3^-$ is also an important metabolic substrate for Hp (5), necessary for its growth, it can be speculated that a VacA-dependent efflux of this compound from the gastric mucosa may increase the efficacy of Hp metabolism.

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